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Developmental regulation of TCR–CD3-dependent $[Ca^{2+}]_i$ responses of individual normal and pp59^{fyn}-deficient T lymphocytes

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SUMMARY

The aim of this study was to ascertain whether different types of T-cell receptor (TCR)-mediated $[Ca^{2+}]_i$ signals could begin to explain the different cellular responses of mature and immature T cells to ligation of the TCR–CD3 complex. Using a digital fluorescence imaging system, we measured and compared $[Ca^{2+}]_i$ of individual cells from immature and mature murine T-cell populations following application of CD3-ε monoclonal antibody (mAb). Our approach revealed distinctions among developmental subsets which were not seen by previous measurements of $[Ca^{2+}]_i$ in bulk cell populations. The CD3-mediated $[Ca^{2+}]_i$ responses of individual thymocytes were very complex. Latencies to peak $[Ca^{2+}]_i$ varied greatly among thymocytes, but the responses of splenic T cells were synchronized, novel evidence that the timing of $[Ca^{2+}]_i$ responses may be an important informative parameter for TCR–CD3 signalling. In addition, among cells responding to CD3 mAb, higher peak $[Ca^{2+}]_i$ responses correlated with maturity ($CD4^+$ $CD8^+$ thymocytes < single-positive thymocytes < splenic T cells). Examination of cells from pp59^{fyn}-deficient mice showed that pp59^{fyn} deficiency affects the amplitude and probability, but not the latency or synchrony, of CD3-mediated $[Ca^{2+}]_i$ responses of $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocytes. All subsets showed equivalent receptor-independent mobilization of $[Ca^{2+}]_i$. These developmentally distinct $[Ca^{2+}]_i$ features most probably reflect meaningful developmental changes in how the TCR–CD3 complex couples to intracellular signalling machinery including pp59^{fyn}. By clearly showing how $[Ca^{2+}]_i$ responses change during development, these results support the hypothesis that distinctive types of $[Ca^{2+}]_i$ responses drive thymocyte differentiation.

INTRODUCTION

Ligation of the T-cell receptor (TCR)–CD3 receptor complex by a cognate combination of antigenic peptide and major histocompatibility complex (MHC), or by monoclonal antibody (mAb) to TCR–CD3 components, provokes increased $[Ca^{2+}]_i$ in the vast majority of mature and immature T cells.[1–8] For mature T cells, TCR–CD3 signalling involving elevated $[Ca^{2+}]_i$ results in cellular activation, a process marked by proliferation, production of cytokines such as interleukin-2 (IL-2) and/or expression of other immune functions such as cytotoxicity.[9] Paradoxically, elevated $[Ca^{2+}]_i$ is also associated with the inactivation (negative selection) of immature thymic T cells,[10] a TCR–CD3-dependent process probably involving apoptosis.[11,12] Since the majority of immature and mature T lymphocytes apparently utilize the same TCR–CD3 structures,[13] it has been suggested that at different developmental stages the TCR–CD3 receptor couples to different intracellular messengers. For example, distinct cellular outcomes have been proposed to derive from different types of $[Ca^{2+}]_i$ responses[5,7,14] and/or activation of different tyrosine kinases.[15,16]

Both tyrosine kinases and $[Ca^{2+}]_i$ are important for TCR–CD3 signal transduction. Tyrosine kinase inhibitors abrogate $[Ca^{2+}]_i$ increases, proliferation and interleukin-2 (IL-2) secretion when applied to mature cells.[17] When added with phorbol esters, Ca^{2+} ionophores provide a powerful receptor-independent means to stimulate T lymphocytes *in vitro*.[3] Although none of the six or seven transmembrane proteins that comprise the TCR–CD3 receptor possesses intrinsic enzymatic activity,[14] several, including ζ, γ and ε, appear capable of independent signalling via interactions with cytoplasmic tyrosine kinases such as pp59^{fyn}[18–20] and ZAP-70.[21] Additionally, the TCR–CD3 colocalizes during antigen recognition with other T-cell surface molecules, which can influence tyrosine kinase signal transduction, for instance the CD4 and CD8

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} ion concentration; DMSO, dimethylsulphoxide; HBSS, Hank's balanced salt solution; PLC, phosphotidlyinositol-specific phospholipase C; SP, single-positive (expressing either CD4 or CD8).

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coreceptors that are linked to the pp56^{lck} tyrosine kinase, and the CD45 receptor tyrosine phosphatase.[16,22,23] Tyrosine phosphorylation activates a phosphatidylinositol-specific phospholipase C (PLC) (PLC-γ),[24,25] leading to Ca^{2+} release from intracellular stores. The T-cell $[Ca^{2+}]_i$ response also involves Ca^{2+} influx across the plasma membrane, at least for mature cells.[26,28] In addition to $[Ca^{2+}]_i$, the TCR–CD3 couples to pathways involving p21^{ras} and phosphatidylinositol-3-kinase via tyrosine kinases.[16,23]

For mature T cells, TCR–CD3 ligation provokes intriguingly complex $[Ca^{2+}]_i$ responses. Individual human and mouse T-cell lines[8,26,29,30] and peripheral blood lymphocytes[31] often respond with $[Ca^{2+}]_i$ spikes or oscillations instead of simply elevating $[Ca^{2+}]_i$ to a higher plateau level. Previously, these complexities were obscured by the use of cuvette[1,2] or flow cytometric [4] methods, which measure only the average $[Ca^{2+}]_i$ responses of cell populations. The meaning of these complex $[Ca^{2+}]_i$ signals is not clear. Complex $[Ca^{2+}]_i$ responses have been observed in many other cell types following hormone stimulation.[32] In some cases, characteristic $[Ca^{2+}]_i$ oscillations correlate with ligand concentration or receptor subtype,[32,33] raising the possibility that receptors might employ time-dependent $[Ca^{2+}]_i$ changes to encode physiologically relevant signalling information. Oscillation frequency has been proposed to transmit important signalling information[32] but no direct evidence implicates this $[Ca^{2+}]_i$ response feature over others such as the amplitude, the overall duration of the response, or the time-integrated $[Ca^{2+}]_i$ level. Even less is known about how information is encoded by irregular $[Ca^{2+}]_i$ signals such as those of T cells responding to TCR–CD3 ligation.

It has been proposed that distinctive $[Ca^{2+}]_i$ responses regulate lymphocyte development.[5,7] To address this issue and to understand better the meaning of the complex TCR–CD3-derived $[Ca^{2+}]_i$ signals, we compared the TCR–CD3-mediated $[Ca^{2+}]_i$ responses of normal murine T lymphocytes at different developmental stages. The complex $[Ca^{2+}]_i$ responses of individual cells cannot be accurately determined by flow cytometric measures of $[Ca^{2+}]_i$, therefore we used a fluorescent imaging system capable of resolving the $[Ca^{2+}]_i$ changes of individual cells. We found that, during T-cell development, changes occur in certain features of the single-cell $[Ca^{2+}]_i$ responses which are provoked by CD3 monoclonal antibody (mAb) application. Most remarkably, the latency decreases and the synchrony of the peak CD3-dependent $[Ca^{2+}]_i$ responses increases as single-positive (SP) thymocytes mature into splenic T cells. Other characteristics of CD3-dependent, single-cell $[Ca^{2+}]_i$ responses depend on pp59^{fyn} at specific developmental stages, shown by parallel analyses on cells from pp59^{fyn}-deficient mice.[34] These developmentally specific features of $[Ca^{2+}]_i$ responses characterize signalling by the TCR–CD3 complex, since receptor-independent mobilization of $[Ca^{2+}]_i$ showed no developmental distinctions. The fact that these TCR–CD3-specific $[Ca^{2+}]_i$ response features correlate with stages of T-cell development argues that they transmit distinct signals important for cellular responses at different developmental stages.

MATERIALS AND METHODS

Cell preparation

Thymocytes and splenocytes were prepared fresh each day from *fyn*(−) and BL6 control mice as described elsewhere,[35] except that the medium was Hanks' balanced salt solution (HBSS) supplemented with 2·5% bovine serum albumin (BSA) and 25 mM HEPES, pH 7·4 (HBSS media). *Fyn*(−) mice[34] were a generous gift of Dr R. Perlmutter (University of Washington, Seattle, WA). BL6/KH or BL6/J control mice were obtained from Dr C. David (Mayo Foundation, Rochester, MN). For herbimycin A experiments, cells were cultured at 3·5–5·0 × 10^5/ml for 21 hr at 37° in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 2 μM β-mercaptoethanol, 200 U/ml penicillin, 60 μg/ml streptomycin and either 3 μM herbimycin A in dimethyl sulphoxide (DMSO) (a generous gift of Dr P. Leibson, Mayo Foundation) or an equivalent amount of DMSO alone. This treatment protocol prevents 95% of TCR-induced proliferation and tyrosine phosphorylation.[36] Cells were incubated for 10 min at 25° with anti-CD4–phycoerythrin (PE) and anti-CD8–fluorescein isothiocyanate (FITC) mAb (0·03 ml/5 × 10^7 cells) and sorted using a FACSTAR Plus or Vantage FACS (instruments and mAb from Becton Dickinson, San Jose, CA). HBSS, RPMI-1640 and FCS were from Gibco/BRL (Gaithersburg, PA).

$[Ca^{2+}]_i$ measurements

Cells were incubated in HBSS medium with 3 μM Fura-2AM (Molecular Probes, Eugene, OR) at 37° for 30 min (45 min for $CD4^+$ $CD8^+$ thymocytes), rinsed, and used within 6 hr. Calibration tests (see below) showed no significant dye compartmentalization during this time. Ten minutes before each $[Ca^{2+}]_i$ analysis, cells were attached to a coverglass pretreated with 50 μg/ml Cell-Tak, a mixture of mussel glue proteins (Collaborative Biomedical Products, Bedford, MA) which does not by itself provoke increased $[Ca^{2+}]_i$ (data not shown).

$[Ca^{2+}]_i$ was visualized in individual Fura-2-loaded cells using an inverted microscope outfitted with internal quartz optics (IM-35; Zeiss, Oberkochen, Germany). Co-ordinated data acquisition, storage and analysis was provided by Image-1 digital image processing software and hardware (Universal Imaging Corporation, West Chester, PA) run on a 386 computer (Reason Technology, Minneapolis, MN). Cells were exposed to 340 or 380 nm light from a 75-watt xenon lamp passed through excitation and quartz neutral density filters in a computer-controlled, dual filter wheel and shutter assembly. Light from the cells was collected by a 25X PlanNeoFluor 0·8 n.a. oil-immersion objective (Zeiss), reflected by a 400DCLPO2 dichroic mirror and selected by a 510BW40EM emmission filter. Filters were from Omega (Brattleboro, VT). Images at each wavelength, consisting of 512 × 480 pixels and derived from on average 16 consecutive video frames, were collected using a CCD video camera with intensifier head (models C2400–60 and −68; Hammamatsu Photonics, Bridgewater, NJ).

Background-subtracted images at 340 and 380 nm were acquired every 4–7 seconds. 340/380 emission ratios were determined for each cell in the field, and $[Ca^{2+}]_i$ calculated according to the method of Grynkiewicz *et al.*[37] assuming a K_d for Fura-2 of 224 nM. R_{min} or R_{max} were derived from measurements of primary murine T lymphocytes in calibration baths containing 10 mM EGTA and 50 μM MAPTAM (Molecular Probes), or 10 mM $CaCl_2$ and 100 μM ionomycin, respectively. Responsive cells were identified by the following

criteria. The first 1–2 min of each experiment established a baseline $[Ca^{2+}]_i$ level for each cell, and at the end of the experiment a cell was considered responsive if $[Ca^{2+}]_i$ rose at least 200% above this baseline (usually > 150 nM) at any time between 0 and 14 min after CD3 mAb addition. P values were calculated using *Primer of Biostatistics* version 3.01 software (McGraw Hill, New York, NY). Results of normal subsets (Figs 2 and 3) were tested for differences using a one-way analysis of variance and Student–Neuman-Keul's test. Responses of *fyn*(−) cells (Fig. 5) were tested for differences from matched controls using Student's paired *t*-test.

Unless otherwise indicated, the bath was HBSS medium, kept at 37° using a Peltier device. The bath was changed with each stimulus. Stimuli included 2–40 μg/ml biotinylated mAb to CD3-ε (CD3-bio; 500AA.2 and 145·2C11 were used interchangeably), or unmodified CD3 mAb (145·2C11). 500AA.2-bio was a generous gift of Dr D. McKean (Mayo Foundation); 145·2C11 reagents were from Pharmingen (San Diego, CA). Other stimuli were 0·1 μM thapsigargin (Sigma, St Louis, MO) or 0·5 mM pervanadate. Ten millimoles of pervanadate stock was prepared just before each use by combining 10 μl 100 mM $NaVO_4$, 10 μl 100 mM H_2O_2 and 80 μl water and incubating for 15 min at 25°. Residual H_2O_2 was removed by treatment with 50 μg/ml bovine liver catalase (Sigma, St Louis, MO) for 3 min at 25°.

Baseline ratios of unstimulated thymocytes remained stable throughout the 15–45 min duration of each experiment. No dye leakage was detected during this time, and afterwards ionomycin application could still increase $[Ca^{2+}]_i$ (data not shown).

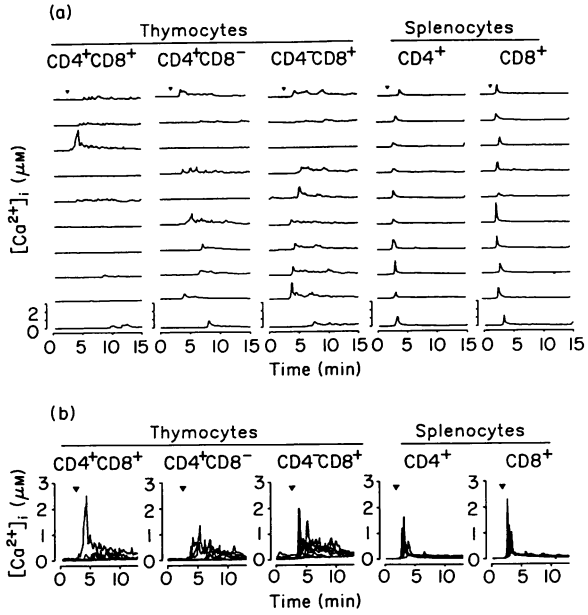

Figure 1. $[Ca^{2+}]_i$ responses of individual T-lineage cells following TCR–CD3 stimulation. Freshly isolated mouse thymocytes and splenocytes were incubated with mAb to CD4 and CD8, sorted, and loaded with Fura-2. $[Ca^{2+}]_i$ was assayed following sequential application of baths containing saturating amounts of biotinylated CD3-ε mAb (10–20 μg/ml; CD3-bio) at the times indicated by the triangles. (a) Each trace shows the average $[Ca^{2+}]_i$ for a single cell versus time. (b) Same data as (a), except the responses of eight cells are overlaid to compare response synchrony.

RESULTS

Calcium responses of splenic T lymphocytes are shorter and more synchronized than those of thymocytes

Figure 1a shows representative $[Ca^{2+}]_i$ responses of individual cells from different FACS-sorted T-lineage subsets of normal mice following application of soluble CD3-ε mAb. The $[Ca^{2+}]_i$ responses of cells from all populations were irregular, usually consisting of one primary peak and sometimes also involving a series of subsequent smaller $[Ca^{2+}]_i$ peaks or plateaux. Like the Jurkat T-cell tumour line[30] and resting peripheral blood T cells,[31] primary thymocytes responded only rarely with a series of regular $[Ca^{2+}]_i$ oscillations. Cells from all the subsets showed similar resting $[Ca^{2+}]_i$ (20–70 nM on different days) and similar responses to receptor-independent $[Ca^{2+}]_i$ mobilizing stimuli (Figs 7 and 8 and data not shown).

By analysing such single-cell $[Ca^{2+}]_i$ responses, we identified $[Ca^{2+}]_i$ response characteristics that correlated with stages of T-cell development. Figure 1b shows that the timing of peak $[Ca^{2+}]_i$ responses to CD3 mAb varied significantly and consistently among developmental subsets: $[Ca^{2+}]_i$ responses of splenic $CD4^+$ and $CD8^+$ T cells occurred earlier, were more synchronous, and were often less prolonged than those of any thymocyte subset. This result was obtained routinely when using thymic and splenic T cells prepared from a single mouse and assayed the same day. Increasing the CD3 mAb concentrations (to 40 or 100 μg/ml) increased the synchrony of thymocyte responses only slightly (data not shown). Soluble CD3-ε mAb, or biotinylated CD3-ε mAb, provoked these $[Ca^{2+}]_i$ responses. Subsequent cross-linking of biotinylated CD3 mAb using streptavidin generated additional $[Ca^{2+}]_i$ increases in all the cells that were responsive initially; however, these secondary $[Ca^{2+}]_i$ responses showed no developmental variations (data not shown). Because we wished to study the $[Ca^{2+}]_i$ signals generated by the TCR–CD3 itself, and since cross-linking might trap and stimulate non-TCR–CD3 receptors, we used only the primary $[Ca^{2+}]_i$ responses evoked by soluble CD3-ε mAb for the following studies.

We employed several parameters to quantify and compare the synchrony of single-cell $[Ca^{2+}]_i$ responses to saturating concentrations (10 or 20 μg/ml) of CD3 mAb among different experiments and cell preparations. The mean time to peak $[Ca^{2+}]_i$ (mean latency) was calculated from the single-cell $[Ca^{2+}]_i$ response data of the responsive cells in each experiment, and the variation in this value was estimated from the standard deviation (SD) and coefficient of variation (CV) of the mean latency of the $[Ca^{2+}]_i$ responses of responsive cells. Criteria for identifying responsive cells were as described in the Materials and Methods. Figure 2 summarizes the results of three to five separate experiments on each cell subset. Figure 2a shows the decreased latency of splenic compared to thymic responses ($P < 0.05$). Splenic T cells consistently responded < 1·5 min after CD3 mAb application, whereas in a single experiment the peak responses of cells within any thymic subset might occur anytime between 1 and 13 min. Peak $[Ca^{2+}]_i$ responses of splenic T cells were also significantly more synchronized than those of thymocytes: for the mean latency to peak $[Ca^{2+}]_i$, groups of splenic T cells exhibited

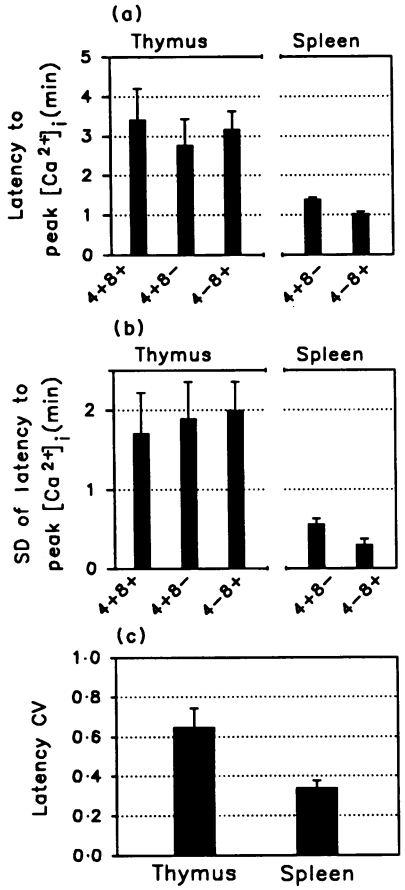

Figure 2. Synchrony of TCR–CD3-mediated $[Ca^{2+}]_i$ responses of individual normal T-lineage cells. Cells were prepared and stimulated as in Fig. 1, and $[Ca^{2+}]_i$ responses to saturating doses of CD3 or CD3-bio mAb were measured for $CD4^+$ $CD8^+$, $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymocytes, and for $CD4^+$ and $CD8^+$ splenic T cells. Only cells that responded were included in this analysis. (a) Latency to peak response. The mean time between CD3 mAb addition and peak $[Ca^{2+}]_i$ (latency) was calculated using 13 responsive cells for each subset of each mouse. Each bar shows the mean of these values ± SEM for five mice. (b) SD comparison of response Synchrony. The SD of the mean time between CD3 mAb addition and peak $[Ca^{2+}]_i$ (SD of latency) was calculated using 13 responsive cells for each subset of each mouse. Each bar shows the mean of these values ± SEM for four mice. (c) CV comparison of response synchrony. The coefficient of variation (CV) for latency to peak $[Ca^{2+}]_i$ response for cells from thymus or spleen was calculated from data in (b). Each bar shows the mean of these values ± SEM for four mice.

significantly smaller SD ($P < 0.05$) and CV ($P < 0.05$) values (Fig. 2b, c).

Peak $[Ca^{2+}]_i$ of responders and percent responding also vary among T-cell developmental subsets

Although each of the murine T-cell populations contained cells with widely varying response types (Fig. 1a), the average peak $[Ca^{2+}]_i$ values of cells responsive to CD3 mAb varied significantly among developmental subsets. Figure 3a summarizes the results of three to five separate experiments on each cell subset. The peak $[Ca^{2+}]_i$ attained by individual cells that responded anytime between 0 and 14 min was used to calculate the mean peak $[Ca^{2+}]_i$ for each experiment. The mean peak $[Ca^{2+}]_i$ of responsive cells among $CD4^+$ $CD8^+$ thymocytes was 0·6 μM, significantly different from that of $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymocytes (0·9 μM, $P < 0.05$), and from that of $CD4^+$ and $CD8^+$ splenocytes (1·2 μM, $P = 0.07$). The peak $[Ca^{2+}]_i$ levels of splenic T cells might be even higher than reported here since $[Ca^{2+}]_i$ quantification using Fura-2 became less precise above 2 μM. Despite this limitation, our peak $[Ca^{2+}]_i$ values reflected intracellular conditions much more accurately than those methods which measure the average $[Ca^{2+}]_i$ of bulk cell populations at each time. This was especially true for the thymocyte subsets, which generally responded with asynchronous $[Ca^{2+}]_i$ peaks lower than 2 μM. In addition to peak $[Ca^{2+}]_i$ differences, we found that significantly fewer $CD4^+$ $CD8^+$ thymocytes than SP thymocytes or splenocytes responded to CD3 mAb (60% versus 90%, $P < 0.05$; Fig. 3b), corresponding to the proportion of cells within these subsets expressing cell-surface TCR–CD3 (approximately 50% versus approximately 100%).[6]

These results demonstrate that TCR–CD3 receptors mobilize $[Ca^{2+}]_i$ differently at different differentiative stages. Furthermore, for the responsive cells, they identify the latency to peak $[Ca^{2+}]_i$, and the peak $[Ca^{2+}]_i$ value itself, as potentially important characteristics of the complex $[Ca^{2+}]_i$ signal that is experienced by individual T-lineage cells following TCR–CD3 stimulation.

The developmentally regulated $[Ca^{2+}]_i$ responses partly depend on the $pp59^{fyn}$ tyrosine kinase

Overexpression of the tyrosine kinase $pp59^{fyn}$ in T cells enhances $[Ca^{2+}]_i$ increases, proliferation and IL-2 secretion following TCR–CD3 stimulation.[38,39] Moreover, thymus cells of $pp59^{fyn}$-deficient (*fyn*(−)) mice are refractory to activation via CD3 mAb.[39,40] To characterize further physiologically relevent parameters of the CD3-dependent, single-cell $[Ca^{2+}]_i$ responses of individual T cells, and to address how developmentally regulated $[Ca^{2+}]_i$ responses might depend on $pp59^{fyn}$, we measured the single-cell $[Ca^{2+}]_i$ responses of sorted T-lineage cells from *fyn*(−) mice.[34]

Fyn(−) cells maintained the normal pattern of more synchronized $[Ca^{2+}]_i$ responses from splenic compared to thymic populations (Fig. 4b and data not shown), however, individual $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocytes from *fyn*(−) mice were often abnormally unresponsive to CD3 mAb stimulation (compare Figs 1 and 4). Despite normal development and normal cell-surface CD3, CD4 and CD8 expression by these cells,[34] only approximately 70% the normal number of *fyn*(−) $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocytes responded to uncross-linked CD3 mAb ($P < 0.08$; Fig. 5a). Responsive cells were sometimes under represented among *fyn*(−) $CD4^-$ $CD8^+$ thymocytes as well as $CD4^+$ and $CD8^+$ splenocytes; however, these differences were not significantly abnormal ($P = 0.31, 0.72, 0.67$, respectively). The peak $[Ca^{2+}]_i$ responses of individual *fyn*(−) cells were, like those of normal cells, highly variable within each population. Nevertheless, the mean peak $[Ca^{2+}]_i$ levels attained by populations of

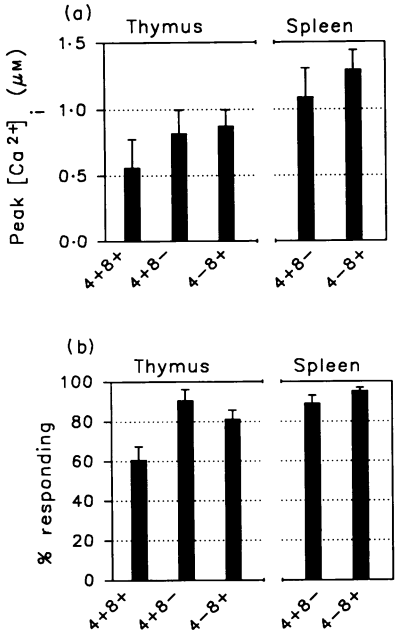

Figure 3. Developmental regulation of TCR–CD3-mediated $[Ca^{2+}]_i$ responses of individual normal T-lineage cells. (a) Mean peak $[Ca^{2+}]_i$ of responsive cells in each subset. The mean peak $[Ca^{2+}]_i$ was calculated using 12 responsive cells for each subset of each mouse. Each bar shows the means of these values ± SEM for four to six mice. (b) Proportion of responsive cells in each subset. Cells were prepared and stimulated as in Fig. 1, and $[Ca^{2+}]_i$ responses to saturating doses of CD3 or CD3-bio mAbs were measured as in Fig. 2. The proportion of responsive cells (%) was calculated using 16 total cells for each subset of each mouse. Each bar shows the means of these values ± SEM for four mice.

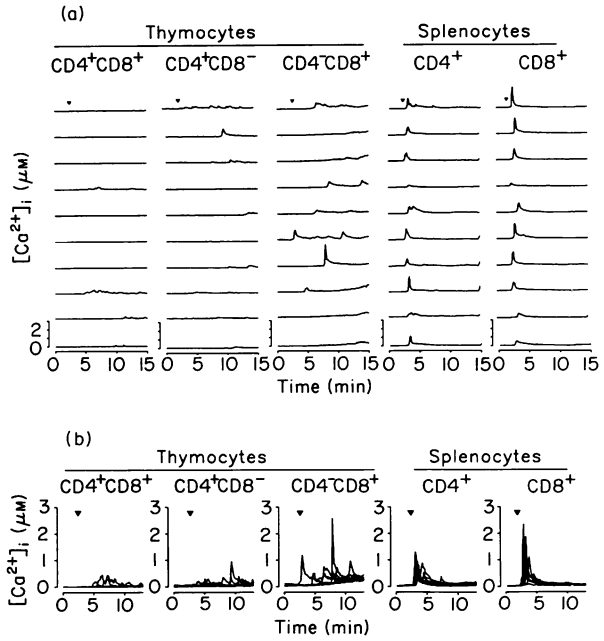

Figure 4. $[Ca^{2+}]_i$ responses of individual *fyn*(−) T-lineage cells following TCR–CD3 stimulation. Cells from *fyn*(−) mice were prepared and stimulated as in Fig. 1. Results for corresponding cell subsets shown here (*fyn*(−)) and in Fig. 1 (normal) were collected on the same day. (a) Each trace shows the average $[Ca^{2+}]_i$ for a single *fyn*(−) cell versus time. (b) Each graph shows the overlaid traces of eight cells from (a).

$CD4^+$ $CD8^+$ thymocytes were significantly lower for *fyn*(−) than for normal populations prepared and assayed in tandem (mean = 70% of normal; $p = 0{\cdot}01$; Fig. 5b). The other *fyn*(−) T-cell populations also showed peak $[Ca^{2+}]_i$ defects, but these varied from mouse to mouse and were more subtle than the defects of $CD4^+$ $CD8^+$ thymocytes. Considering results from all non-$CD4^+$ $CD8^+$ *fyn*(−) populations together indicates, however, that the peak $[Ca^{2+}]_i$ responses of these populations were significantly lower than normal (mean = 83% of normal, $P = 0{\cdot}03$; Fig. 5b).

Thus, $pp59^{fyn}$ deficiency significantly decreases the number of CD3-responsive cells only within $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocyte populations. In contrast, the peak $[Ca^{2+}]_i$ levels attained by responding cells following CD3 stimulation were abnormally low for all *fyn*(−) T-cell populations examined, an effect especially noticeable for $CD4^+$ $CD8^+$ thymocytes.

Tyrosine kinases in addition to $pp59^{fyn}$ regulate the $[Ca^{2+}]_i$ responses of normal and *fyn*(−) thymocytes

Next we asked whether $pp59^{fyn}$ was the only tyrosine kinase involved in developmentally regulated $[Ca^{2+}]_i$ responses. Normal and *fyn*(−) total thymocytes (each of which is composed of approximately 85% $CD4^+$ $CD8^+$ thymocytes (data not shown)[34] were cultured overnight with the tyrosine kinase inhibitor, herbimycin A, under conditions that abrogate TCR-mediated IL-2 secretion,[17] and then tested for $[Ca^{2+}]_i$ responses to uncross-linked biotinylated CD3 mAb. Herbimycin A treatment prevented the $[Ca^{2+}]_i$ responses of both normal and *fyn*(−) thymocytes, while cells incubated with the DMSO solvent alone responded normally (Fig. 6). Herbimycin A apparently blocks TCR–CD3 mobilization of intracellular Ca^{2+}, because changing to extracellular EGTA and ionomycin demonstrated that the contents of intracellular Ca^{2+} stores were equivalent in both cell populations. A final change to a bath containing high Ca^{2+} and ionomycin concentrations showed adequate dye loading by all cells. Clearly, thymocytes, like splenic T cells,[34] contain tyrosine kinases in addition to $pp59^{fyn}$ that participate in TCR–CD3-dependent $[Ca^{2+}]_i$ responses.

Similarly, all T-lineage subsets exhibited comparable $[Ca^{2+}]_i$ responses when tyrosine kinases were activated in a receptor-independent manner with pervanadate, a potent tyrosine phosphatase inhibitor which stimulates tyrosine kinase activity when applied to intact T cells.[36] Treatment of $CD4^+$ $CD8^-$ and $CD4^+$ $CD8^+$ thymocytes and $CD4^+$ splenocytes from normal and *fyn*(−) mice with 0·5 mM H_2O_2-free pervanadate (see the Materials and Methods) led to increased $[Ca^{2+}]_i$ within 1–2 min in nearly 100% of the cells (Fig. 7). Two types of responses were observed, a slow $[Ca^{2+}]_i$ rise to a plateau, or an early spike followed by an increase to a similar plateau level. Although the latter were less frequent among $CD4^+$ $CD8^+$ populations, overall $[Ca^{2+}]_i$ responses to pervanadate were very similar among total thymocytes, $CD4^+$ $CD8^+$ and SP thymocytes, and splenocytes (Fig. 7 insets and data not shown). In addition, mature and immature

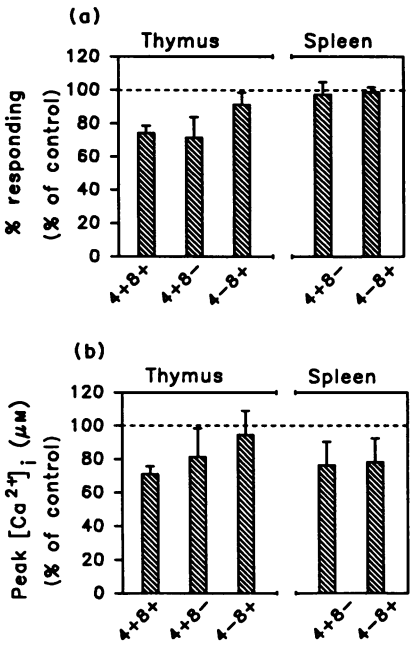

Figure 5. Summary of TCR–CD3-mediated $[Ca^{2+}]_i$ responses of individual *fyn*(−) versus normal T-lineage cells. Cells from *fyn*(−) mice were prepared and stimulated as in Figs 2 and 3. (a) Proportion of responsive cells in each subset. For each experiment, the proportions of responding cells (%) in *fyn*(−) and control populations were calculated using 16 total cells for each subset of each mouse. *Fyn*(−) results are expressed as percentage of parallel control results, which were defined as 100% (dashed line). Each bar shows the means of these values ± SEM for four to six mice. (b) Mean peak $[Ca^{2+}]_i$ of responsive cells in each subset. For each experiment, the mean peak $[Ca^{2+}]_i$ values for *fyn*(−) and control populations were calculated using nine responsive cells for each subset of each mouse. *Fyn*(−) results are expressed as a percentage of parallel control results. Each bar shows the means of these values ± SEM for four or five mice.

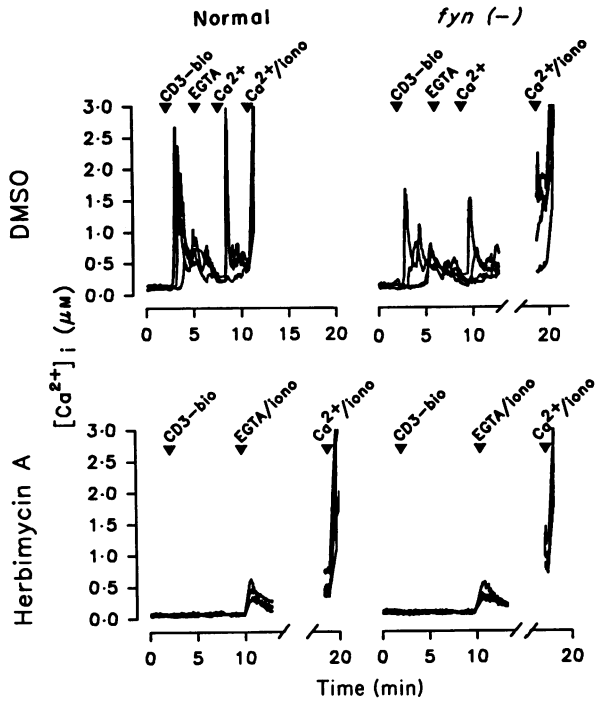

Figure 6. TCR–CD3-mediated $[Ca^{2+}]_i$ responses of normal and *fyn*(−) thymocytes are sensitive to herbimycin A. Total thymocytes from normal and *fyn*(−) mice were cultured with 3 μM herbimycin A or DMSO solvent alone for 21 hr, then $[Ca^{2+}]_i$ responses were measured as in Fig. 1. For DMSO controls (top), baths containing 10 μg/ml CD3-bio and 1·3 mM Ca^{2+}; 2·5 mM EGTA and 0 added Ca^{2+}; 1·3 mM Ca^{2+}; and 10 mM Ca^{2+} and 100 μM ionomycin were added at the indicated times. Bath changes for herbimycin-treated cells (bottom) were 10 μg/ml CD3-bio mAb, 1·3 mM Ca^{2+}; 2·5 mM EGTA and 10 μM ionomycin; and 10 mM Ca^{2+} and 100 μM ionomycin. Each graph shows the overlaid traces of the average $[Ca^{2+}]_i$ for each of four cells versus time.

thymocyte subsets from normal and *fyn*(−) mice responded identically with respect to percentage of responding cells and $[Ca^{2+}]_i$ plateau levels.

Thapsigargin stimulates similar Ca^{2+} influx by immature, mature, and *fyn*(−) T cells

Ca^{2+} influx is an important part of the mature T-cell $[Ca^{2+}]_i$ response.[3,42,42] Immature T-cell responses also involve Ca^{2+} influx, because the absence of free extracellular Ca^{2+} dramatically attentuates the CD3-mediated $[Ca^{2+}]_i$ responses of both normal and *fyn*(−) thymocytes (data not shown). Moreover, as previously shown for mature T cells,[8,26,29,31] ongoing $[Ca^{2+}]_i$ responses of individual normal and *fyn*(−) thymocytes to TCR–CD3 stimulation were arrested by chelation of extracellular Ca^{2+} (Fig. 6) or by membrane depolarization with high extracellular K^+ concentrations (data not shown). The TCR–CD3-stimulated Ca^{2+} influx, as studied in Jurkat and other T cells,[26–28] resembles the macrophage I_{CRAC} current which arises when the intracellular stores are depleted of Ca^{2+}.[43] We used thapsigargin, a Ca^{2+}/ATPase inhibitor which specifically prevents stores refilling,[44] to assess the stores-dependent Ca^{2+} influx mechanisms in mature, immature and *fyn*(−) T cells. 0·1 μM thapsigargin elevated $[Ca^{2+}]_i$ to equivalent plateau levels when applied to thymic or splenic T cells from normal mice. Responses of *fyn*(−) cells were similar (Fig. 8 and data not shown).

DISCUSSION

To begin to address the differences in TCR–CD3 signal transduction among immature and mature T-lymphocyte subsets, we examined the coupling between TCR–CD3 and $[Ca^{2+}]_i$ at the level of individual cells. Since the $[Ca^{2+}]_i$ levels of individual cells change irregularly with time following TCR–CD3 stimulation,[8,26,29–31] and since antigen recognition and cellular responses depend on signals that are at first interpreted only by that cell, the measurement and analysis of the $[Ca^{2+}]_i$ signal received by a single cell are crucial for a full understanding of that cell's response. We used CD3-ε mAb to investigate directly the relationship of TCR–CD3 and $[Ca^{2+}]_i$, without the complexity introduced by also stimulating other receptors. While additional cell-surface structures certainly play an important role in TCR–CD3 signalling *in vivo*,[22,23,45,46] we consider this study of $[Ca^{2+}]_i$ regulation by TCR–CD3 in isolation an important initial step for dissecting this complicated signalling pathway.

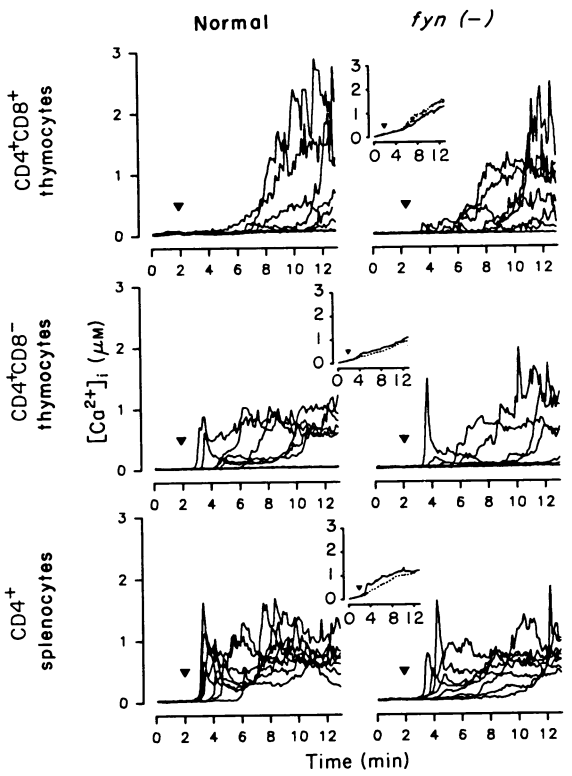

Figure 7. pp59^{fyn} is not required for pervanadate to elevate $[Ca^{2+}]_i$ of mature and immature T cells. Sorted $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocytes and $CD4^+$ splenic T cells were isolated from normal and *fyn*(−) mice the same day. $[Ca^{2+}]_i$ was assayed following application of 0·5 mM pervanadate at the times indicated by the triangles. Each large graph shows the responses of 10 cells. Insets show the average responses of 20 cells from normal (solid lines) and *fyn*(−) (dashed lines) populations.

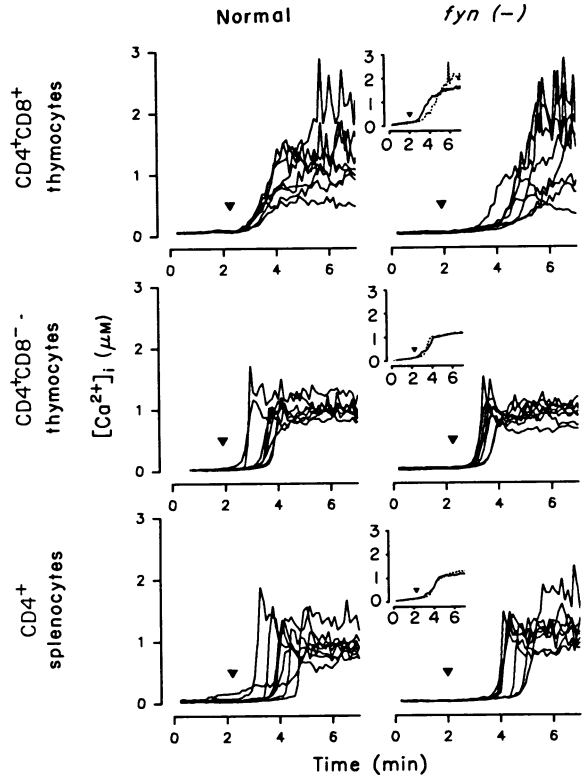

Figure 8. Thapsigargin provokes equivalent $[Ca^{2+}]_i$ responses from normal and *fyn*(−) T cells. Sorted $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocytes and $CD4^+$ splenic T cells were isolated from normal and *fyn*(−) mice the same day. $[Ca^{2+}]_i$ was assayed following application of 100 nM thapsigargin at the times indicated by the triangles. Each large graph shows the responses of eight individual cells. Insets show the average responses of 20 cells from normal (solid lines) and *fyn*(−) (dashed lines) populations.

By analysing freshly isolated cells from different thymocyte and mature peripheral T-cell populations, we identified certain characteristics of the CD3 mAb-mediated $[Ca^{2+}]_i$ responses of individual cells that change during T-cell development. These are: (1) whether or not the cell gives any $[Ca^{2+}]_i$ response; and, for the responsive cells, (2) the time to the highest $[Ca^{2+}]_i$ elevation following CD3 stimulation (the latency); (3) the timing of the peak $[Ca^{2+}]_i$ spike relative to other cells in the population receiving the same stimulus (the synchrony); and (4) the peak $[Ca^{2+}]_i$ value itself. pp59^{fyn}-deficiency affected the peak $[Ca^{2+}]_i$ levels and the numbers of responsive cells among $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocyte populations exposed to CD3 mAb, but not the synchrony or latencies of $[Ca^{2+}]_i$ responses. All T-cell developmental subsets exhibited equivalent receptor-independent mobilization of $[Ca^{2+}]_i$, including Ca^{2+} influx, shown by use of thapsigargin and the tyrosine phosphatase inhibitor, pervanadate. These distinctive CD3 mAb-mediated $[Ca^{2+}]_i$ response features most probably reflect meaningful developmental changes in how the TCR–CD3 complex, in the absence of deliberate coreceptor stimulation, couples to intracellular signalling machinery including pp59^{fyn}. Since $[Ca^{2+}]_i$ is important for the immune responses of mature T cells[3,41,42] and probably also for thymocyte development,[10] it is likely that these distinctive $[Ca^{2+}]_i$ responses participate in determining the cellular outcome of TCR–CD3 ligation on both mature and immature T cells.

These results provide new information for understanding the $[Ca^{2+}]_i$ responses of T cells. The latency and synchrony of $[Ca^{2+}]_i$ responses, in particular, cannot be adequately detected by flow cytometric or cuvette methods used previously,[5,6] and the $[Ca^{2+}]_i$ responses of immature T cells have not previously been measured with single-cell resolution. Uncross-linked CD3-ε mAb revealed the systematic variations in single-cell $[Ca^{2+}]_i$ responses. Cross-linked CD3 mAb generated large $[Ca^{2+}]_i$ increases that were not characteristic of T-cell developmental stages, thus the extensive CD3 cross-linking typically used in many T-cell stimulation protocols might mask developmentally regulated changes in TCR–CD3 signalling.

Among the $[Ca^{2+}]_i$ response distinctions, the most striking is our finding that the peak $[Ca^{2+}]_i$ responses of normal murine splenic T cells occur earlier and are much more synchronized than those of any thymocyte subset. To our knowledge, this is the first description of a $[Ca^{2+}]_i$ response distinction among normal T lymphocytes that implicates time as a meaningful variable. It is unlikely that the latency and synchrony differences arise from variations in cell-surface TCR–CD3

expression since cells from all thymic subsets exhibited similar variations in peak $[Ca^{2+}]_i$ latency times despite 5–10-fold differences in cell-surface TCR–CD3.[6] Previous flow cytometry studies suggested the overall unresponsiveness of immature T cells,[5,6] but only our results clearly show how, compared with more mature T cells, both low numbers of responding cells and low peak $[Ca^{2+}]_i$ levels characterize the responses of $CD4^+$ $CD8^+$ thymocytes to TCR–CD3 stimulation.[5,6] In addition, we obtained accurate values for both these parameters for responding cells from different subsets, information which is essential for understanding how these cells interpret $[Ca^{2+}]_i$ signals.

Since the peak $[Ca^{2+}]_i$ responses were usually also the first responses detected (especially for splenic T cells; Fig. 1), developmental variations in the probability, latency and amplitude of $[Ca^{2+}]_i$ responses probably reflect the timing and/or efficiency of early signalling events, particularly TCR–CD3 regulation of PLC. Receptor-independent stimulation of $[Ca^{2+}]_i$ influx, and release of $[Ca^{2+}]_i$ via activation of tyrosine kinases and PLC-γ,[36] were similarly effective for all developmental subsets, supporting this idea. TCR–CD3 stimulation provokes a rapid, transient activation of associated $pp59^{fyn}$,[18,19] and a major target of $pp59^{fyn}$ appears to be PLC-γ1.[20] Overexpression of $pp59^{fyn}$ [20,38,39] enhances average proliferation, IL-2 production, and $[Ca^{2+}]_i$ following TCR–CD3 stimulation, and thymocytes from $pp59^{fyn}$-deficient mice are profoundly unresponsive to *in vitro* TCR stimulation.[34,40] We found that the average peak $[Ca^{2+}]_i$ values and the number of responding cells among $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ thymocyte subsets showed partial sensitivity to $pp59^{fyn}$-deficiency, suggesting a role for this tyrosine kinase in regulating these parameters of the TCR–CD3-mediated $[Ca^{2+}]_i$ signal. Previous analysis of $[Ca^{2+}]_i$ mobilization by cells from *fyn*(−) mice suggested that $pp59^{fyn}$ deficiency primarily affected $CD4^+$ $CD8^-$ rather than $CD4^+$ $CD8^+$ thymocytes,[34] however, our single-cell analysis is unbiased by response asynchrony and capable of unambiguously identifying non-responding cells.

Developmental variations in TCR–CD3 coupling to $[Ca^{2+}]_i$ might also arise from variable expression and/or localization of signalling molecules, including $pp59^{fyn}$ and ZAP-70, which interact with cytosolic portions of the TCR–CD3. Cell-surface molecules associating with TCR–CD3 transmembrane or extracellular structures can also, even without their deliberate stimulation, affect TCR–CD3 signalling. Such influences might explain how $pp59^{fyn}$ deficiency affects the TCR–CD3-mediated $[Ca^{2+}]_i$ responses of $CD4^+$ but not $CD8^+$ SP thymocytes, despite their normally equivalent $pp59^{fyn}$ expression[38] and CD3-mediated $[Ca^{2+}]_i$ responses (Figs 2 and 3). In a T-cell tumour line, the synchrony of $[Ca^{2+}]_i$ responses provoked by CD3 mAb was shown to depend on levels of CD45 expressed at the cell surface.[29] The expression of high molecular weight isoforms of this receptor tyrosine phosphatase increases as T cells mature,[47] providing one possible explanation for the synchronized $[Ca^{2+}]_i$ responses of splenic T cells. It is also possible that the observed variations in $[Ca^{2+}]_i$ responses derive from prior ligation of cell-surface structures *in vivo*, for instance through interactions with the thymic microenvironment.[48]

Besides TCR–CD3 signalling, additional signals from coreceptors,[22,23,46] adhesion molecules,[49] cytokines,[45] and hormones[50] accompany antigen recognition *in vivo*. Each of these signals could modify TCR–CD3 coupling to $[Ca^{2+}]_i$ regulation, adding layers of complexity to the results described here. Clearly this should be a topic of future work. Nevertheless, mAb to CD3-ε alone have long been used *in vitro* to mimic the activation of mature T cells[25] and the negative selection of $CD4^+$ $CD8^+$ thymocytes.[11] The $[Ca^{2+}]_i$ response features described above might therefore presage or determine the responses of cells to TCR–CD3 ligation *in vivo*. Our results suggest a correlation between low peak $[Ca^{2+}]_i$ values and negative selection processes, since SP thymocytes mainly contain postselection cells while most of their $CD4^+$ $CD8^+$ precursors have not yet undergone negative selection.[51] In addition, the $[Ca^{2+}]_i$ responses of SP thymocytes and splenic T cells are distinguished by long/variable and short/invariant latencies, respectively, possibly reflecting the presence of unselected cells within SP thymocyte populations. Alternatively, since stimulated $CD4^+$ $CD8^-$ thymocytes proliferate less vigorously and secrete less IL-2,[52] latency differences might influence the efficiency of cellular activation. $[Ca^{2+}]_i$ response latencies could affect cellular responses by changing the timing of the $[Ca^{2+}]_i$ signal relative to other TCR–CD3-derived signals, such as those involving activation of $p21^{ras}$ and phosphatidlyinositol-3-kinase. By clearly showing how $[Ca^{2+}]_i$ responses change during development, our results support the hypothesis that distinctive types of $[Ca^{2+}]_i$ responses drive thymocyte differentiation.[5,7]

Although receptor-dependent $[Ca^{2+}]_i$ responses are used ubiquitously for signal transduction, single-cell $[Ca^{2+}]_i$ measurements are just beginning to reveal the possibility that $[Ca^{2+}]_i$ signals might encode much complex information using time as a variable. Much further work will be necessary to understand how receptors such as TCR–CD3 generate such signals, and how cells interpret and react to them.

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