Calcium Oscillations in Human T and Natural Killer Cells Depend upon Membrane Potential and Calcium Influx

Stephen D. Hess, Marga Oortgiesen, and Michael D. Cahalan

ABSTRACT. With the use of single-cell digital imaging of the fluorescent Ca$^{2+}$ indicator dye fura-2 we investigated Ca$^{2+}$ signaling in human T lymphocytes and NK cells during activation by a variety of stimuli. A low percentage of resting T cells or T cell blasts displayed oscillations in cytosolic Ca$^{2+}$ when stimulated with the mitogenic lectin PHA or by the addition of OKT3 mAb followed by a secondary cross-linking antibody. Lymphokine-activated T killer cells were more responsive than resting cells. A comparison of PHA, cross-linked anti-CD3, and a heteroconjugate mAb showed that at least 20% of the cells from these T cell preparations oscillated. Addition of PHA or cross-linked anti-CD16 caused NK cells to oscillate. In contrast, thapsigargin, a microsomal ATPase blocker, resulted in a relatively uniform, slowly rising and sustained Ca$^{2+}$ response in all cell types studied. The maintenance of both thapsigargin- and receptor-induced responses required Ca$^{2+}$ influx driven by a negative membrane potential. Because Ca$^{2+}$ oscillations occurred in response to stimuli which mimic the normal activation of lymphocytes, and inasmuch as the percentage of oscillating cells increases with state of activation, these oscillations may play an important role in mitogenic activation.

Calcium plays a crucial role in the activation of T lymphocytes. Cross-linking surface proteins with mitogenic lectins or mAb to the CD3/TCR complex elicits a series of intracellular biochemical events (reviewed in Ref. 1), including the activation of tyrosine kinases (for reviews see Refs. 2–4) and PLC$^5$ (5–7). Both inositol-1,4,5-triphosphate (IP$_3$) and diacylglycerol (8) are generated, resulting in the activation of protein kinase C and release of Ca$^{2+}$ from internal stores (8–10). In addition, Ca$^{2+}$ influx from the external medium is triggered (11–13) through voltage-independent Ca$^{2+}$ channels (14, 15). Both Ca$^{2+}$ release and influx contribute to the rise in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) (15–17).

The early [Ca$^{2+}$], transient is necessary for a variety of later events in T cell activation, and is temporally correlated with the transcriptional activation of so-called immediate genes including c-fos and c-myc (18), as well as subsequent activation of c-myb, IL-2 and its receptor (reviewed in Ref. 19). Calcium may stimulate the activity of PLC (20, 21) (but see also Ref. 22), in addition to activating protein kinase C and calcium-calmodulin-dependent protein kinases. Blocking Ca$^{2+}$ influx prevents normal activation of T cells (23), underscoring the requirement for this ion in the signaling cascade.

To date most studies of [Ca$^{2+}$], transients in T cells have employed intracellular Ca$^{2+}$ indicator dyes such as indo-1.
quin-2 (24, 25), or fura-2 (26) in conjunction with flow cytometry or cuvette measurements. These methods sample the average response of the population and do not provide details of single-cell responses. When fura-2 measurements are coupled with digital fluorescence ratio-imaging methods, [Ca\(^{2+}\)]\(_i\) signals in individual cells are resolved (15, 16, 27). With the use of the human Jurkat leukemic T cell line, Lewis and Cahalan (15) found that PHA-stimulated Jurkat cells display oscillations in [Ca\(^{2+}\)]\(_i\), that are dependent on Ca\(^{2+}\) influx and a negative membrane potential and are correlated with the current carried through a mitogen-gated Ca\(^{2+}\)-selective conductance. Similar [Ca\(^{2+}\)]\(_i\) oscillations have been observed in a human CD4\(^+\) T cell clone after contact with APC (27). Such oscillations may be important in signal transduction (22, 28). Our goal therefore was to extend the approach of single-cell [Ca\(^{2+}\)]\(_i\), imaging to describe the temporal and spatial patterning of [Ca\(^{2+}\)]\(_i\) transients in normal human T cells.

In the present study, we used digital fluorescence ratio imaging of fura-2 to describe the dynamics of Ca\(^{2+}\) signals in individual resting and activated human T cells in response to addition of PHA or mAb to appropriate surface proteins. We report that [Ca\(^{2+}\)]\(_i\) oscillations occur in freshly isolated T cells, peripheral blast cells, and T-LAK cells stimulated through the CD3/TCR complex, and in CD3\(^-\)NK cells stimulated with PHA or cross-linked anti-CD16 mAb. These findings suggest that [Ca\(^{2+}\)]\(_i\) oscillations may play an important role in the activation of a variety of human lymphocyte populations.

**Materials and Methods**

**Human lymphocyte preparations**

Lymphocytes were purified from peripheral blood obtained from consenting healthy adult donors. Venous blood was collected in heparinized tubes and diluted 50% with RPMI 1640 (GIBCO/BRL, Gaithersburg, MD) containing 25 mM HEPES. This suspension was then centrifuged at 400 \(\times\) g through a Ficoll-Paque density gradient (Pharmacia LKB, Piscataway, NJ) for 30 min at room temperature (25–27°C). The interface was removed, washed three times with RPMI containing 20% FCS (JR Scientific, Woodland, CA), and applied to a sterile nylon wool column pre-equilibrated with RPMI/20% FCS. After 45 min at 37°C, the column was eluted with prewarmed RPMI/20% FCS, and the eluted cells were washed three times with this medium. FACS analysis of cells isolated from four healthy donors showed that 76.9% of the cells were CD3\(^+\) T cells, 7.6% were CD19\(^+\) B cells, and 0.6% were CD14\(^+\) monocytes. The cells were used within 6 h for experiments with freshly isolated cells or cultured at an initial density of 0.3 \(\times\) 10\(^5\) cells/ml in RPMI/10% FCS in a humidified incubator at 37°C with 5% CO\(_2\).

A T cell blast preparation was obtained by culturing fresh T cells in RPMI/10% FCS with 10 \(\mu\)g/ml PHA (Difco Laboratories, Detroit, MI). After 72 to 96 h in culture, the medium was changed to fresh RPMI/10% FCS with PHA. These cells were usually used after 4 to 8 days in culture.

NK cells were isolated from fresh blood by negative-selection panning (29) of cells eluted from a nylon wool column as described above. Plastic petri plates were coated overnight at 4°C with 10 \(\mu\)g/ml GAM IgG (Caltag, South San Francisco, CA). The plates were washed twice with PBS, allowed to stand at room temperature for 30 min in PBS containing 0.2% BSA, and then washed once again with PBS. Approximately 20 \(\times\) 10\(^6\) T cells were suspended in 280 \(\mu\)l of RPMI/10% FCS along with 40 \(\mu\)l each of anti-CD3, CD4, and CD8 mAb (Becton Dickinson, San Jose, CA) for 45 min on ice. The excess antibodies were washed off five times with ice-cold RPMI/10% FCS. The cells were then resuspended at 5 to 10 \(\times\) 10\(^6\) cells/ml, and 4 ml of this suspension were allowed to settle on a precoated plate for 45 min at 40°C, with a gentle swirl after 25 min. The plate was swirled again, and the cells in suspension were removed by pipetting, washed twice with PBS/BSA, and either stained with anti-CD16 and anti-CD3 mAb as above or resuspended in RPMI/FCS and used in experiments within 6 h. These cells were 75 to 85% CD16\(^+\) and CD3\(^-\).

T-LAK cells were obtained from PBMC by culturing for 48 to 72 h with 200 U/ml human rIL-2 (Hoffmann LaRoche, Nutley, NJ) and 400 ng/ml PHA (Burroughs Wellcome, Research Triangle Park, NC) followed by growth in rIL-2 alone by using methods described elsewhere (30).

**Reagents and antibodies**

PHA-P (Difco or Burroughs Wellcome) was stored at -20°C in aliquots at 10 mg/ml, then thawed and diluted immediately before use in experiments. Unconjugated OKT3 mAb (Ortho Diagnostics, Raritan, NJ) and conjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD16 mAb (Becton Dickinson) were kept refrigerated until just before use. Goat anti-mouse IgG (Organon Teknika, West Chester, PA) was used to cross-link primary mAb and kept cold until just before use. Heteroconjugate antibodies anti-CD3/CD4 and CD3/CD8 were a kind gift of Dr. Jeffrey Ledbetter (Bristol Myers Squibb, Seattle, WA), and were stored as aliquots containing 1 mg/ml BSA at -70°C until just before use.

**Digital video-imaging**

All experiments were performed on an IM-35 inverted microscope (Zeiss, Oberkochen, Germany). Cells were loaded with fura-2 AM (Molecular Probes, Eugene, OR) at 1 \(\mu\)M in RPMI/10% FCS for 25 min at room temperature in the dark. Longer loading times or loading at higher temperatures resulted in loading of dye into intracellular organelles.
that did not exhibit the changes in [Ca\(^{2+}\)], seen in the bulk cytoplasm. The cells were washed thrice with RPMI/FCS and used within 4 h. Separate whole-cell patch-clamp experiments with pipettes containing fura-2 free acid showed that the concentration of fura-2 AM in cells following the above loading procedure was 50 to 100 μM. Lymphocytes were then plated at 3 to 4 \( \times 10^6 \) cells/ml on poly-d-lysine-coated glass coverslips, and rinsed to remove nonadherent cells with mammalian Ringer solution containing (in mM): 160 NaCl, 4.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES-NaOH (pH 7.4), and 11 glucose.

The experiments were controlled, recorded, and analyzed with the use of VideoProbe software and hardware (ETM Systems, Irvine, CA) with a 386 computer (AST Research, Irvine, CA). Cells were alternately illuminated at 350 and 385 nm by a xenon light source (Zeiss) passing through excitation filters (Omega Optical, Brattleboro, VT) housed in a motorized filter wheel and electronic shutter (Sutter Instruments, Novato, CA). The light beam was then reflected by a 405-nm dichroic mirror and passed through a 63× oil immersion objective (Zeiss) to illuminate the cells. Emitted light at greater than 510 nm was then measured with a SIT camera (Hamamatsu Photonics, Bridgewater, NJ).

Typically, [Ca\(^{2+}\)] was estimated every 10 s by dividing the background-subtracted 350- and 385-nm raw fluorescence images pixel-by-pixel, and displaying the result with a pseudocolor scale. Data were analyzed in more detail offline by averaging the nonzero 350/385 ratio values within an approximately 20 × 20 pixel rectangle positioned over each cell in the field (usually 90–150 cells with the 63× objective). Drift of the cells during the 20-min experiments was usually not a problem; deliberately positioning rectangles at the edges of cells did not affect the analysis until only a very small area of the cytoplasm (<10%) remained under the rectangle. Cells that drifted substantially during the experiment were not included in the analysis. To obtain information on the period of the oscillations, the [Ca\(^{2+}\)] responses of cells that produced sustained oscillations (5 or more peaks in [Ca\(^{2+}\)]) during the experiment) were further analyzed using a FFT algorithm implemented in a commercial spreadsheet/analysis program (Igor v. 1.2, WaveMetrics, Inc., Lake Oswego, OR).

In some cases, cells were phenotyped at the end of the experiment by the addition of 20 μl of anti-CD4 and anti-CD8 mAb conjugated to FITC or phycoerythrin directly to cells on the microscope stage. The excess antibodies were washed off after 8 min, and the images obtained by exciting phycoerythrin and FITC separately were later superimposed over the fura-2 images to score the phenotype of cells. Whenever a GAM secondary antibody had been used as a cross-linker, excess antibody was first adsorbed by the addition of excess mouse IgG or mouse serum.

Fura-2 calibration

Fura-2 signals were calibrated with an intact-cell method. Estimates of \( R_{\text{min}} \) were obtained by exposing loaded cells to Ringer solution containing 10 mM EGTA, and 1 μM ionomycin, but no added Ca\(^{2+}\). After collection of these images, the Ringer solution was changed to one containing 10 mM Ca\(^{2+}\) and 1 μM ionomycin, and the cells were rinsed twice before obtaining the \( R_{\text{max}} \) estimates. Typical values obtained were 0.7 for \( R_{\text{min}} \), 10 for \( R_{\text{max}} \), and 9 for the scale factor. Using a \( K_d \) of 350 nM obtained for fura-2 inside cells (31), the resting [Ca\(^{2+}\)] of cells was usually 60 to 120 nM. This calibration technique has the advantage of measuring dye responses in situ, although the necessary condition that no Ca\(^{2+}\) be bound to the dye at \( R_{\text{min}} \) is difficult to achieve in a living cell. Therefore, these calibrations are reasonable estimates of [Ca\(^{2+}\)].

Results

[Ca\(^{2+}\)], signaling induced by a mitogenic lectin

Video-imaging experiments have previously demonstrated that Jurkat cells often exhibit oscillations in [Ca\(^{2+}\)], after PHA stimulation (15). To extend these results to normal human T cells, we examined [Ca\(^{2+}\)] responses at the single-cell level in resting T cells and in a population of T-LAK cells. Freshly isolated human T cells responded to PHA stimulation with an increase in [Ca\(^{2+}\)], after approximately 60 s at 25°C. In the experiment shown in Figure 1, 29% of the cells demonstrated [Ca\(^{2+}\)] oscillations, but the pattern of the [Ca\(^{2+}\)] signal was quite variable between individual cells. Some cells displayed no response or only a transient increase in [Ca\(^{2+}\)] (Fig. 1, A, and B), whereas others showed a continual increase in [Ca\(^{2+}\)] after activation (Fig. 1C). Figure 1, D and E, shows two examples of sustained oscillations; the peak [Ca\(^{2+}\)] in the cells varied from 400 to 850 nM. When the population average is examined (Fig. 1F), the lack of response in some cells, variable latency to the first response, and asynchrony of individual cell [Ca\(^{2+}\)], oscillations together produce a smooth rise in [Ca\(^{2+}\)], similar to that seen in flow cytometry or cuvette experiments.

To determine if [Ca\(^{2+}\)] oscillations induced by PHA are a general property of T cell subsets, the [Ca\(^{2+}\)] transients in a preparation of T-LAK cells prepared by culturing blast cells in PHA and IL-2 were also examined, as illustrated in Figure 2. In this experiment, 33% of the cells showed no response to 10 μg/ml PHA (Fig. 2A), whereas others showed a large (1 μM) rise in [Ca\(^{2+}\)] (Fig. 2B). The [Ca\(^{2+}\)] level of 26% of the cells in this experiment oscillated (Fig. 2, C, D, and E). We conclude that [Ca\(^{2+}\)] oscillations can be induced in normal T cells by PHA. The results also point to the possibility that [Ca\(^{2+}\)] oscillations occur more frequently in cells that have been previously activated. This
FIGURE 1. Responses of human resting T cells to addition of 10 ng/ml PHA (arrow). (A–E) intracellular [Ca^{2+}]; in single cells measured with fura-2. Here and in the following figures the examples were chosen to approximate the proportion of cells in the total population that displayed similar responses. (F) Average response of the cells in the experiment.

The conclusion is substantiated in experiments with mAb stimulation (described below).

[Ca^{2+}]; signaling induced by mAb to surface receptors: correlation with state of activation

Although mitogenic lectins provide a convenient method for activating T cells, their binding specificity is not clearly defined (1). Recent results have focused attention on the role of the ζ-chain of the CD3/TCR complex in signal transduction (1, 32–34). In addition to being expressed in T cells, the ζ-chain is present in NK cells in association with CD16 (35). We have examined [Ca^{2+}]; signals in these cell types in response to more specific stimuli believed to be physiologically relevant. We have also compared [Ca^{2+}]; signaling in cell types at various stages of their activation to determine whether the tendency to produce [Ca^{2+}]; oscillations increases with the state of activation.

Resting T cells. Figure 3 illustrates responses in resting T cells to soluble OKT3 mAb followed by GAM H and L chain secondary cross-linking antibody. Addition of soluble OKT3 alone had little effect on [Ca^{2+}]; in individual cells or in the average of the 120 cells in the field (Fig. 3F). Upon addition of the secondary cross-linking GAM, several cells displayed an abrupt increase in [Ca^{2+}]; which approached 700 nM (Fig. 3, B–D); 16% of the cells oscillated (see Fig. 3E for an example). The apparent decrease in [Ca^{2+}]; following addition of the GAM mAb (Fig. 3F) is an occasional artifact caused by a change in background fluorescence at one excitation wavelength.

Table I summarizes the experiments on fresh T cells. For PHA stimulation at 25°C, the percentage of cells showing a [Ca^{2+}]; increase exhibited a weak dose dependence, although the percentage of cells that oscillated appeared to peak at 25 μg/ml PHA. Soluble OKT3 mAb applied alone was not very effective in eliciting [Ca^{2+}]; transients. Cross-linking the OKT3 mAb with a GAM secondary antibody
was approximately as effective as 10 μg/ml PHA in eliciting \([Ca^{2+}]_i\) transients. Fifty to sixty percent of the cells in this mixed population responded to PHA or cross-linking CD3 with a \([Ca^{2+}]_i\) increase.

**Activated T cells.** To correlate the occurrence of \([Ca^{2+}]_i\) oscillations with the state of activation of T cells, we examined \([Ca^{2+}]_i\) transients in a primary blast cell culture prepared by growing human T cells in medium containing 10 μg/ml PHA. These cells exhibit increased numbers of voltage-gated K⁺ channels (36) and Ca²⁺-dependent K⁺ channels (37). Both channel types might be expected to influence \([Ca^{2+}]_i\) signaling in normal human T cells, as has been shown in Jurkat T cells (15, 38). When soluble OKT3 mAb was applied alone to these cells, fewer than 1% of the cells oscillated and 46% showed an increase in \([Ca^{2+}]_i\) (Table II). Cross-linking CD3 on these cells with a GAM secondary antibody caused 20% of the cells to oscillate, and 77% of the cells showed an increase in \([Ca^{2+}]_i\) (Table II). Comparison of Tables I and II shows that although cross-linking CD3 was apparently more effective in eliciting both increases in \([Ca^{2+}]_i\) and oscillations in blast cells than in fresh cells, the differences were not statistically significant (for oscillations \(p = 0.446, t = -0.781\); for \([Ca^{2+}]_i\) increase \(p = 0.095, t = -1.78\)).

Because CD4 or CD8, in conjunction with CD3, is normally engaged in a complex by peptide bound to MHC during Ag presentation, we examined the ability of two heteroconjugate mAb to stimulate blast cells. Table II shows that the CD3/CD4 heteroconjugate applied at 1 μg/ml caused 16% of blast cells to oscillate, and 54% showed an increase in \([Ca^{2+}]_i\). An experiment in which the conjugate was particularly effective is shown in Figure 4. After addition of the CD3/CD4 conjugate, 82% of the cells in this experiment responded with an abrupt increase in \([Ca^{2+}]_i\) which approached 700 nM in some cells (Fig. 4, A–E). The types of responses we observed included one or more large peaks in \([Ca^{2+}]_i\), that progressively decayed (Fig. 4, A and B), a large peak followed by several smaller

**FIGURE 2.** Addition of 10 μg/ml PHA (arrow) triggers \([Ca^{2+}]_i\) increases in individual T-LAK cells (B–E) and in the average response (F).
Addition of mAb to CD3 (OKT3, 2.5 µg/ml) elicits little \([\text{Ca}^{2+}]_i\) change in individual human resting T cells (A-E) or in the average response (F). Addition of a cross-linking secondary antibody (GAM, 1.0 mg/ml) elicits transient responses in some cells (B and C) and oscillations in others (D and E).

Table I

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>n</th>
<th>Total No. of cells</th>
<th>([\text{Ca}^{2+}]_i), % increase, percent of cells*</th>
<th>([\text{Ca}^{2+}]_i), % increase, percent of cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA 10 µg/ml</td>
<td>5</td>
<td>359</td>
<td>17.3±4.5</td>
<td>46.6±10.7</td>
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<tr>
<td>25 µg/ml</td>
<td>2</td>
<td>191</td>
<td>32.5±2.9</td>
<td>54.8±1.6</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>7</td>
<td>790</td>
<td>21.9±3.4</td>
<td>63.3±3.0</td>
</tr>
<tr>
<td>OKT3, 2.5 µg/ml</td>
<td>7</td>
<td>417</td>
<td>4.2±3.9</td>
<td>28.3±9.4</td>
</tr>
<tr>
<td>GAM after OKT3</td>
<td>6</td>
<td>366</td>
<td>12.8±3.6</td>
<td>50.9±8.1</td>
</tr>
</tbody>
</table>

* Mean ± SEM.

Table II

<table>
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<tr>
<th>Stimulus</th>
<th>n</th>
<th>Total No. of cells</th>
<th>([\text{Ca}^{2+}]_i), % increase, percent of cells*</th>
<th>([\text{Ca}^{2+}]_i), % increase, percent of cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT3, 2.5 µg/ml</td>
<td>6</td>
<td>617</td>
<td>0.6±0.4</td>
<td>46.4±11.9</td>
</tr>
<tr>
<td>GAM 1/10, after OKT3</td>
<td>9</td>
<td>939</td>
<td>20.8±7.9</td>
<td>77.1±10.7</td>
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<tr>
<td>CD3/CD4, 1 µg/ml</td>
<td>14</td>
<td>1045</td>
<td>16.7±3.7</td>
<td>54.1±6.0</td>
</tr>
<tr>
<td>CD3/CD8, 1 µg/ml</td>
<td>8</td>
<td>711</td>
<td>2.2±0.9</td>
<td>16.0±6.6</td>
</tr>
</tbody>
</table>

* Mean ± SEM.

Table II also shows that the CD3/CD8 heteroconjugate was much less effective than the CD3/CD4 conjugate in eliciting \([\text{Ca}^{2+}]_i\) transients. To explore possible differences in subset responsiveness, as has been reported for fresh human T cells activated with anti-CD3 mAb (39), we phenotyped cells at the end of experiments by adding anti-CD4 mAb conjugated to phycoerythrin and anti-CD8 mAb conjugated to FITC. We found that 94% of the CD4+ blast cells showed a \([\text{Ca}^{2+}]_i\) transient in response to cross-linking
Ca²⁺ Oscillations in Human Lymphocytes

FIGURE 4. The addition of a heteroconjugate of CD3 and CD4 mAb elicits large increases in [Ca²⁺], in individual human blast cells (A–E) and in the average response (F). Several cells (B–E) displayed [Ca²⁺] oscillations.

CD3, whereas 97% of the CD8⁺ cells responded. This suggests that the CD3/CD8 heteroconjugate antibody is not as effective as the CD3/CD4 antibody in triggering [Ca²⁺] transients, since the CD8⁺ blast cells were competent to respond to CD3 cross-linking. In addition, we found that in 99% of the cells the phenotype, as determined with anti-CD4 and anti-CD8 mAb, corresponded exactly to the heteroconjugate antibody that elicited a response in an individual cell. This correlation demonstrates appropriate specificity of the heteroconjugate antibodies and allowed us to use both heteroconjugates in a single experiment to stimulate both helper/inducer and cytotoxic/suppressor subsets.

T-LAK. PHA was an effective stimulus in eliciting [Ca²⁺] transients in T-LAK cells (Fig. 2, Table III). Although either purified OKT3 or mAb obtained from a hybridoma supernatant applied alone caused [Ca²⁺] transients and even some oscillations, both were much more effective when cross-linked with a GAM secondary antibody (Table III).

We examined the subset responsiveness of T-LAK cells stimulated by PHA or cross-linked CD3. We found no significant difference between CD4⁺ and CD8⁺ subsets in either the fraction of responsive cells or the fraction of cells that exhibited [Ca²⁺] oscillations when stimulated with PHA, the OKT3 hybridoma supernatant alone, or the supernatant followed by a GAM secondary antibody (not shown). The increased tendency of T-LAK cells to respond with [Ca²⁺] oscillations, compared with resting or PHA-activated T cells, suggests that culturing these cells in IL-2 indirectly potentiates [Ca²⁺] signaling. We did not examine this possibility directly, but it has been reported that IL-2 does not by itself stimulate phosphatidylinositol turnover (40).

Because 20 to 60% of these T-LAK cells are CD16⁺ (30), and a ζ-chain similar to that associated with CD3 is coupled to CD16 in NK cells (35), we determined whether CD16 could transduce [Ca²⁺] signals in T-LAK cells by adding an anti-CD16 mAb to the cells. As shown in Table III, CD16
mAb alone was not effective in eliciting [Ca\textsuperscript{2+}]\textsubscript{i} transients. After addition of a secondary GAM antibody, 20% of the cells oscillated, and 61% showed an increase in [Ca\textsuperscript{2+}]. This result suggests that the CD16 molecule on T-LAK cells is able to transduce signals which lead to elevations in [Ca\textsuperscript{2+}].

**NK cells.** A population of NK cells was obtained from human peripheral blood by negative-selection panning with anti-CD3, CD4, and CD8 mAb. These cells were approximately 80% CD-16\textsuperscript{+}. Figure 5 shows that application of an anti-CD16 mAb (before time 0) followed by a GAM secondary cross-linking antibody elicited [Ca\textsuperscript{2+}]\textsubscript{i} transients in these cells. In this experiment, 73% of the cells showed an increase in [Ca\textsuperscript{2+}], (Fig. 5, A and B) and 47% of the cells oscillated (Fig. 5, C, D, and E) with the peak [Ca\textsuperscript{2+}], exceeding 500 nM in some cells. The average of the 193 cells in the field is shown in Figure 5F.

When a GAM secondary antibody was used to cross-link CD16 on the surface of NK cells, 51 ± 7% of the cells oscillated, and 72 ± 11% of the cells showed an increase in [Ca\textsuperscript{2+}] \textsubscript{i}; (mean ± SEM, n = 5 experiments, 644 cells). PHA at 25 \mu{g}/ml was also an effective stimulus, as 58 ± 7% of the cells oscillated and 70 ± 8% had increased [Ca\textsuperscript{2+}] \textsubscript{i}; (n = 2 experiments, 174 cells). These results show that NK cells display [Ca\textsuperscript{2+}]\textsubscript{i} oscillations when activated either with PHA or with anti-CD16 mAb.

**Analysis of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations**

We examined the relationship between the latency of the first response and the period of the subsequent oscillations to test whether these two processes might share a common mechanism. To measure the period of the oscillations, we analyzed the Fourier transform of 640-s segments of the data. Figure 6 shows examples of the [Ca\textsuperscript{2+}] \textsubscript{i} responses and the complex conjugate of the FFT in two T cell blasts responding to the CD3/CD4 heteroconjugate antibody. In this analysis, the low frequency peak at a period of 640 s corresponds to a slowly relaxing component of the signal. At higher frequencies, one or more clear peaks of variable amplitude occurred at periods corresponding to approximately 90 to 160 s. For six blasts cells, the mean period was 143 ± 21 s, whereas the latency to the first response after antibody application was 57 ± 8 s. For both T cell blasts (Fig. 6E) and for resting T cells (data not shown), no correlation exists between the latency and the period ($r^2 = 0.006$).

We also compared the latency and timing of [Ca\textsuperscript{2+}]\textsubscript{i} transients from those cells in which [Ca\textsuperscript{2+}]\textsubscript{i} oscillated when stimulated by PHA or by mAb. In resting T cells, the latency to the first [Ca\textsuperscript{2+}]\textsubscript{i} response was 67 ± 9 s (mean ± SEM of 6 cells) when cross-linked OKT3 was the stimulus and 63 ± 12 s when PHA was used. Fourier analysis yielded similar peaks corresponding to periods of 137 ± 18 s for OKT3-stimulated cells, and 164 ± 20 s for PHA-treated cells. The similarity in the latencies and periods suggests that both stimuli produce a rise in [Ca\textsuperscript{2+}], through the same biochemical pathway. For comparison, [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in Jurkat cells and an Ag-specific T cell clone P28D have been reported to exhibit periods of 92 ± 12 s and 116 ± 28 s, respectively (15, 27).

**Temperature dependence**

Additional experiments on resting T cells were performed at 37°C to evaluate the temperature dependence of the response latency and oscillation period. Raising the temperature decreased the latency after cross-linking CD3 with GAM antibody from 67 to 24.0 ± 5.1 s, but the mean period of the oscillations (121 ± 14 s, n = 6) and the percentage of oscillating cells were not significantly affected. Together these data suggest that the steps coupling receptor stimulation to the initial rise in [Ca\textsuperscript{2+}], are more temperature dependent than are the mechanisms that control the subsequent [Ca\textsuperscript{2+}]\textsubscript{i} oscillations.

**Calcium influx is reduced by depolarization of the membrane potential**

The results described above demonstrate a heterogeneous response pattern in individual resting and activated T cells, as well as in NK and T-LAK cells. Oscillations in [Ca\textsuperscript{2+}]\textsubscript{i} can occur in each of these cell types in response to appropriate stimuli, but the tendency to oscillate increases in activated cells. Because the initial large increase in [Ca\textsuperscript{2+}]\textsubscript{i} occurs in the absence of added external Ca\textsuperscript{2+}, release of Ca\textsuperscript{2+} from internal stores is thought to underlie the initial transient increase in [Ca\textsuperscript{2+}], in lymphocytes (9, 10). In Jurkat T cells and P28D cells examined with single-cell Ca\textsuperscript{2+} imaging, release from internal stores has been shown to produce the initial rise in [Ca\textsuperscript{2+}], but sustained responses and oscillations require Ca\textsuperscript{2+} entry from the outside (15, 16,
FIGURE 5. 

[Ca\textsuperscript{2+}] responses in human NK cells mediated through CD16. After a 10-min incubation with a CD16 mAb (before time 0), addition of GAM secondary antibody (arrow) caused [Ca\textsuperscript{2+}] increases in individual cells (A–E) and in the population average (F). In this experiment, 47% of the cells oscillated (C, D, and E).

To determine the relative contribution of Ca\textsuperscript{2+} from internal stores versus that obtained from influx in peripheral blood T cells, we removed external Ca\textsuperscript{2+} during the oscillations. As seen in Figure 7, A and B, for blast cells, changing the bath solution from one containing 2.0 mM Ca\textsuperscript{2+} to a solution containing 10 mM EGTA, with no added Ca\textsuperscript{2+}, caused the oscillations to cease immediately and the [Ca\textsuperscript{2+}] to decrease to below that seen at rest. Upon return to normal Ringer solution, the [Ca\textsuperscript{2+}] level showed an abrupt transient increase similar to the initial response seen in other cells (e.g., Fig. 1E). Because this concentration of EGTA empties thapsigargin-sensitive internal stores within 90 s in these cells (see below), the large increase in [Ca\textsuperscript{2+}], upon return to normal Ringer solution may reflect an increased plasma membrane permeability to Ca\textsuperscript{2+} due to depletion of intracellular pools (41–44). After the initial [Ca\textsuperscript{2+}] increase, the oscillations in some cells resumed after addition of normal (Ca\textsuperscript{2+}-containing) Ringer solution. These results show that influx of external Ca\textsuperscript{2+} sustains the oscillations, and suggest that the pathway by which Ca\textsuperscript{2+} enters the cells, perhaps a mitogen-gated Ca\textsuperscript{2+} channel similar to one described in Jurkat cells (15), remains activated after cross-linking of CD3. Similar results were obtained for both resting and activated T cells.

A negative membrane potential facilitates Ca\textsuperscript{2+} entry in many nonexcitable cells including mast cells (45) and Jurkat T lymphocytes (15). We therefore examined the role of membrane potential in the maintenance of the oscillations in normal T cells. Soon after exposure of the cells to 160 mM K\textsuperscript{+} Ringer solution, which should depolarize human T cells to 0 mV, the [Ca\textsuperscript{2+}] declined in each of the cell types investigated here, as illustrated for activated T cells in Figure 7, C and D. The overshoot in [Ca\textsuperscript{2+}] upon return to normal Ringer is similar to that seen after removal of external Ca\textsuperscript{2+} (Fig. 7, A and B). Comparable results were seen with resting T cells, NK and T-LAK cells (not shown). These results suggest that the normal membrane potential facilitates Ca\textsuperscript{2+} influx in human lymphocytes.
Thapsigargin induces a smooth rise in [Ca\textsuperscript{2+}], sustained by the membrane potential and inhibited by the K\textsuperscript{+}-channel blocker, charybdotoxin.

Thapsigargin, a specific blocker of the Ca\textsuperscript{2+} ATPase which pumps Ca\textsuperscript{2+} ions into intracellular stores, is a useful tool in the analysis of [Ca\textsuperscript{2+}], regulation (46, 47). In contrast to the highly variable [Ca\textsuperscript{2+}], responses obtained by stimulation through surface receptors, application of thapsigargin results in a smoothly graded, relatively uniform, and long lasting rise of [Ca\textsuperscript{2+}], in individual T cells (Fig. 8). The rise of [Ca\textsuperscript{2+}], after thapsigargin treatment, at an average rate of 2 to 3 nM/sec, suggests an ongoing "leak" and re-uptake of Ca\textsuperscript{2+} ions moving between intracellular stores and cytoplasm in resting cells. Rapid turnover of the intracellular stores is also indicated by the rapid (<90 s) depletion of the pools after incubating cells in Ringer solution containing EGTA with no added Ca\textsuperscript{2+} (data not shown). The slow decline of [Ca\textsuperscript{2+}], after treatment with thapsigargin may reflect either increased activity of the Ca\textsuperscript{2+} pump at the surface membrane or inactivation of Ca\textsuperscript{2+} influx. Figure 8 also shows that the rise in [Ca\textsuperscript{2+}], induced by thapsigargin is inhibited by membrane depolarization, in a manner similar to results shown in Figure 7 for T cells stimulated through the TCR complex.

Both voltage-gated and calcium-activated K\textsuperscript{+} channels may participate in the generation of [Ca\textsuperscript{2+}], signals (48, 49) by maintaining the membrane potential and thereby providing the electrical driving force for Ca\textsuperscript{2+} entry. Because the Ca\textsuperscript{2+} signals induced by receptor stimulation are highly variable from cell to cell and are spiky in individual cells, we have used the relatively consistent thapsigargin-induced [Ca\textsuperscript{2+}], signal to address the role of K\textsuperscript{+} channels in controlling Ca\textsuperscript{2+} influx. Charybdotoxin, a peptide scorpion toxin, blocks both voltage- and calcium-activated K\textsuperscript{+} channels in human T cells with an affinity in the 1 to 5 nM range (37, 50, 51). Figure 9 demonstrates that 100 nM charybdotoxin lowers [Ca\textsuperscript{2+}], following thapsigargin stimulation. These results are consistent with the idea that thapsigargin treatment, by emptying intracellular stores, results in calcium influx down an electrochemical gradient sustained, in part, by charybdotoxin-sensitive K\textsuperscript{+} channels.

**Discussion**

With video-imaging of [Ca\textsuperscript{2+}], we have characterized the responses of normal human resting and activated T lymphocytes, T-LAK cells, and NK cells to a variety of activation stimuli at the single-cell level. Our results indicate that human T lymphocytes display a temporally heterogeneous pattern of [Ca\textsuperscript{2+}], responses when stimulated with PHA or mAb. Individual cells within each cell type are capable of displaying oscillations in [Ca\textsuperscript{2+}],. We found that the proportion of I-LAK cells that display both increases in [Ca\textsuperscript{2+}], and [Ca\textsuperscript{2+}], oscillations when activated with crosslinked anti-CD3 was greater than for fresh cells. In the T cell preparations, we compared the [Ca\textsuperscript{2+}], responses to various stimuli and found that PHA, cross-linked anti-CD3, and heteroconjugate CD3/CD4 mAb each produced oscillations in at least 20% of the responding cells. Human NK cells also displayed [Ca\textsuperscript{2+}], increases and oscillations when triggered with PHA or specifically through CD16. These oscillations are dependent on the presence of external Ca\textsuperscript{2+} and are reduced by membrane depolarization. Because [Ca\textsuperscript{2+}], oscillations can occur in all of the subsets we examined in response to stimuli designed to mimic the normal parameters of in vivo activation, and the expression of the oscillations increases with the activation state of the cells, the oscillations in [Ca\textsuperscript{2+}], may well play an important role in early activation events in T cells and NK cells.

Our results on human T cells are generally consistent with previous investigations with cell lines. Similar response amplitudes of [Ca\textsuperscript{2+}], into the low micromolar
**FIGURE 7.** [Ca\(^{2+}\)]\(_i\) oscillations are dependent upon a membrane potential-sensitive Ca\(^{2+}\) influx. Oscillations triggered by cross-linking CD3 with GAM antibody (arrows) terminated abruptly upon changing the external medium to one with no added Ca\(^{2+}\) and 10 mM EGTA (arrow). [Ca\(^{2+}\)]\(_i\), rapidly increased upon return to normal Ringer solution (arrow) in the example cell (A) and in the average (B). (C and D) When the Ringer solution was changed to one containing 160 mM K\(^+\) (arrow), the [Ca\(^{2+}\)]\(_i\) of individual cells (C) and the average (D) quickly decreased. After return to normal Ringer solution, the oscillations resumed in some cells, and an overshoot in [Ca\(^{2+}\)]\(_i\), was seen in the cell and the average response.

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range and oscillation periods of 90 to 160 s are observed in fresh T cells stimulated with PHA or cross-linking antibodies (Figs. 1 and 3), Jurkat cells stimulated with PHA (15), and a T cell clone in response to contact with APC (27). Our results on the effects of temperature are different from those of Donnadieu et al. (27), who reported a substantial temperature dependence of the periodicity of APC-induced oscillations in P28D cells. In addition, response latencies in human T cells are 20 to 40% of those reported for P28D cells in response to soluble antibody stimulation (27). It is unclear whether these differences reflect the properties of the cell line or the mode of stimulation.

**Mechanism of [Ca\(^{2+}\)]\(_i\) oscillations.** Cross-linking of surface receptors associated with the \(\xi\)-chain, either the CD3/TCR complex or CD16, results in [Ca\(^{2+}\)]\(_i\) signals that are often oscillatory in lymphoid cell lines. Recent evidence using chimeric proteins that link the intracellular domain of the \(\xi\)-chain of CD3 to the extracellular and transmembrane regions of CD8 suggests that the \(\xi\)-chain functionally couples the CD3/TCR complex in Jurkat T cells (33). Antibodies which cross-link the CD3/TCR complex or which bring together CD4 and CD3 were especially effective stimuli in eliciting [Ca\(^{2+}\)]\(_i\) responses and oscillations (Figs. 3 and 4). Since CD16 is associated with the \(\xi\)-chain in CD3-NK cells (35), we investigated whether stimulation through CD16 could cause [Ca\(^{2+}\)]\(_i\) oscillations. We found that cross-linking CD16 on both NK and T-LAK cells elicited [Ca\(^{2+}\)]\(_i\) transients and oscillations (Fig. 5). These findings suggest that CD16, possibly through the \(\xi\)-chain, interacts with intracellular enzymes necessary for [Ca\(^{2+}\)]\(_i\) signaling in these cell types.

The pattern of [Ca\(^{2+}\)]\(_i\) signaling stimulated through surface receptors is very different from that induced by thapsigargin, an inhibitor of the intracellular Ca-ATPase (Fig. 8). Thapsigargin treatment results in a relatively smooth and uniform rise in [Ca\(^{2+}\)]\(_i\), compared with the fluctuating and highly variable [Ca\(^{2+}\)]\(_i\) signals when monoclonal antibodies or lectins are used as ligands. Stimulation via surface receptors appears to be necessary for eliciting oscillatory [Ca\(^{2+}\)]\(_i\) responses.

The oscillations observed in human lymphocytes typically followed an abrupt increase in [Ca\(^{2+}\)]\(_i\), due to release of Ca\(^{2+}\) from internal stores, and were dependent upon both influx of Ca\(^{2+}\) from the external medium and the maintenance of a negative membrane potential. Because Ca\(^{2+}\) influx in T lymphocytes is inhibited by depolarization (Fig. 7), it has been proposed that potassium channels, by maintaining a negative membrane potential, play a crucial role in T cell activation (reviewed in Refs. 48 and 49). Several lines of evidence indicate that voltage-gated type \(n\) channels control the resting membrane potential of T cells (reviewed in Refs. 48 and 49). After activation, the subsequent rise in [Ca\(^{2+}\)]\(_i\) would open Ca\(^{2+}\)-dependent K\(^+\) channels, hyperpolarize the membrane potential, and potentiate Ca\(^{2+}\) entry (52, 53). Recent patch-clamp experiments have demonstrated a dramatic increase in the expression of Ca\(^{2+}\)-dependent K\(^+\) channels in PHA-activated blast cells relative to resting cells (37). The additional hyperpolarizing outward current provided by these channels may provide an element of positive feedback to augment Ca\(^{2+}\) influx. K-channel blockers, including charybdotoxin, have been reported to inhibit T cell activation (reviewed in Refs. 48 and 49) (51, 54) (but see Refs. 55 and 56). Several of these blockers, including charybdotoxin, inhibit both voltage-gated and calcium-activated K\(^+\) channels (37, 50, 51). Ca\(^{2+}\) influx stimulated by thapsigargin is similarly inhib-
FIGURE 8. Thapsigargin-induced rise in [Ca$^{2+}$]. The top five traces show the response of individual cells; the average response is shown in the bottom trace. Addition of 1 μM thapsigargin produced a prolonged elevation in [Ca$^{2+}$], in a majority of the cells. Addition of 40 mM K$^+$-containing Ringer solution (arrow) caused a transient decrease in [Ca$^{2+}$], which was reversed by restoring the bath solution to normal Ringer solution.

FIGURE 9. Inhibition of the thapsigargin-induced [Ca$^{2+}$], signal by charybdotoxin (100 nM) added at the indicated times. The lower trace shows the average response. A further decrease in [Ca$^{2+}$], was observed upon raising [K$^+$], to 160 mM and was reversed by changing the solution to Ringer solution.

The lack of correlation between the latency to the first [Ca$^{2+}$], increase and the period of the subsequent oscillations in T cells suggests that different biochemical pathways regulate these events (Fig. 6) (see also Refs. 15 and 57). Lewis and Cahalan (15) concluded that a mitogen-gated Ca$^{2+}$ conductance, which waxed and waned preceding [Ca$^{2+}$], changes in Jurkat T cells, induces the oscillations and that [Ca$^{2+}$], regulates both the Ca$^{2+}$ influx pathway and the oscillations. Some of the possible feedback loops include the modulation by [Ca$^{2+}$], of the Ca$^{2+}$ influx pathway (15) or the ability of IP$_3$ to release internal stores (58), the existence of multiple internal stores of Ca$^{2+}$ with different [Ca$^{2+}$] sensitivities (58), or Ca$^{2+}$ stimulation of PLC activity (21, 59).

Are [Ca$^{2+}$], oscillations physiologically important? Our results have demonstrated the ubiquity of [Ca$^{2+}$], oscillations in lymphoid cells in response to a variety of in vitro stimuli. Similar oscillations in [Ca$^{2+}$], have been observed in T cell clones that are dependent on contact with APC (27) (S. D. Hess, D. Choquet, and M. D. Cahalan, manuscript in preparation). These data suggest that the oscillations observed in human T cells may occur during antigen presentation in vivo.

Despite speculations about the physiological role of [Ca$^{2+}$], oscillations in a variety of cell types, few correlations between the amplitude or frequency of the oscillations and [Ca$^{2+}$],-dependent events have been demonstrated (22, 28, 60). It is possible that signaling using
[Ca$$^2+$$]i oscillations may represent an energy savings compared to using changes in the basal level of [Ca$$^2+$$]i (28) and may increase the fidelity of the signal (20). In our experiments, the rise in [Ca$$^2+$$]i appeared to be uniform throughout the cell, including the nucleus. Using the single-cell approach it should be possible to correlate functional consequences of different types of [Ca$$^2+$$]i signals on IL-2 secretion or early gene activation events in T cells, using a reporter gene (61). Our characterization of [Ca$$^2+$$]i signals in individual human lymphocytes provides an important framework for future attempts to correlate these events with single-cell functional assays of [Ca$$^2+$$]i-dependent events in the activation cascade.

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