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Physiology

Intracellular calcium dependence of gene expression in single T lymphocytes

(thapsigargin/calcium signaling/T-cell activation/*lacZ*/interleukin 2)

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ABSTRACT **In T lymphocytes, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rises within seconds of T-cell antigen-receptor stimulation and initiates the synthesis and secretion of interleukin 2, a cytokine essential for T-cell proliferation and the immune response. Using video-imaging techniques, we tracked $[Ca^{2+}]_i$ signals in individual T cells and measured subsequent expression of a β-galactosidase reporter gene (*lacZ*) controlled by the NF-AT element of the interleukin 2 enhancer. $[Ca^{2+}]_i$ spikes elicited by monoclonal antibody binding to the CD3ε subunit of the T-cell receptor were positively correlated with gene expression, but varied widely between individual cells and were therefore difficult to relate quantitatively to *lacZ* expression. The $[Ca^{2+}]_i$ dependence of NF-AT-regulated gene expression was determined by elevating $[Ca^{2+}]_i$ with either thapsigargin or ionomycin and then "clamping" $[Ca^{2+}]_i$ to various, stable levels by altering either extracellular $[Ca^{2+}]$ or extracellular $[K^+]$. Raising $[Ca^{2+}]_i$ from resting levels of 70 nM to between 200 nM and 1.6 μM increased the fraction of cells expressing *lacZ*, with $K_d \approx 1$ μM. Activation of protein kinase C enhanced the $[Ca^{2+}]_i$ sensitivity of gene expression ($K_d = 210$ nM), whereas stimulation of protein kinase A inhibited $[Ca^{2+}]_i$-dependent gene expression. The experiments described here provide single-cell measurements linking a second messenger to gene expression in individual cells.**

Video-imaging experiments have demonstrated heterogeneous patterns of T-cell intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) responses to mitogenic lectins, antibodies to the T-cell antigen receptor (TCR), or antigen presentation. Individual cells can exhibit transient, sustained, or oscillatory $[Ca^{2+}]_i$ signals, as well as intracellular Ca^{2+} gradients (1–6). The $[Ca^{2+}]_i$ increase is essential for T-cell activation, a process involving gene expression, differentiation, and proliferation (7). Of particular interest is the $[Ca^{2+}]_i$-dependent production of interleukin 2 (IL-2), a cytokine required for the immune response (7–9). Thus far it has not been possible to establish a quantitative link between the $[Ca^{2+}]_i$ signal and gene expression because of the variability of $[Ca^{2+}]_i$ responses in individual cells and the inability to correlate those responses with gene expression at the single-cell level.

We approached this problem by coupling $[Ca^{2+}]_i$-imaging techniques with the *lacZ* assay, a method for measuring gene expression in individual cells (10, 11). The *lacZ* assay uses cells expressing the bacterial β-galactosidase reporter gene (*lacZ*) linked to a specific promoter element. In our study, *lacZ* was under transcriptional control of NF-AT (nuclear factor of activated T cells), the key response element regulating the IL-2 gene (12). The NF-AT-responsive *lacZ* construct mimics many aspects of IL-2 transcriptional regulation, including time course of activation, synergistic control by Ca^{2+} and protein kinase C (PKC) (10–12), and inhibition by immunosuppressive drugs (13). We used a T-cell hybridoma (B3Z) transfected with the NF-AT-responsive *lacZ* construct (14) and loaded with the Ca^{2+} indicator fura-2. $[Ca^{2+}]_i$ signals were induced by settling cells onto coverslips coated with antibodies to the CD3ε subunit of the TCR (immobilized anti-CD3). Although more robust $[Ca^{2+}]_i$ signals were positively correlated with gene expression, the variability of the ligand-induced $[Ca^{2+}]_i$ signals prevented quantitation of the $[Ca^{2+}]_i$ sensitivity of gene expression. Therefore, we developed a $[Ca^{2+}]_i$-clamp protocol using the microsomal Ca^{2+}-ATPase inhibitor thapsigargin (TG). TG depletes intracellular Ca^{2+} stores, thereby triggering Ca^{2+} influx and a sustained $[Ca^{2+}]_i$ elevation in T cells (15–20), without activating other signal transduction pathways (15, 17, 21, 22). Unlike the response to anti-CD3, the $[Ca^{2+}]_i$ response to TG was consistent between cells, allowing correlation of $[Ca^{2+}]_i$ with *lacZ* expression in T-cell populations. Furthermore, $[Ca^{2+}]_i$ in TG-treated cells could be manipulated by altering the driving forces for Ca^{2+} influx, permitting measurement of gene expression at various $[Ca^{2+}]_i$ values.

METHODS

Cell Culture. $CD8^+$, IL-2-producing murine T-cell hybridoma (B3Z) cells carrying the NF-AT-responsive *lacZ* construct NFATZ (14) were grown in RPMI 1640 containing 10% fetal bovine serum (RPMI/FBS), 10 mM Hepes, 2 mM glutamine, 1 mM pyruvate, 50 μM 2-mercaptoethanol, and 1% (vol/vol) penicillin/streptomycin stock. Cells were cultured in 25-ml flasks (Costar) at 37°C in a 5% CO_2/95% air atmosphere in a humidified incubator. Chemicals were obtained from Sigma unless otherwise noted. Cyclosporin A (SandImmune) was a gift of Lewis M. Slater (University of California, Irvine).

$[Ca^{2+}]_i$ Measurements. Logarithmic-phase B3Z cells were loaded in suspension with 1 μM fura-2 acetoxymethyl ester (AM) (Molecular Probes) for 20 min at 37°C in RPMI/FBS. Cells were then washed with RPMI/FBS and settled onto coverslips mounted on a heated (37°C) microscope stage. For experiments lasting >1 hr, cells were continuously incubated with 300 nM fura-2 AM. Eight-bit intensity images at 350 and 380 nm were captured and analyzed with a video-image processor and software (Videoprobe, ETM Systems, Irvine, CA) as described (1). Calibration was performed by measuring fluorescence intensities at 0 and saturating $[Ca^{2+}]_i$ in cells and applying the equation of Grynkiewicz *et al.* (23). Fura-2 had no effect on *lacZ* expression.

Cell Activation. For TCR stimulation on the microscope, sterile coverslips were coated with anti-CD3ε (10 μg/ml; PharMingen) overnight and rinsed briefly with phosphate-buffered saline before use. For TG stimulation, cells were first

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Abbreviations: $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}; $[K^+]_o$ and $[Ca^{2+}]_o$, extracellular concentrations of K^+ and Ca^{2+}; TCR, T-cell antigen receptor; TG, thapsigargin; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin 2; PKA, protein kinase A; PKC, protein kinase C; AM, acetoxymethyl ester.

settled onto poly(D-lysine)-coated coverslips and then stimulated with 1 μM TG (LC Services, Woburn, MA) in RPMI/FBS. In flow cytometry experiments (FACScan, Becton Dickinson), 5×10^4 cells were placed in 24-well plates, and 1 μM TG was added. After 10 min, either EGTA or K^+ (exchanged for Na^+ by mixing with K^+-containing medium) was added. Unless otherwise noted, cells were activated for a total of 4 hr before loading with the fluorogenic substrate fluorescein di-β-galactopyranoside (FDG, Molecular Probes). There was no difference in the percentage of *lacZ*$^+$ cells for stimulation on plastic wells vs. coverslips.

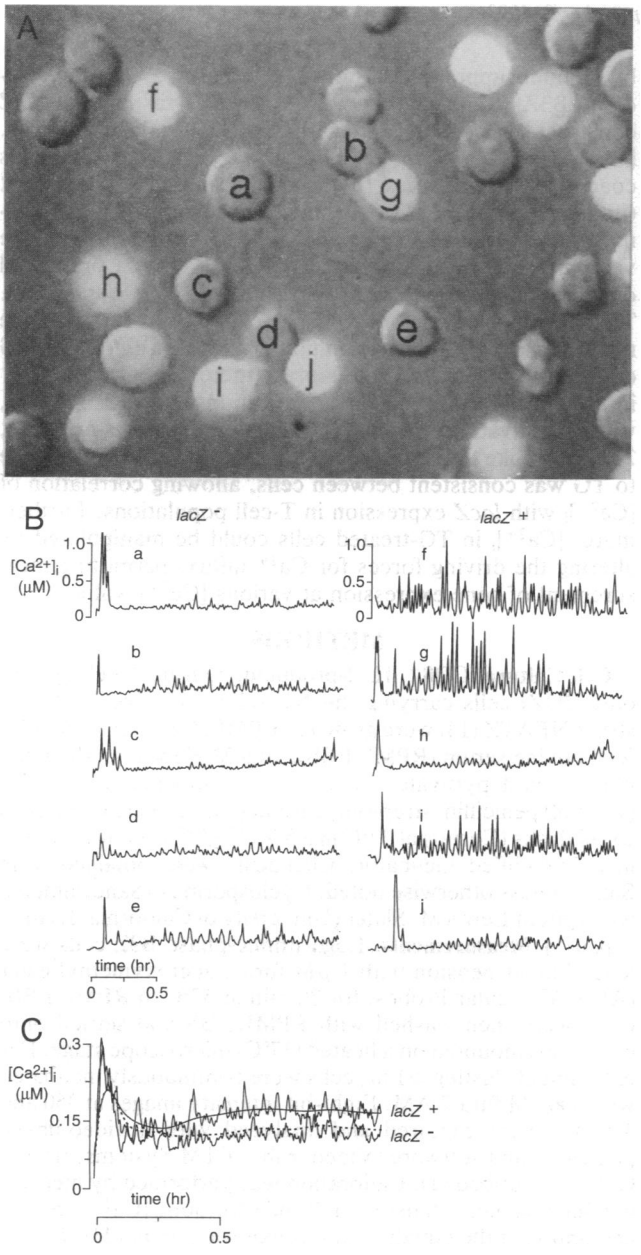

FIG. 1. $[Ca^{2+}]_i$ signals and *lacZ* expression in B3Z cells activated by settling cells onto anti-CD3-coated coverslips. (*A*) Simultaneous differential interference contrast and fluorescence image of a field of cells after 3.5 hr of activation and subsequent hypotonic loading of β-galactosidase substrate, showing *lacZ*$^-$ (a–e) and *lacZ*$^+$ cells (f–j). (*B*) $[Ca^{2+}]_i$ responses of the same 10 cells showing variability in signaling patterns between individual cells. (*C*) Average $[Ca^{2+}]_i$ responses of *lacZ*$^+$ and *lacZ*$^-$ cells from five experiments, showing that $[Ca^{2+}]_i$ was maintained at higher levels in the *lacZ*$^+$ population. Jagged lines indicate the averages based on 10-sec intervals. Smoother lines represent averages taken over 300-sec intervals.

β-Galactosidase Assay. After activation on the microscope stage, cells were perfused with 0.5 mM FDG in 35% tonicity for 3 min at 37°C and then returned to 80% tonicity at room temperature. Cells remained adherent throughout the loading procedure. Since the loading protocol terminates cell activation, the assay provides a "snapshot" of *lacZ* gene expression rather than a continuous measurement. Loading occurred in ≈95% of the cells, as determined in control experiments with 1 mM Lucifer yellow, a dye similar in molecular weight to FDG. Hypotonic loading did not damage cells (>97% viability by trypan-blue exclusion). FDG-loaded cells were incubated at room temperature for 20 min. Cells were then illuminated at 490 nm, and green fluorescence was assayed. Cells were judged *lacZ*$^+$ if fluorescence was at least 3 times the average autofluorescence. For FACScan assays, cells were resuspended, spun down, and loaded by osmotic shock with FDG (10, 11). The fluorescence of *lacZ*$^+$ cells was at least 5 times the autofluorescence. Quantitation of *lacZ* expression with either video microscopy (see Figs. 1*A* and 2*A*) or FACScan (see Fig. 4) showed two populations of cells: (*i*) extremely bright *lacZ*$^+$ cells and (*ii*) nonresponders. The bimodal distribution supports previous evidence that NF-AT-regulated *lacZ* expression is all-or-none (10, 11).

RESULTS

Tracking Anti-CD3-Induced $[Ca^{2+}]_i$ Signals and *lacZ* Expression in Single Cells. Settling cells onto immobilized anti-CD3 stimulated *lacZ* expression in ≈70% of B3Z cells, whereas adding soluble anti-CD3 did not induce *lacZ* expression. Parallel $[Ca^{2+}]_i$-imaging experiments showed that immobilized anti-CD3 caused a prolonged period of $[Ca^{2+}]_i$ signaling characterized by irregular spikes, each lasting ≈1 min (Fig. 1*B*; Table 1), whereas soluble anti-CD3 produced only a single, relatively short $[Ca^{2+}]_i$ transient (data not shown). Because this difference suggested that the intensity of the $[Ca^{2+}]_i$ signal might control *lacZ*-mediated gene expression, we assayed both $[Ca^{2+}]_i$ responses and *lacZ* expression in fura-2-loaded cells. Fig. 1*A* shows a representative field with both *lacZ*$^-$ (a–e) and *lacZ*$^+$ (f–j) cells. The $[Ca^{2+}]_i$ responses of the same cells are shown in Fig. 1*B*. Thirty to 90 sec after contact with immobilized anti-CD3, ≥80% of B3Z cells responded with a large initial $[Ca^{2+}]_i$ transient. This response was similar in both *lacZ*$^+$ and *lacZ*$^-$ cells (Fig. 1 *B* and *C*) and was usually followed by a prolonged period of $[Ca^{2+}]_i$ spikes which were particularly vigorous during the first 90 min and often continued for >2 hr. Both the amplitude and the frequency of the $[Ca^{2+}]_i$ responses of individual cells varied (Fig. 1*B*); it was often difficult to predict whether a given cell would be *lacZ*$^+$ based on the $[Ca^{2+}]_i$ response. However, analysis of the average $[Ca^{2+}]_i$ response of all cells (Fig. 1*C*; Table 1) showed that larger and more frequent $[Ca^{2+}]_i$ spikes were characteristics of *lacZ*$^+$

Table 1. Properties of CD3-induced $[Ca^{2+}]_i$ signals in *lacZ*$^-$ and *lacZ*$^+$ B3Z cells

Group	Mean $[Ca^{2+}]_i$, nM	Spike amplitude, nM	Spikes per hr, no.	Spike duration, sec
lacZ$^-$	128 ± 13	168 ± 12	5.9 ± 2.1	73 ± 5
lacZ$^+$	166 ± 18	208 ± 28	16.1 ± 3.0	64 ± 4

Values were determined by pooling results from *lacZ*$^+$ ($n = 63$) and *lacZ*$^-$ ($n = 48$) cells from five separate experiments. Mean $[Ca^{2+}]_i$ was determined by averaging the amplitude of all the points (taken at 10-sec intervals) during the first 90 min following CD3 stimulation. *lacZ*$^+$ cells displayed significantly higher average $[Ca^{2+}]_i$ ($P < 0.01$, two-tailed *t* test), spike amplitude ($P < 0.04$), and no. of spikes per hr ($P < 0.0006$). The average duration was calculated by fitting the shape of each spike to a Gaussian curve and calculating the duration of the central 66% of the curve. The spikes in *lacZ*$^-$ cells were significantly longer than those of *lacZ*$^+$ cells ($P < 0.009$).

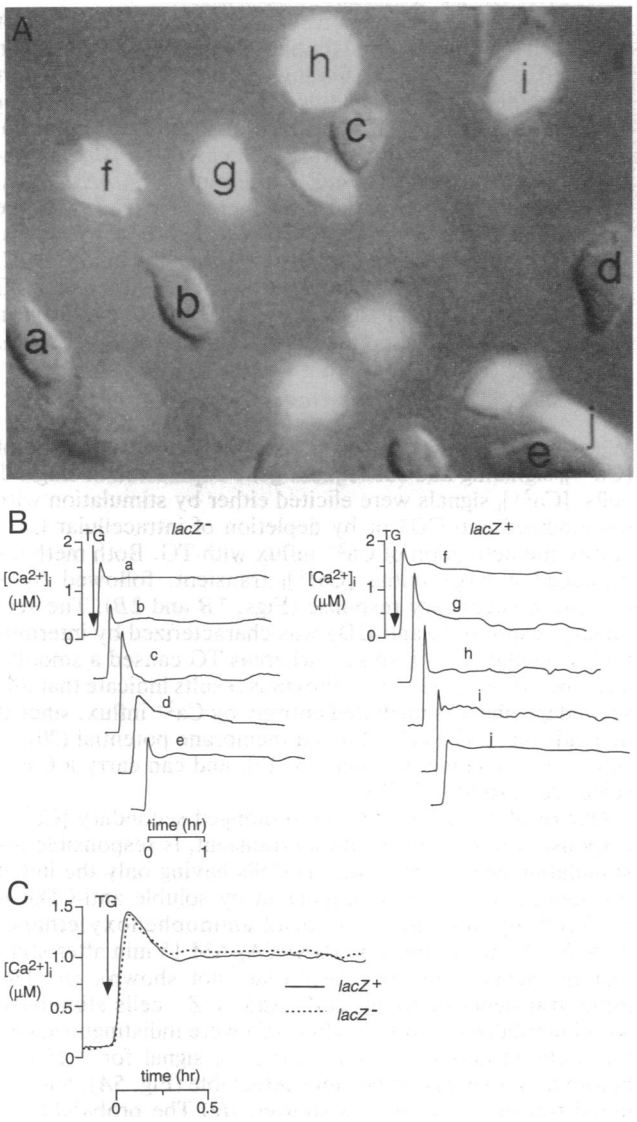

FIG. 2. $[Ca^{2+}]_i$ signals and *lacZ* expression in B3Z cells stimulated with 1 μM TG. (*A*) Image of cells 4 hr after activation and subsequent loading of β-galactosidase substrate. Cells a–e were $lacZ^-$, whereas cells f–j were $lacZ^+$. (*B*) $[Ca^{2+}]_i$ response of the same cells (a–j) to TG, illustrating the uniformity of responses between cells. (*C*) Average of $lacZ^+$ and $lacZ^-$ cells from four experiments shows that $[Ca^{2+}]_i$ was the same in both responsive and nonresponsive cells. Averaging and smoothing are as in Fig. 1*C*.

cells. The average $[Ca^{2+}]_i$ in $lacZ^+$ cells (166 ± 18 nM) was significantly higher than in $lacZ^-$ cells (128 ± 13 nM). Most of this difference was due to a secondary rise in average $[Ca^{2+}]_i$ which occurred about 20 min after CD3 stimulation and persisted for the first hour (Fig. 1*C*).

$[Ca^{2+}]_i$ Clamp of T-Cell Populations with TG. TG stimulated *lacZ* expression in ≈60% of B3Z cells (Fig. 2*A*; see also Fig. 4*B*). The TG-stimulated $[Ca^{2+}]_i$ increase (Fig. 2*B* and Fig. 3 *A* and *C*) was consistent both in the percentage of responding cells (≥95%, 34 experiments) and in the level of $[Ca^{2+}]_i$ attained between cells (1.1 ± 0.18 μM, 718 cells). In contrast to the experiments with anti-CD3, variability in $[Ca^{2+}]_i$ cannot explain the difference between $lacZ^+$ and $lacZ^-$ cells (Fig. 2 *B* and *C*). Thus, the response of individual cells to a given $[Ca^{2+}]_i$ is heterogeneous. Also, a sustained $[Ca^{2+}]_i$ signal is sufficient to stimulate gene expression, because TG does not activate other biochemical events triggered by anti-CD3 (17).

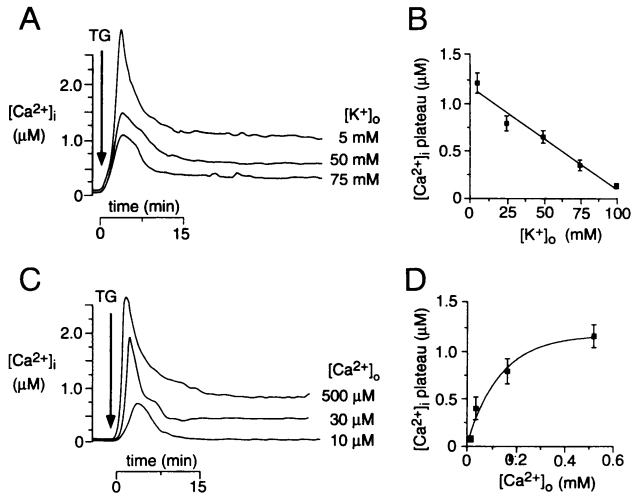

FIG. 3. Clamping of $[Ca^{2+}]_i$ in TG-treated cells. Cells were pretreated for 3 min with various concentrations of either external K^+ (*A*) or EGTA (*C*) prior to TG addition. Distinct, stable $[Ca^{2+}]_i$ levels were obtained at different concentrations (each trace is the average of 12 cells in a single experiment). The average (five separate experiments) of the sustained $[Ca^{2+}]_i$ plateaus is shown for various external K^+ (*B*) and EGTA (*D*) concentrations.

The quantitative relation between $[Ca^{2+}]_i$ and gene expression was determined by altering $[Ca^{2+}]_i$ following TG stimulation. The sustained $[Ca^{2+}]_i$ plateau can be reduced in discrete steps by decreasing either the electrical gradient (elevated extracellular K^+ concentration, $[K^+]_o$) or the concentration gradient (lowered $[Ca^{2+}]_o$) driving Ca^{2+} influx (Fig. 3 *A* and *C*). The $[Ca^{2+}]_i$ plateau showed an inverse linear dependence on $[K^+]_o$ and was nearly abolished at 100 mM $[K^+]_o$ (Fig. 2*B*); it approached saturation at 500 μM $[Ca^{2+}]_o$ and was eliminated below 10 μM $[Ca^{2+}]_o$. Using the $[Ca^{2+}]_i$-clamp protocol, we assayed *lacZ* expression in single cells by flow cytometry in parallel with video-imaging $[Ca^{2+}]_i$ measurements (Fig. 4). Decreases in $[Ca^{2+}]_i$, obtained by either raising $[K^+]_o$ or lowering $[Ca^{2+}]_o$, corresponded with similar decreases in the percentage of $lacZ^+$ cells, confirming that *lacZ* expression was sensitive to the $[Ca^{2+}]_i$ plateau. The histograms resemble a pattern observed previously: a peak of $lacZ^-$ cells and a second peak of uniformly $lacZ^+$ cells (qualitatively similar to Figs. 1*B* and 2*B*). This pattern suggests that changes in $[Ca^{2+}]_i$ affect the percentage of responding cells. The effects of sustained $[Ca^{2+}]_i$ elevation on gene expression are summarized in Fig. 5*B*. The percentage

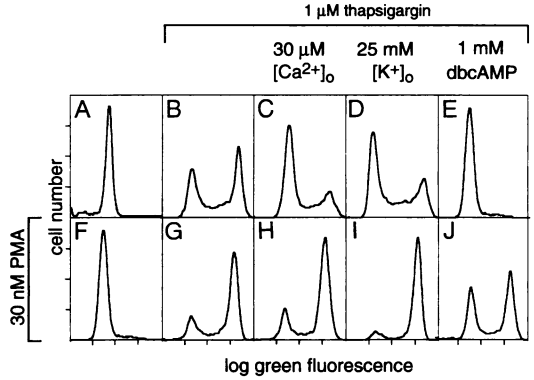

FIG. 4. *lacZ* expression in populations of $[Ca^{2+}]_i$-clamped B3Z cells. Flow cytometric analysis of TG-stimulated gene expression in B3Z cells in either the absence (*A–E*) or presence (*F–J*) of 30 nM phorbol 12-myristate 13-acetate (PMA). Each trace represents 10^4 cells and is typical of at least three experiments. dbcAMP, dibutyryl cAMP.

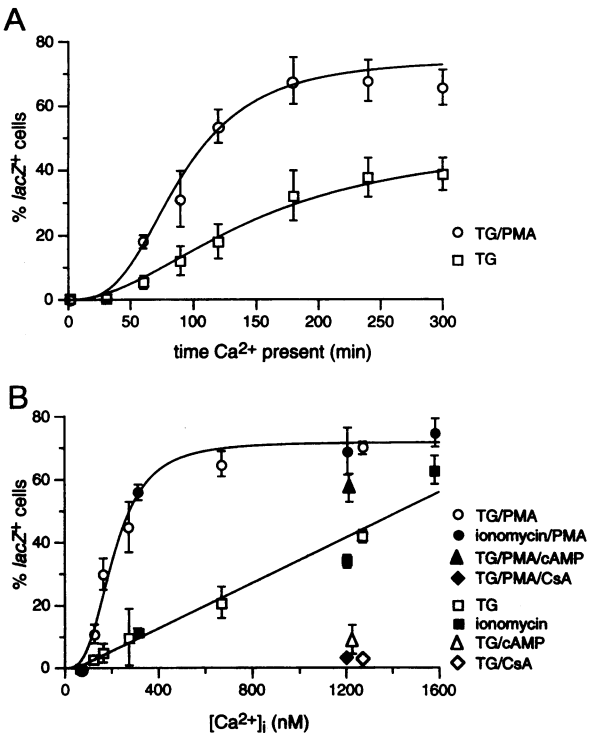

FIG. 5. Modulation of *lacZ* expression by $[Ca^{2+}]_i$, PKA (protein kinase A), PKC, and calcineurin. (*A*) Time course of *lacZ* expression measured as described for Fig. 2. Cells were activated with either TG (1 μM) or TG (1 μM)/PMA (30 ng/ml) in RPMI 1640 (containing 0.52 mM Ca^{2+}). At various times, 0.8 mM EGTA was added to reduce $[Ca^{2+}]_o$ and arrest *lacZ* expression. Cells were then assayed after 4 hr of stimulation. Data were fitted by eye to the equation $\%\ lacZ^+ = A[1 - \exp(-t/\tau)]^n \times 100$, where A is the maximal value, t is time, τ is the time constant, and n describes the number of binding sites and accounts for the initial delay. For the curves shown, $A = 75$ and 50, $\tau = 150$ and 90, and $n = 2$ and 3 for cells without and with PMA, respectively. Each point is the average of at least three experiments and shows the mean ± SD. (*B*) $[Ca^{2+}]_i$ dependence of *lacZ* expression. TG data points were obtained by changing either the EGTA or K^+ concentration. In some experiments, 0.3, 1.0, or 5 μM ionomycin was used to raise $[Ca^{2+}]_i$ to approximately the levels elicited by TG. For either ionomycin or TG, the percentage of $lacZ^+$ cells was similar for a given change in $[Ca^{2+}]_i$. Also included are cells treated with 30 nM PMA, 1 mM dibutyryl cAMP, or 60 nM cyclosporin A (CsA). Data obtained in the absence of PMA were fitted to a straight line crossing the abscissa at resting $[Ca^{2+}]_i$. PMA data were fitted by eye to a modified Hill equation: $\%\ lacZ^+ = A/\{1+(K_d/[Ca^{2+}])^n\} \times 100$, where A represents the maximal value and n represents the number of binding sites. For the curve shown, $A = 73$, $n = 3$, and $K_d = 210$ nM. This function suggests positive cooperativity of Ca^{2+} ions bound to at least three sites.

of $lacZ^+$ cells increases with a shallow slope and a $K_d \approx 1$ μM. At a resting $[Ca^{2+}]_i$ of 70 nM, $[Ca^{2+}]_i$ must rise 15-fold in order to activate half of the responsive cells.

PKC, PKA, and Calcineurin Modulate the $[Ca^{2+}]_i$ Dependence of Gene Expression. Stimulating PKC with a phorbol ester, or PKA with a cell-permeant derivative of cAMP, can potentiate or inhibit T-cell responses, respectively (24, 25). Neither PMA (30 ng/ml) nor dibutyryl cAMP (1 mM) affected the TG-stimulated $[Ca^{2+}]_i$ increase (data not shown; see refs. 16 and 20), but they influenced the $[Ca^{2+}]_i$ threshold for gene expression (Fig. 4 *E–J*). PMA alone was not stimulatory, but cotreatment with PMA and TG increased the percentage of $lacZ^+$ cells to ≈80%, the highest fraction observed (Fig. 4 *F* and *G*). PMA treatment reduced the K_d by a factor of 5 (to ≈210 nM) compared with TG and steepened the $lacZ^+$–$[Ca^{2+}]_i$ relationship such that a 3-fold increase in $[Ca^{2+}]_i$ activated half of the responsive cells. In addition, PMA decreased the time required for half-maximal *lacZ* expression from 150 to 90 min. These findings support a previous report that sustained $[Ca^{2+}]_i$ signaling for ≈2 hr was required for maximal stimulation of IL-2 production in Jurkat T cells (26). Treatment with dibutyryl cAMP decreased the $[Ca^{2+}]_i$ sensitivity of TG-dependent *lacZ* expression (Fig. 4*E*). Thus, cAMP inhibits at a step distal to the increase in $[Ca^{2+}]_i$. In the presence of PMA, dibutyryl cAMP was less effective (Figs. 4*J* and 5*B*), suggesting a complex relationship among PKC, PKA, and $[Ca^{2+}]_i$. Gene expression stimulated by either TG or TG/PMA was inhibited by coincubation with cyclosporin A (EC_{50} = 18 nM), implying that calcineurin was essential.

DISCUSSION

We used video-imaging techniques to evaluate patterns of $[Ca^{2+}]_i$ signaling and subsequent gene expression in single T cells. $[Ca^{2+}]_i$ signals were elicited either by stimulation with immobilized anti-CD3 or by depletion of intracellular Ca^{2+} stores and activation of Ca^{2+} influx with TG. Both methods produced a large initial $[Ca^{2+}]_i$ transient, followed by a prolonged secondary response (Figs. 1*B* and 2*B*). The secondary response to anti-CD3 was characterized by intermittent, irregular $[Ca^{2+}]_i$ spikes, whereas TG caused a smooth, sustained $[Ca^{2+}]_i$ elevation. Previous results indicate that this secondary phase is mediated entirely by Ca^{2+} influx, since it depends on $[Ca^{2+}]_o$ (17–20) and membrane potential (20), is sensitive to channel blockers (3, 19), and can carry a Ca^{2+}-selective current (27, 28).

Our results indicate that the prolonged secondary $[Ca^{2+}]_i$ response, rather than the initial transient, is responsible for stimulating gene expression. (*i*) Cells having only the initial transient, such as those stimulated by soluble anti-CD3 or buffered by loading with bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetracetic acid (BAPTA) AM 15 min after stimulation, never expressed *lacZ* (data not shown). (*ii*) The initial transients between $lacZ^+$ and $lacZ^-$ cells stimulated with immobilized anti-CD3 (Fig. 1*C*) were indistinguishable. (*iii*) Cells required a sustained $[Ca^{2+}]_i$ signal for >25 min before *lacZ* expression became detectable (Fig. 5*A*), and the initial transient was always shorter. (*iv*) The probability of gene expression was the same whether EGTA or K^+ was added before TG (to blunt the initial transient as in Fig. 3 *A* and *C*) or 10 min after TG addition (data not shown).

Thus, we focused on the relation between the secondary phase of the $[Ca^{2+}]_i$ signal and gene expression. The single-cell approach was the only way to evaluate the link between the complex and heterogeneous $[Ca^{2+}]_i$ signals generated by CD3 engagement and gene expression. We found a positive correlation between the level of the anti-CD3-induced $[Ca^{2+}]_i$ response and the percentage of $lacZ^+$ cells (Fig. 1; Table 1). Cells with higher amplitudes and/or frequency of spikes were more likely to express *lacZ*. This suggests that T cells can integrate the intensity of these irregular signals. It remains to be seen whether selective modulation of either the amplitude or the frequency of spikes can produce predictable changes in gene expression. Despite the correlations summarized in Table 1, it is clear from the experiments with TG that $[Ca^{2+}]_i$ spikes or oscillations *per se* are not required for stimulation of gene expression. Also, since gene expression can result from a diverse range of $[Ca^{2+}]_i$ patterns (e.g., compare Fig. 1 *B* cells g and j), it appears that the $[Ca^{2+}]_i$ requirements leading to gene expression vary between cells.

Although we could neither manipulate nor synchronize the $[Ca^{2+}]_i$ signals of individual cells during CD3 stimulation, with TG treatment we showed that gene expression varied considerably in cells with nearly identical $[Ca^{2+}]_i$ signals (Figs. 2 and 3). This variation was bimodal: cells were either $lacZ^-$ or $lacZ^+$. Earlier results (11) indicate $lacZ^+$ cells have

a uniform number of β-galactosidase molecules; therefore the threshold is not an artifact of the assay (although it may be related to the nature of the NFATZ construct; see ref. 10). Therefore, we propose that, at a given $[Ca^{2+}]_i$, a certain fraction of cells will be activated. Since the percentage of *lacZ*$^+$ cells increased as $[Ca^{2+}]_i$ rose from 200 nM to 1.6 μM, the $[Ca^{2+}]_i$ requirement for activation lies within this range.

Kinases and the phosphatase calcineurin strongly modulate the $[Ca^{2+}]_i$ required for gene expression. Coactivation of PKC decreased the $[Ca^{2+}]_i$ threshold for activation into the range observed during CD3 stimulation (compare Table 1 and Fig. 5*B*) or antigen presentation (6). In contrast, dibutyryl cAMP potently inhibited TG-stimulated *lacZ* expression, although this action was overcome by costimulation with PMA. cAMP also decreases Ca^{2+} influx stimulated by mitogenic lectins (29) and $[Ca^{2+}]_i$ signals induced by anti-CD3 (ref. 30; P.A.N. and M.D.C., unpublished work). Since cAMP inhibits T-cell activation at both pre- and post-Ca^{2+} steps in the TCR transduction pathway, cAMP may be a more powerful inhibitor when the TCR is engaged than when TG is used to raise $[Ca^{2+}]_i$.

We speculate that cell-to-cell differences in the activities of kinases or phosphatases can explain the variable $[Ca^{2+}]_i$ sensitivity during TG treatment. For example, calcineurin levels influence the ability of Ca^{2+} ionophores to stimulate NF-AT-mediated gene expression (31, 32). Therefore, cell-to-cell differences in the levels of calcineurin or other mediators such as the NF-AT protein (10) may be limiting factors in different cells (33). Indeed, the immunosuppressant cyclosporin A decreased the percentage of responders rather than the intensity of the response in individual cells (Fig. 5*B*), suggesting that the threshold of individual cells can be affected by interfering with the calcineurin/NF-AT pathway. Comparing these results with *lacZ* expression controlled by the intact IL-2 promoter, which contains additional regulatory elements (7, 9), could reveal more complex regulation.

The $[Ca^{2+}]_i$ dependence and time course of *lacZ* expression summarized in Fig. 5 represent quantitative measurements of the relationship between a second messenger and gene expression in individual cells. Both video imaging of single-cell signals and the $[Ca^{2+}]_i$-clamp method will be essential for investigating the roles of dynamic and cell-specific $[Ca^{2+}]_i$ phenomena such as gradients and oscillations in regulating the expression of IL-2 and other genes.

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