T-Cell Stimulation through the T-Cell Receptor/CD3 Complex Regulates CD2 Lateral Mobility by a Calcium/Calmodulin-Dependent Mechanism

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ABSTRACT T lymphocyte activation through the T cell receptor (TCR)/CD3 complex alters the avidity of the cell surface adhesion receptor CD2 for its ligand CD58. Based on the observations that activation-associated increases in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) strengthen interactions between T cells and antigen-presenting cells, and that the lateral mobility of cell surface adhesion receptors is an important regulator of cellular adhesion strength, we postulated that $[Ca^{2+}]_i$ controls CD2 lateral mobility at the T cell surface. Human Jurkat T leukemia cells were stimulated by antibody-mediated cross-linking of the TCR/CD3 complex. CD2 was labeled with a fluorescently conjugated monoclonal antibody. Quantitative fluorescence microscopy techniques were used to measure $[Ca^{2+}]_i$ and CD2 lateral mobility. Cross-linking of the TCR/CD3 complex caused an immediate increase in $[Ca^{2+}]_i$ and, 10–20 min later, a decrease in the fractional mobility of CD2 from the control value of 68 ± 1% to 45 ± 2% (mean ± SEM). One to two hours after cell stimulation the fractional mobility spontaneously returned to the control level. Under these and other treatment conditions, the fraction of cells with significantly elevated $[Ca^{2+}]_i$ was highly correlated with the fraction of cells manifesting significantly reduced CD2 mobility. Pretreatment of cells with a calmodulin inhibitor or a calmodulin-dependent kinase inhibitor prevented Ca^{2+}-mediated CD2 immobilization, and pretreatment of cells with a calcineurin phosphatase inhibitor prevented the spontaneous reversal of CD2 immobilization. These data suggest that T cell activation through the TCR/CD3 complex controls CD2 lateral mobility by a Ca^{2+}/calmodulin-dependent mechanism, and that this mechanism may involve regulated phosphorylation and dephosphorylation of CD2 or a closely associated protein.

INTRODUCTION

T lymphocyte activation, proliferation, and differentiation are induced upon ligation of antigen-specific T cell receptors (TCRs) by antigen/major histocompatibility complex (MHC) proteins on antigen-presenting cells (APCs). Other T cell surface molecules also contribute to TCR-mediated activation by increasing the adhesion strength between T cells and APCs and by providing costimulatory signals to T cells (Springer, 1990). The T cell adhesion receptor CD2 binds CD58 (also known as lymphocyte function-associated antigen 3, LFA-3), CD59 (Deckert et al., 1992; Hahn et al., 1992), and CD48 (Arulanandam et al., 1993; Kato et al., 1992) on APCs, whereas T cell CD11a/CD18 (LFA-1) binds CD54, CD102, and CD50 (also known as intercellular adhesion molecules (ICAMs) 1, 2, and 3, respectively) (Springer, 1990). By stimulating intracellular signal transduction pathways, TCR-mediated T cell activation transiently up-regulates the avidity of CD2 for CD58 and the avidity of CD11a/CD18 for CD54 (Dustin and Springer, 1989; Hahn et al., 1993). Interactions between T cells and APCs are also dynamic, involving cycles of cell-cell adhesion and detachment (Martz, 1977; Martz et al., 1983; Poenie et al., 1987). Intracellular signals may regulate such adhesion and detachment cycles by coupling TCR ligation to adhesion molecule avidity.

Adhesion strength is determined both by the intrinsic affinity of binding between receptors and ligands and by the density of these adhesion molecules at contact sites. Redistributions of adhesion molecules to and from sites of contact between lymphocyte and target membranes may be important steps in cell-cell adhesion and detachment events (Boniface and Davis, 1995; Dustin et al., 1996; Koyasu et al., 1990). Adhesion molecules must be capable of lateral movement in the plane of the plasma membrane for such molecules to redistribute to contact sites. Experiments in which Jurkat T leukemia cells were found to adhere more strongly to laterally mobile CD58 than to immobile CD58 reconstituted in glass-supported planar bilayer membranes have demonstrated the importance of adhesion molecule lateral mobility for cellular adhesion strength (Chan et al., 1991).

T cell stimulation through the TCR/CD3 complex initiates a series of intracellular events, including activation of protein tyrosine kinases and of phospholipase C (see Weiss and Littman, 1994). Phospholipase C mediates hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate inositol-1,4,5-trisphosphate and 1,2-diacylglycerol, which induce, respectively, an increase in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC). Although both tyrosine kinase and PKC activation are involved in the TCR-mediated enhancement of CD2 avidity for CD58 (Hahn et al., 1993), several lines of evidence suggest that intracellular Ca^{2+} plays an important role in regulating T

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cell interactions with APCs. The kinetics and magnitude of Ca^{2+} signals appear to correlate with those of T cell activation (Wulfing et al., 1997). An increase in intracellular $[Ca^{2+}]$ stabilizes the interaction of T cells with APCs and reduces the ability of T cells to migrate (Donnadieu et al., 1994; Negulescu et al., 1996). We hypothesize that one mechanism by which intracellular Ca^{2+} controls adhesion strength involves regulation of CD2 lateral mobility and cell surface distribution. By specifically immobilizing CD2 molecules that accumulate at cell-cell contact sites, for example, intracellular Ca^{2+} could increase the probability of formation of adhesive molecular contacts and decrease the chemical activity of the stabilized CD2-CD58 adhesion complexes (Zhu et al., manuscript in preparation) and thereby increase cellular adhesion strength, stabilize T cell-APC interactions, and reduce T cell migration. We have previously demonstrated that T cell stimulation by pairs of anti-CD2 monoclonal antibodies (mAbs) causes immobilization of cell surface CD2, and that this CD2 immobilization is mediated in part by increases in $[Ca^{2+}]_i$ (Liu et al., 1995). In this report, we extend this hypothesis to the more physiological case by quantifying the degree to which TCR-mediated changes in $[Ca^{2+}]_i$ regulate CD2 lateral mobility. We also investigate the intracellular events that mediate changes in CD2 lateral mobility upon cell stimulation through the TCR/CD3 complex. These studies may provide the molecular link by which T cell activation is coupled to changes in CD2 mobility and distribution, and thereby to alterations in CD2 avidity.

MATERIALS AND METHODS

Cell lines and antibodies

Jurkat T leukemia cells were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin-100 μg/ml streptomycin (Sigma), 3 mM glutamine (Gibco BRL, Gaithersburg, MD), and 50 μM 2-mercaptoethanol (Sigma) at 37°C in a 5% CO_2 atmosphere. The anti-CD2 mAb TS2/18 (subclass IgG1; Sanchez-Madrid et al., 1982) was conjugated to fluorescein isothiocyanate (FITC), as described (Golding, 1976; Stolpen et al., 1988). OKT3 (subclass IgG2a) is an anti-CD3 mAb (Sigma Immunochemicals). Goat anti-mouse IgG2a (GaM; Caltag Laboratories, San Francisco, CA) was used to cross-link OKT3. 9–1 is an anti-CD2R mAb (Bernard et al., 1986). Antibodies were centrifuged at 16,000 $\times$ *g* (model 5412; Eppendorf, Hamburg, Germany) for 1 h immediately before experiments.

Cell preparation

Cells were washed with HEPES-buffered saline (HBS) (140 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM HEPES, 5.6 mM glucose, pH 7.4). For fluorescence photobleaching recovery (FPR) experiments, cell surface CD2 molecules were labeled with FITC-conjugated TS2/18 (FITC-TS2/18) by incubating cells (5×10^6/ml) with FITC-TS2/18 (50 μg/ml) for 30 min at 0°C in the dark. For intracellular Ca^{2+} measurements, cells were incubated with 10 μM fluo-3-acetoxymethyl ester (Molecular Probes, Eugene, OR) for 1 h at room temperature, as described (Liu et al., 1995).

Cell pretreatment

W-7 (Seikagaku America, Rockville, MA), cyclosporine A (CsA) (Sandoz Research Institute, Hanover, NJ), and KN-62 (Seikagaku America) were dissolved in H_2O, ethanol, and dimethyl sulfoxide to make 5 mM, 1 mM, and 10 mM stock solutions, respectively. Other reagents were dissolved in HBS.

Jurkat cells were incubated in the dark with trifluoperazine (TFP) (Sigma) for 15 min at 37°C; with 100 μM W-7, 50 μM KN-62, or CsA for 1 h at 37°C; or with 500 nM okadaic acid (Calbiochem, La Jolla, CA) for 30 min at room temperature. For some experiments, intracellular Ca^{2+} was depleted by pretreating cells with 10 mM EGTA and 10 μM ionomycin (EGTA + ionomycin) in HBS for 1 h at room temperature. None of these pretreatments caused a change in cell morphology by phase-contrast microscopy. By trypan blue exclusion, >90% of cells remained viable after each pretreatment. All pretreatments were carried out before fluorescent labeling and mAb stimulation of cells.

Cell stimulation

Cells (5×10^6/ml) were incubated with TS2/18 or FITC-TS2/18 (50 μg/ml) in HBS for 10 min on ice, then incubated with OKT3 (50 μg/ml) + TS2/18 or FITC-TS2/18 in HBS for 20 min on ice, then washed with HBS. Unless otherwise indicated, GaM (50 μg/ml) was added to the cell suspension immediately before lateral mobility or $[Ca^{2+}]_i$ measurements. In some experiments (see Fig. 6), cells were incubated with FITC-TS2/18 for 30 min on ice, then OKT3 was added to the cell suspension immediately before lateral mobility or $[Ca^{2+}]_i$ measurements. Samples pretreated with EGTA + ionomycin or with a pharmacological inhibitor were maintained in HBS with EGTA + ionomycin or with the inhibitor throughout the incubation, mAb stimulation, and data collection steps. Jurkat cells were alternatively stimulated by using a pair of anti-CD2 mAbs, 9–1 + FITC-TS2/18. Cells were first labeled with FITC-TS2/18, then 9–1 (50 μg/ml) was added to the cell suspension immediately before lateral mobility or $[Ca^{2+}]_i$ measurements.

Fluorescence photobleaching recovery

An ACAS 570 interactive laser cytometer (Meridian Instruments, Okemos, MI) was used to perform fluorescence photobleaching recovery (FPR) experiments (Axelrod et al., 1976), as described (Liu et al., 1995). Briefly, a Gaussian laser beam was focused to a spot on a labeled cell in a fluorescence microscope. After a brief, intense photobleaching pulse, recovery of fluorescence was monitored by periodic pulses of lower intensity. Fluorescence recovery resulted from the lateral diffusion of unbleached fluorophores into the bleached area. The Gaussian beam radius was 1 μm, and excitation and emission wavelengths were 488 nm and 510 $\pm$ 5 nm, respectively. Photobleaching power at the sample was $\sim$0.5 mW, and the bleaching time was 40–50 ms. The fractional mobility (*f* value; the fraction of FITC-labeled protein that was free to diffuse in the plane of the membrane) and the lateral diffusion coefficient (*D*) of the mobile fraction were obtained by nonlinear least-squares analysis of fluorescence recovery curves (Golan et al., 1986).

Intracellular calcium ion concentration

The ACAS 570 interactive laser cytometer was used to image individual fluo-3-loaded cells, using an excitation wavelength of 488 nm and an emission wavelength of 510 nm. Integrated single-cell fluo-3 fluorescence intensities were then measured. Fluo-3 spectral response was calibrated using Mn^{2+}, and $[Ca^{2+}]_i$ was calculated from the integrated single-cell fluo-3 fluorescence intensity, based on the method described by Kao et al. (Kao et al., 1989; Liu et al., 1995). Fluo-3-loaded cells were treated with mAbs (TS2/18 or TS2/18 + OKT3) and washed. GaM (50 μg/ml) was then added to the cell suspension. Single-cell images were taken immediately after fluo-3 loading and after each antibody treatment. Because calcium

dyes such as fluo-3 are rapidly internalized into pinocytic vesicles and other intracellular organelles at 37°C, the calcium imaging experiments were performed at room temperature. To ensure uniformity of cell treatment between CD2 lateral mobility and $[Ca^{2+}]_i$ experiments, lateral mobility measurements were also made at room temperature.

RESULTS

T cell stimulation through the TCR/CD3 complex induces a reversible decrease in the fractional mobility of CD2

Jurkat T cells were stimulated by cross-linking of the TCR/CD3 complex with the anti-CD3 mAb OKT3 (subclass IgG2a) and goat anti-mouse IgG2a (GaM). CD2 was fluorescently labeled with FITC-conjugated TS2/18, an anti-CD2 mAb, and FPR was used to measure CD2 lateral mobility. Fluorescence imaging of FITC-TS2/18-labeled resting and stimulated cells showed the characteristic plasma membrane "rim" stain; 29% of resting cells and 49% of stimulated cells were also found to have one or two fluorescent patches (≤ 2 μm in diameter) in the plasma membrane.

To test the effect of TCR-mediated T cell stimulation on CD2 lateral mobility, cells were first incubated with FITC-TS2/18 and OKT3; then GaM was added to the cell suspension. Control FITC-TS2/18-labeled cells manifested a CD2 fractional mobility of 68 ± 1% (mean ± SEM). Treatment with either OKT3 or GaM did not significantly change the fractional mobility. In contrast, cross-linking of OKT3 by GaM induced time-dependent immobilization of CD2. The fractional mobility decreased significantly 20 min after GaM addition and remained at a level of 45 ± 2% for 21–60 min of incubation (Fig. 1, *a* and *b*, Table 1). The fractional mobility then increased spontaneously ~1 h after GaM addition, returning to baseline levels 2 h after GaM addition and remaining at this level for at least 1.5 additional hours (Fig. 2 *a*). T cell stimulation through the TCR/CD3 complex was therefore associated with a significant, reversible decrease in CD2 fractional mobility.

An apparent decrease in fractional mobility could result from immobilization of fluorescently labeled cell surface receptors or, alternatively, from internalization of labeled receptors (Thatte et al., 1994) upon T cell stimulation. Receptor internalization would be expected to shift the fluorescence distribution from the periphery to the center of cells. The distribution of FITC-TS2/18 fluorescence intensity along the axis of laser scanning was similar on resting cells and on cells stimulated with OKT3 + GaM (data not shown), suggesting that CD2 internalization was not responsible for the decrease in CD2 lateral mobility. Rather, the decrease in fractional mobility was due to CD2 immobilization at the cell surface.

Intracellular calcium ions mediate the TCR-induced CD2 immobilization

We have previously shown that T cell activation through CD2 induces both an increase in $[Ca^{2+}]_i$ and a marked decrease in CD2 lateral mobility, and that the increase in $[Ca^{2+}]_i$ is responsible in part for the CD2 immobilization (Liu et al., 1995). Because T cell stimulation through the TCR/CD3 complex also decreased CD2 fractional mobility, we hypothesized that intracellular Ca^{2+} could act as a common regulator of CD2 lateral mobility and attempted to determine whether the TCR-induced immobilization of CD2 was caused by an increase in $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ was measured in single cells before and after T cell stimulation. Relative fluorescence intensity was used to characterize changes in $[Ca^{2+}]_i$ under various experimental conditions. As previously observed (Yang et al., 1987), treatment of Jurkat T cells with the single anti-CD2 mAb TS2/18 did not cause an increase in $[Ca^{2+}]_i$. Similarly, treatment with TS2/18 and GaM did not increase $[Ca^{2+}]_i$ (data not shown). Treatment with TS2/18 and OKT3 caused a small but significant increase in $[Ca^{2+}]_i$ (Fig. 3); this increase occurred immediately after stimulation with OKT3 and did not change significantly over 20 min of incubation (data not shown). Cross-linking of the TCR/CD3 complex by addition of GaM to OKT3-treated cells caused a more prominent $[Ca^{2+}]_i$ elevation. This increase in $[Ca^{2+}]_i$ occurred immediately after GaM addition, peaked at 11–20 min of incubation, and declined to baseline levels by 41–50 min of incubation (Fig. 3).

To determine whether intracellular Ca^{2+} was required for CD2 immobilization, cells were depleted of intracellular Ca^{2+} by pretreatment with EGTA + ionomycin. This pretreatment lowered the $[Ca^{2+}]_i$ from 115 nM to 18 nM in resting cells and completely eliminated the increase in $[Ca^{2+}]_i$ upon stimulation with OKT3 + GaM. The fractional mobility of CD2 in cells depleted of intracellular Ca^{2+} remained constant (*f* value, 65–70%) for 60 min after the addition of GaM to cells treated with TS2/18 and OKT3 (Table 1, Fig. 1 *c*), suggesting that intracellular Ca^{2+} was required for TCR-induced CD2 immobilization.

The degree of CD2 immobilization correlates directly with the increase in intracellular calcium ion concentration

T cell stimulation, through either the TCR/CD3 complex (the present study) or CD2 (Liu et al., 1995), caused both an increase in $[Ca^{2+}]_i$ and a decrease in CD2 mobility. Because the magnitude of the changes in CD2 mobility and in $[Ca^{2+}]_i$ differed between cells stimulated through the TCR/CD3 complex and cells stimulated through CD2, we could test the hypothesis that CD2 immobilization correlates directly with the increase in $[Ca^{2+}]_i$. The single cell distributions of CD2 mobility and $[Ca^{2+}]_i$ were measured in cells stimulated by various combinations of antibodies. We predicted that if the increase in $[Ca^{2+}]_i$ caused CD2 immobilization, then for each experimental treatment the fraction of cells with high $[Ca^{2+}]_i$ (R_a') would equal the fraction of cells with low CD2 mobility (R_f). (Optimally, measurements of CD2 mobility and $[Ca^{2+}]_i$ would have been per-

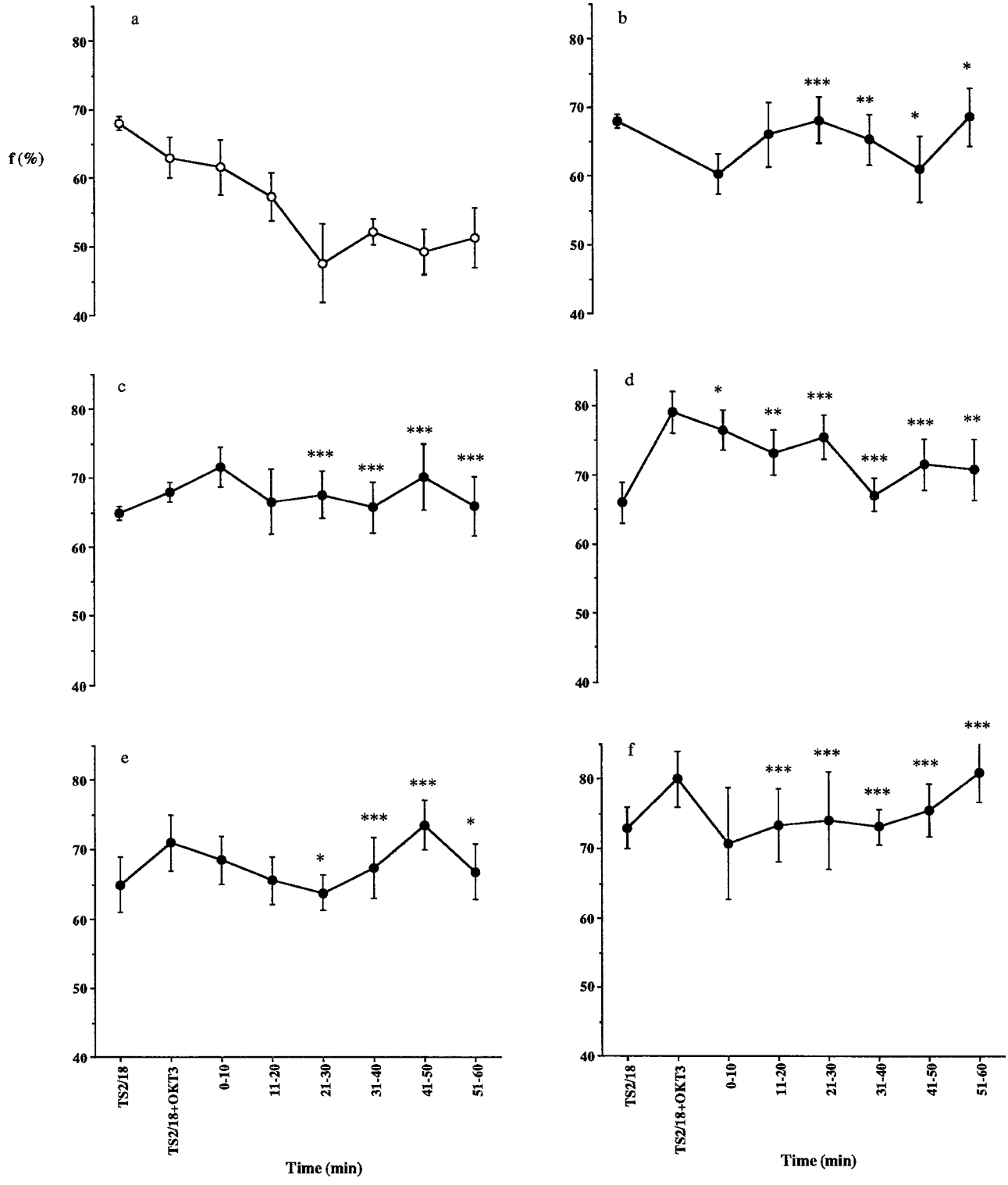

FIGURE 1 Time course of CD2 immobilization induced by stimulation of Jurkat T cells through the TCR/CD3 complex, and effects of intracellular Ca^{2+} depletion, CaM inhibition, and CaM kinase inhibition on the immobilization of CD2. Cells were treated with FITC-TS2/18 + OKT3 (*a*, *c–f*) or FITC-TS2/18 alone (*b*). Goat anti-mouse IgG2a (GaM) was added at time 0 to cross-link the TCR/CD3 complex. In some experiments cells were pretreated with 10 mM EGTA + 10 μM ionomycin (*c*), 100 nM trifluoperazine (TFP) (*d*), 100 μM W-7 (*e*), or 50 μM KN-62 (*f*). Data points represent the mean $\pm$ SEM of 19–39 (*a–c*) or 8–18 (*d–f*) measurements of CD2 fractional mobility (*f*, %) from 8 (*a*), 6 (*b*), 7 (*c*), 3 (*d*), or 2 (*e*, *f*) independent experiments in each time interval. By Student's two-tailed *t*-test, the mean *f* values in *b–f* are significantly different from those in *a* at each of the corresponding time intervals, with $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

TABLE 1 Effect of intracellular Ca^{2+} depletion on immobilization of CD2 induced by stimulation of Jurkat T cells through the TCR/CD3 complex

Pretreatment	mAb treatment	D ($\times 10^{10}$ cm^2 s^{-1})	f (%)	N
None	TS2/18*	7.5 ± 0.3	68 ± 1 (1, 5, 6, 7, 8)	338
None	TS2/18 + OKT3*	7.5 ± 0.8	63 ± 3 (2, 5)	34
None	TS2/18 → GaM*	7.0 ± 0.4	65 ± 2 (3, 6)	161
None	TS2/18 + OKT3 → GaM$^{\#}$	8.9 ± 0.8	45 ± 2 (1, 2, 3, 4)	72
EGTA + ionomycin	TS2/18*	8.6 ± 2.0	65 ± 4 (7, 9)	9
EGTA + ionomycin	TS2/18 + OKT3*	7.8 ± 0.6	68 ± 3 (9, 10)	54
EGTA + ionomycin	TS2/18 + OKT3 → GaM$^{\#}$	7.0 ± 0.9	66 ± 3 (4, 8, 10)	52

After pretreatment, cells were incubated with TS2/18 or TS2/18 + OKT3 and then with GaM. FPR was used to measure CD2 lateral mobility 0–60 min (*) or 21–60 min ($^{\#}$) after the addition of GaM. The time course of CD2 immobilization induced upon addition of GaM is shown in Fig. 1. D, diffusion coefficient; f, fractional mobility; N, number of measurements. D and f values represent the mean ± SEM. 1, 2, 3, 4, $p < 0.001$, Student's two-tailed t-test. 5, 6, 7, 8, 9, 10, $p > 0.05$, Student's two-tailed t-test.

formed in the same cells at the same time for each experimental treatment. We could not identify a pair of fluorescent labels that would allow such simultaneous measurements, however. For example, although both FITC-TS2/18 and the calcium dye fura red could be excited by 488-nm light, the spectral overlap between the weak FITC emission and the strong fura red emission prevented signal separation adequate for this study.)

Objective cutoff values were determined for the $[Ca^{2+}]_i$ and CD2 fractional mobility that corresponded to cell stimulation. The distribution of $[Ca^{2+}]_i$ values in cells treated with TS2/18 and OKT3 followed by GaM (stimulated cells) was compared to that in cells treated with TS2/18 and GaM (control cells). Compared to control cells, stimulated cells showed a lower fraction of cells with $[Ca^{2+}]_i < 200$ nM and a higher fraction of cells with $[Ca^{2+}]_i > 400$ nM (Fig. 4 *a*). The critical value of $[Ca^{2+}]_i$ was taken to be that at which the distribution profiles of $[Ca^{2+}]_i$ for stimulated and control cells intersected. Finer analysis showed that these two profiles intersected at 280 nM (Fig. 4 *b*). Similar experiments were performed to compare the distribution profiles of $[Ca^{2+}]_i$ values in cells treated with the anti-CD2 mAb pair TS2/18 + 9–1 (stimulated cells) and control cells. These two distribution profiles again intersected at 280 nM (Fig. 4, *c* and *d*). An $[Ca^{2+}]_i$ of 280 nM was therefore used as the cutoff value that characterized cell stimulation, and R_a' was defined as the fraction of cells with $[Ca^{2+}]_i > 280$ nM.

Next the distribution of CD2 f values in cells treated with TS2/18 and OKT3 followed by GaM (stimulated cells) was compared to that in cells treated with TS2/18 alone or TS2/18 and GaM (control cells). The distribution profiles for stimulated cells (Fig. 5 *c*) and control cells (Fig. 5, *a* and *b*) intersected at an f value of 50%. Because depletion of intracellular Ca^{2+} prevented the CD2 immobilization induced by cross-linking of the TCR/CD3 complex (Figs. 1 *b* and 5 *e*), we also compared the distribution of f values in stimulated cells (Fig. 5 *c*) with that in cells pretreated with EGTA + ionomycin and then treated with OKT3 followed by GaM (Fig. 5 *e*). The latter two profiles also intersected at an f value of 50%. Therefore, a fractional mobility of 50% was used as the cutoff value that characterized TCR-mediated cell stimulation, and R_f was defined as the fraction of either control cells or cells stimulated through the TCR with a CD2 f value < 50%.

Stimulation of T cells by pairs of anti-CD2 mAbs decreases the fractional mobility of CD2 from 70% to <10%. This effect is mediated in part by mobilization of intracellular Ca^{2+}, because intracellular Ca^{2+} depletion causes the f value of CD2 to decrease to 35% rather than <10% upon stimulation through CD2 (Liu et al., 1995). To determine a cutoff value for the CD2 fractional mobility that corresponded to the Ca^{2+}-sensitive portion of the CD2 immobilization induced by stimulation through CD2, we compared the distributions of f values in cells treated with TS2/18 + 9–1 to those in cells pretreated with EGTA + ionomycin and then treated with TS2/18 + 9–1 (Fig. 5, *d* and *f*, respectively). The two distribution profiles intersected at an f value of 15%. This fractional mobility was therefore used as the cutoff value that characterized the Ca^{2+}-sensitive portion of the CD2 immobilization induced by stimulation through CD2, and R_f was defined as the fraction of cells stimulated by pairs of anti-CD2 mAbs with a CD2 f value < 15%.

The objective criteria defined above were used to calculate R_a' and R_f values for the various control and stimulated cell populations. Control cells included unlabeled cells and cells treated with TS2/18 alone or with TS2/18 and GaM. Stimulated cells included cells treated with TS2/18 and OKT3, with TS2/18 and OKT3 followed by GaM, or with TS2/18 and 9–1. Both R_a' and R_f values increased in the following order: control cells < cells treated with OKT3 < cells stimulated by OKT3 followed by GaM < cells stimulated by TS2/18 + 9–1. For both control and stimulated cells, R_a' values increased in proportion to the corresponding R_f values (Fig. 6), suggesting a strong correlation between the fraction of cells with elevated $[Ca^{2+}]_i$ and the fraction of cells with decreased CD2 mobility. The linear least-squares fit regression line describing the relationship between R_a' and R_f had a slope of 0.92, a y-intercept of −0.04, and a correlation coefficient of 0.95.

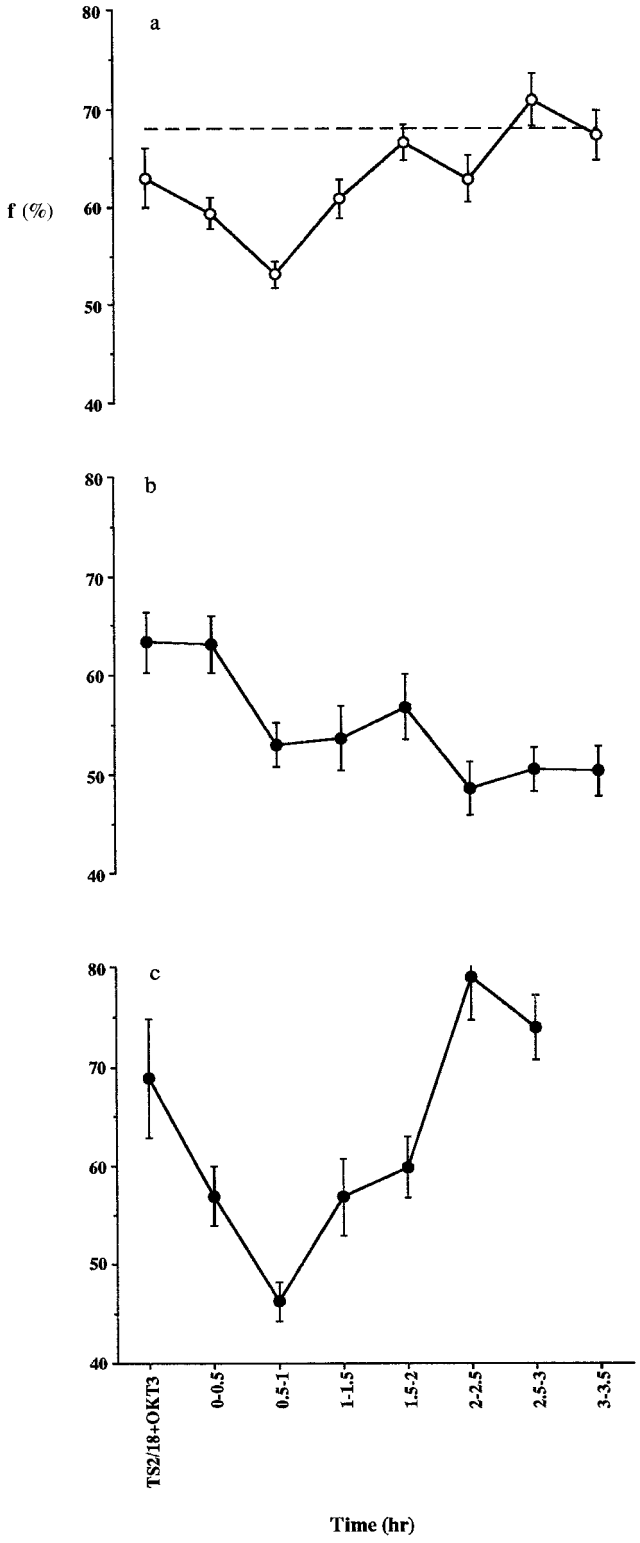

FIGURE 2 Reversal of CD2 immobilization induced by stimulation of Jurkat T cells through the TCR/CD3 complex, and effects of phosphatase inhibition on the reversal of CD2 immobilization. Cells were treated with FITC-TS2/18 + OKT3, then GaM was added at time 0 to cross-link the TCR/CD3 complex. (*a*) Data points represent the mean ± SEM of 40–150 measurements of CD2 fractional mobility (*f*, %), including all data presented in Fig. 1 *a* and data from three additional experiments in which measurements were performed for 3.5 h after GaM addition. By Student's two-tailed *t*-test, the mean *f* value at 1.5–2.0 h after GaM addition is significantly different from that at 0.5–1 h after GaM addition ($p < 0.001$). (*b*, *c*) Cells were pretreated with 100 nM cyclosporine A (*b*) or 500 nM okadaic acid (*c*). Data points represent the mean ± SEM of 26–43 measurements of CD2 fractional mobility from three experiments (*b*) and 13–16 measurements of CD2 *f* value from one experiment (*c*) in each time interval. By Student's two-tailed *t*-test, the mean *f* values of cells pretreated with cyclosporine (*b*) are significantly different from those of control cells (*a*) in the 1.5–2-h time interval ($p < 0.02$) and in all time intervals from 2 to 3.5 h ($p < 0.001$). For comparison the mean fractional mobility of CD2 in cells treated with FITC-TS2/18 alone is shown (*dashed line, a*).

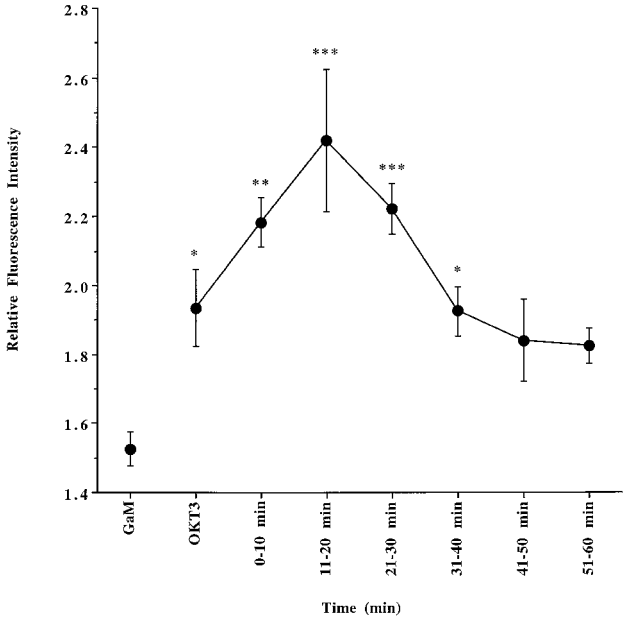

FIGURE 3 Time course of $[Ca^{2+}]_i$ changes induced by stimulation of Jurkat T cells through the TCR/CD3 complex. Cells were loaded with fluo-3, incubated with mAb-free control medium or with TS2/18 + OKT3, and washed. GaM was then added to the cells at time 0. The fluorescence intensity of control and TCR-stimulated cells was normalized by the fluorescence intensity measured in the presence of Mn^{2+}, as described in Materials and Methods, to obtain the relative fluorescence intensity. Data points represent the mean ± SEM of 74–101 measurements from five independent experiments under each treatment condition. By Student's two-tailed *t*-test, GaM alone did not induce a significant increase in $[Ca^{2+}]_i$ ($p > 0.05$), but OKT3 and OKT3 + GaM did induce significant increases in $[Ca^{2+}]_i$ compared to the $[Ca^{2+}]_i$ in control cells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The relative fluorescence intensity of cells 11–20 min after GaM addition was significantly different from that of cells treated with OKT3 alone ($p < 0.01$) and from that of cells at 51–60 min after GaM addition ($p < .01$). The fluorescence intensity of control cells did not vary over the time course of the experiments (not shown).

Inhibition of calmodulin or CaM kinase prevents the CD2 immobilization induced by cross-linking of the TCR/CD3 complex

The increase in $[Ca^{2+}]_i$ induced by OKT3 + GaM occurred immediately after GaM addition and peaked at 11–20 min of incubation (Fig. 3). In contrast, for 10 min after GaM addition, CD2 mobility remained at control levels, then began to decrease between 11 and 20 min of incubation and reached a minimum value ($f \approx 45\%$) at 21–30 min after GaM addition (Fig. 1 *a*). The 10-min delay between the

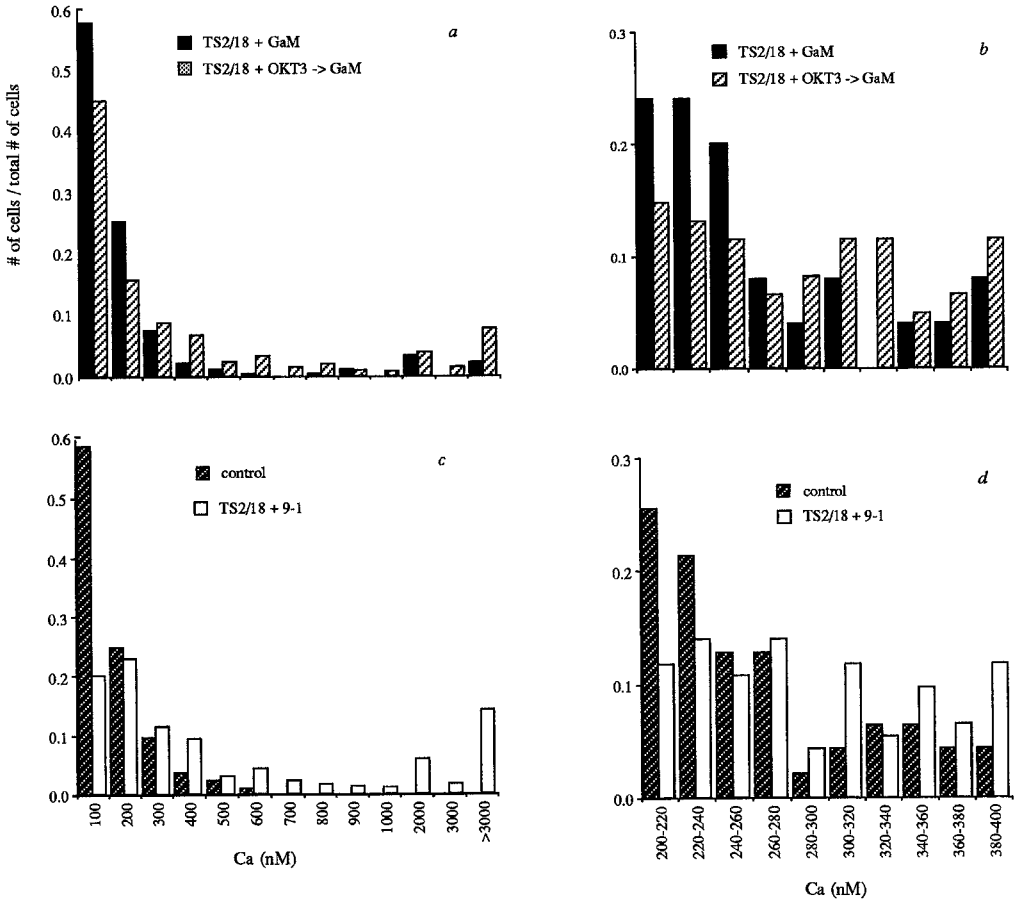

FIGURE 4 Histograms of intracellular $[Ca^{2+}]$ in control and stimulated Jurkat T cells, normalized by the total number of cells. Intracellular $[Ca^{2+}]$ was measured in control cells (*dark stripes; c, d*), cells treated with TS2/18 + GaM (*black; a, b*), cells treated with TS2/18 + OKT3 followed by GaM (*light stripes; a, b*), and cells treated with TS2/18 + 9–1 (*white; c, d*), from 0 to 60 min after the addition of GaM or 9–1. For each experimental condition, the fractions of cells with $[Ca^{2+}]_i$ from 0 to >3000 nM were calculated using data pooled from at least three independent experiments. The total numbers of measurements for each condition were 347 (*dark stripes*); 279 (*black*); 547 (*light stripes*); and 449 (*white*). *b* and *d* represent expanded portions of the data shown in *a* and *c*, respectively. The cutoff $[Ca^{2+}]_i$ value for cell stimulation was determined to be 280 nM for both TS2/18 + OKT3 → GaM and TS2/18 + 9–1-treated samples (see Results).

increase in $[Ca^{2+}]_i$ and the decrease in CD2 mobility suggested that modulation of CD2 mobility by intracellular Ca^{2+} was mediated by intermediate molecular events. To determine whether intracellular Ca^{2+} regulated CD2 lateral mobility by activation of the Ca^{2+}/CaM pathway, Jurkat cells were pretreated with a CaM inhibitor, either trifluoperazine (TFP) or W-7, and then CD2 lateral mobility was measured before and after cell stimulation by OKT3 + GaM or by a pair of anti-CD2 mAbs. Pretreatment with TFP prevented the CD2 immobilization induced by cross-linking of the TCR/CD3 complex, in a dose-dependent manner. The minimum CD2 fractional mobility after stimulation with OKT3 + GaM increased with increasing TFP concentration, reaching the control level (i.e., the level in the absence of cell stimulation) at concentrations of TFP ≥ 100 nM (Figs. 1 *d*, 7). Similarly, pretreatment with 100 μM W-7 completely prevented the CD2 immobilization induced by cross-linking of the TCR/CD3 complex (Fig. 1 *e*). These data suggested that TFP and W-7 interrupted a stimulation-induced signaling pathway leading to CD2 immobilization. Neither TFP nor W-7 inhibited the increase in $[Ca^{2+}]_i$ induced by cross-linking of the TCR/CD3 complex (data not shown). Thus the effects of TFP and W-7 on CD2 immobilization appeared to result from specific inhibition of CaM activity.

We next determined whether CaM activity was required for the Ca^{2+}-sensitive portion of the CD2 immobilization induced by a pair of anti-CD2 mAbs. In cells pretreated with either TFP or W-7, the CD2 fractional mobility decreased from 65–70% to 40–50% after stimulation by TS2/18 + 9–1, whereas in control cells such stimulation resulted in complete immobilization of CD2 ($f < 10\%$) (Table 2). The CaM-dependent (Table 2) and Ca^{2+}-dependent (Fig. 5, *d* and *f*) portions of the CD2 immobilization induced by TS2/18 + 9–1 were in quantitative agreement with one another, providing further support for the hypothesis that intracellular Ca^{2+} regulated CD2 lateral mobility through activation of CaM.

Ca^{2+}/CaM-dependent protein kinase II (CaM kinase) is activated by calmodulin. Based on the observation of a

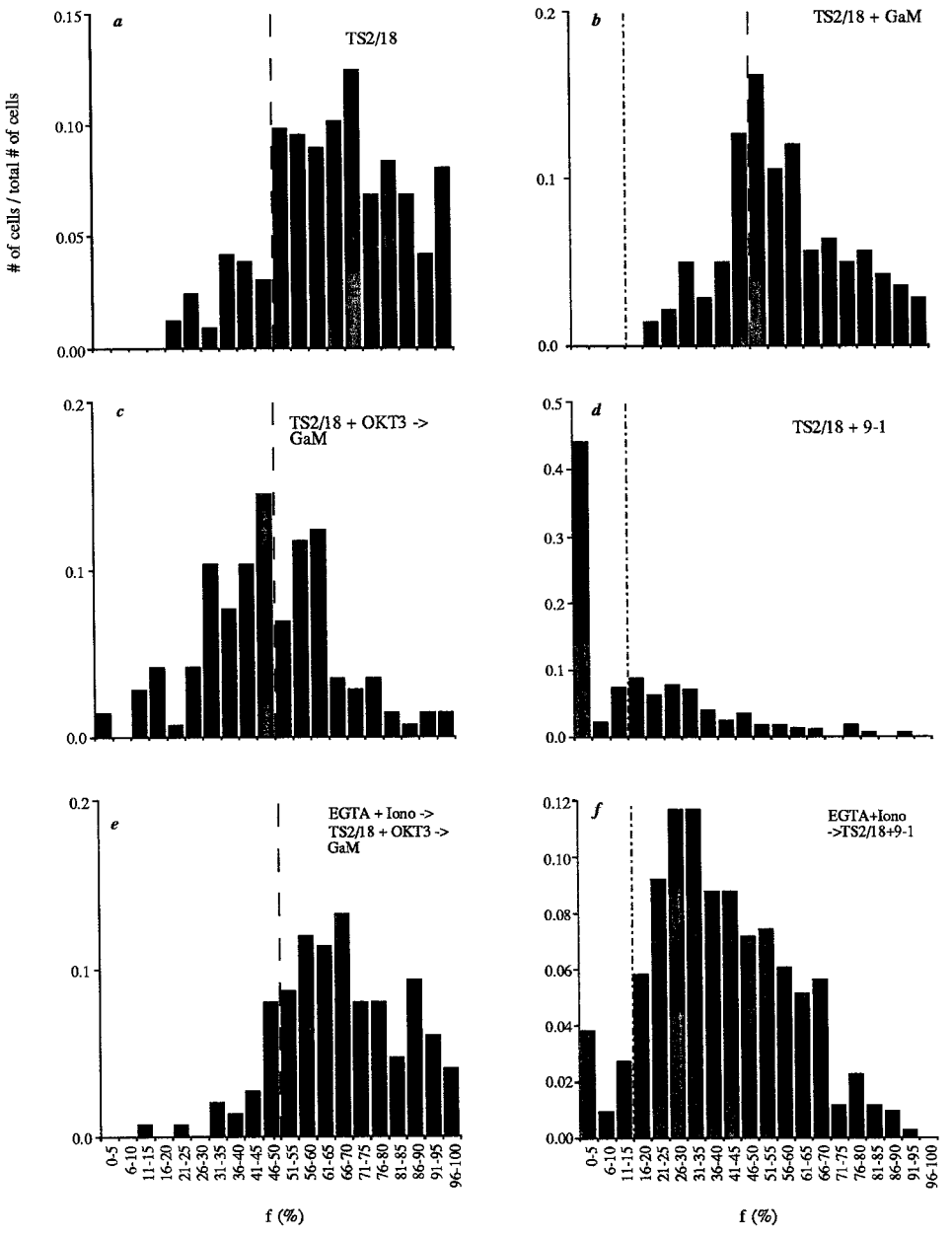

FIGURE 5 Histograms of CD2 fractional mobility in resting cells (*a* and *b*), stimulated cells (*c* and *d*), and cells pretreated with EGTA + ionomycin before stimulation (*e* and *f*), normalized by the total number of measurements. Cells were incubated with FITC-TS2/18 (*a*), FITC-TS2/18 + GaM (*b*), FITC-TS2/18 + OKT3 and then GaM (*c* and *e*), and FITC-TS2/18 + 9–1 (*d* and *f*). Fractional mobility measurements were taken 0–60 min after the addition of GaM or 9–1. The total numbers of measurements were 338 (*a*), 161 (*b*), 145 (*c*), 429 (*d*), 154 (*e*), and 450 (*f*). Data sets shown in *d* and *f* were taken from our previous report (table 1 and figure 2 of Liu et al., 1995). Vertical lines represent the cutoff *f* values for cell stimulation, which were determined to be 50% for TS2/18 + OKT3 → GaM-treated samples and 15% for TS2/18 + 9–1-treated samples (see Results).

significant delay between the return of $[Ca^{2+}]_i$ and that of CD2 fractional mobility toward baseline values after T cell stimulation by OKT3 + GaM (Figs. 1 *a*, 3), we hypothesized that CaM kinase activation was involved in the induction and maintenance of CD2 immobilization. Cells were pretreated with the selective CaM kinase inhibitor KN-62 (Ishikawa et al., 1990; Tokumitsu et al., 1990) and then stimulated by cross-linking of the TCR/CD3 complex or by a pair of anti-CD2 mAbs. Pretreatment with KN-62 completely prevented the CD2 immobilization induced by OKT3 + GaM (Fig. 1 *f*) and partially prevented the CD2 immobilization induced by TS2/18 + 9–1 (Table 2). The effects of this CaM kinase inhibitor were in quantitative agreement with the Ca^{2+}-dependent and CaM-dependent portions of the CD2 immobilization induced by OKT3 + GaM or by TS2/18 + 9–1, suggesting that intracellular Ca^{2+} and CaM regulated CD2 lateral mobility by activating CaM kinase.

Inhibition of calcineurin phosphatase prevents the reversal of CD2 immobilization induced by cross-linking of the TCR/CD3 complex

Calcineurin is a Ca^{2+}/CaM-dependent protein serine/threonine phosphatase that dephosphorylates many substrates of the Ca^{2+}/CaM-dependent kinases (Klee, 1991; Klee et al., 1988; Premack and Gardner, 1992). We hypothesized that calcineurin phosphatase activity was responsible for the reversal of CD2 fractional mobility from ~45% to ~70%

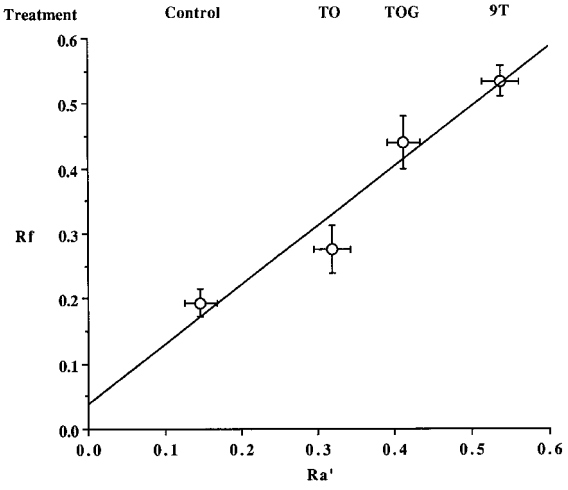

FIGURE 6 Linear correlation between fractional $[Ca^{2+}]_i$ elevation (R_a') and fractional CD2 lateral immobilization (R_f). R_a' represents the fraction of cells with $[Ca^{2+}]_i$ greater than 280 nM for each experimental treatment. R_f represents the fraction of cells with a CD2 f value less than 50% (control, TO, TOG) or less than 15% (9T). R_a' and R_f values (mean ± SEM) were calculated using data pooled from at least three independent experiments. R_f values were determined for cells treated with FITC-TS2/18 and FITC-TS2/18 + GaM (control), FITC-TS2/18 + OKT3 (TO), FITC-TS2/18 + OKT3 followed by GaM (TOG), and FITC-TS2/18 + 9–1 (9T). These R_f values were significantly different from each other with $p < 0.05$ by Student's two-tailed t-test; the total numbers of cells per sample were 338 (cont), 149 (TO), 145 (TOG), and 429 (9T). R_a' values were calculated for cells without antibody treatment, cells treated with TS2/18, and cells treated with TS2/18 + GaM (control), and for cells treated with TO, TOG, and 9T. These R_a' values were significantly different from each other with $p < 0.01$ by Student's two-tailed t-test; the total numbers of cells per sample were 278 (control), 377 (TO), 548 (TOG), and 447 (9T). Data were fitted by linear least-squares regression. The slope of the regression line was 0.92, the y-intercept was 0.04, and the correlation coefficient R^2 was 0.95.

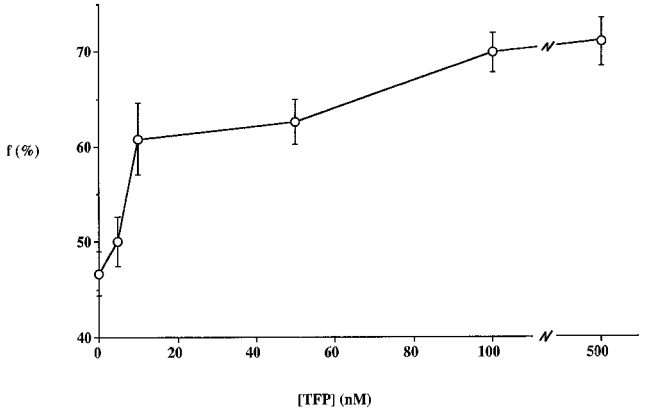

FIGURE 7 Concentration dependence of inhibition of CD2 immobilization by trifluoperazine. Jurkat cells were pretreated with various concentrations of TFP and then stimulated by cross-linking the TCR/CD3 complex with OKT3 + GaM. CD2 fractional mobility was measured 31–60 min after the addition of GaM. Data points represent the mean ± SEM of 54–97 measurements of CD2 fractional mobility (f) from at least two experiments at each TFP concentration.

1–2 h after stimulation of T cells by OKT3 + GaM. To test this hypothesis, Jurkat cells were pretreated with the calcineurin phosphatase inhibitor cyclosporine A (CsA), and CD2 lateral mobility was measured before and after cell stimulation by OKT3 + GaM. Pretreatment with CsA did not affect the immobilization of CD2 induced by OKT3 + GaM. In a dose-dependent manner, however, CsA did prevent the recovery of CD2 fractional mobility for up to 3.5 h after OKT3 + GaM treatment. The CD2 fractional mobility 1.5–3.5 h after stimulation with OKT3 + GaM decreased with increasing CsA concentration, reaching a minimum value at concentrations of CsA ≥ 100 nM (Figs. 2 *b*, 8).

Calcineurin can activate protein phosphatase 1 (PP1) and thereby trigger a protein phosphatase cascade (Cohen, 1989; Ingerbritsen and Cohen, 1983) that leads to dephosphorylation and activation of multiple proteins, including CaM kinase (Klee, 1991). To determine whether the Ca^{2+}/CaM-independent phosphatases were involved in regulation of CD2 lateral mobility, Jurkat cells were pretreated with the PP1/PP2A inhibitor okadaic acid, and CD2 lateral mobility was measured before and after cell stimulation by OKT3 + GaM. Pretreatment with 500 nM okadaic acid did not change the kinetics of either the decrease or the spontaneous reversal in CD2 fractional mobility induced by cross-linking of the TCR/CD3 complex (Fig. 2 *c*), suggesting that neither PP1 nor PP2A was involved in initiating CaM kinase-mediated CD2 immobilization or in reversing the effects of CaM kinase. Taken together, these data suggested that specific activation of calcineurin phosphatase by Ca^{2+}/CaM was responsible for the reversal of CD2 immobilization induced by cross-linking of the TCR/CD3 complex.

DISCUSSION

In this report we show that T cell stimulation by cross-linking of the TCR/CD3 complex reduces CD2 fractional mobility from ~70% to ~45%. This result is consistent with our previous observation that T cell stimulation by pairs of anti-CD2 mAbs causes CD2 immobilization (Liu et

TABLE 2 Effects of CaM and CaM kinase inhibitors on the immobilization of CD2 induced by stimulation of Jurkat T cells through CD2

	mAb treatment			
	TS2/18		TS2/18 + 9-1	
Pretreatment	f (%)	N	f (%)	N
None*	68 ± 1	338	<10 (1, 2, 3)	149
TFP	66 ± 3	41	50 ± 2(1)	100
W-7	65 ± 4	14	40 ± 2(2)	62
KN-62	73 ± 3	23	42 ± 2(3)	107

After pretreatment, cells were incubated with TS2/18 and then with 9-1. FPR measurements of CD2 lateral mobility were made for 60 min after the addition of 9-1. The time course of CD2 immobilization induced upon the addition of 9-1 is shown in figure 2 of Liu et al. (1995). f, fractional mobility; N, number of measurements. f values represent the mean ± SEM. *Control data are reproduced from Table 1 of this study and from table 2 of Liu et al. (1995). 1, 2, 3, $p < 0.001$, Student's two-tailed t-test.

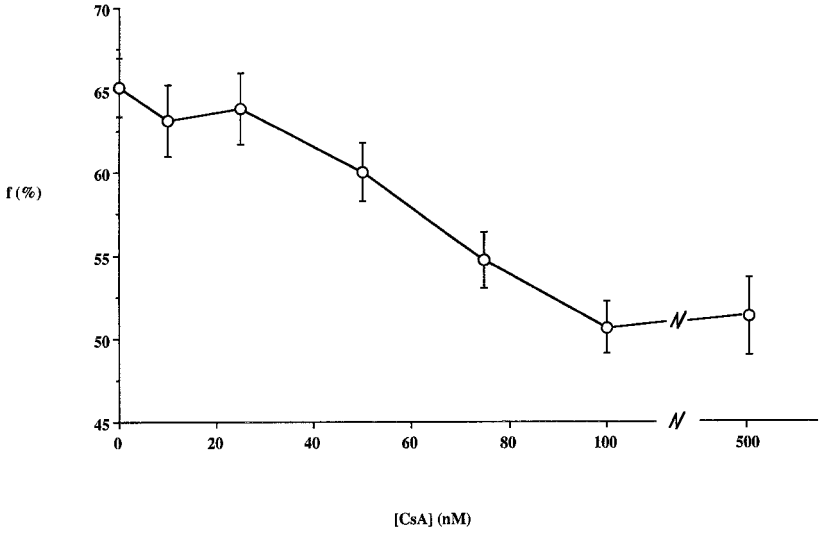

FIGURE 8 Concentration dependence of inhibition of the reversal of CD2 immobilization by cyclosporine A. Jurkat cells were pretreated with various concentrations of CsA and then stimulated by cross-linking the TCR/CD3 complex with OKT3 + GaM. CD2 fractional mobility was measured 1.5–3.5 h after the addition of GaM. Data points represent the mean ± SEM of 47–103 measurements of CD2 fractional mobility (*f*) from one or two experiments at each CsA concentration.

al., 1995). Similar correlations between cell activation and surface receptor immobilization have also been found in neutrophils stimulated with fMLP (Johansson et al., 1993) and in the human T cell line HPB-ALL stimulated with bivalent anti-CD3 mAb (Hashemi et al., 1992).

Results from this and our previous report (Liu et al., 1995) support the hypothesis that increased intracellular $[Ca^{2+}]$ is required for lateral immobilization of CD2. First, depletion of intracellular Ca^{2+} prevents the decrease in CD2 fractional mobility in cells stimulated by cross-linking of the TCR/CD3 complex (the present study) and prevents a significant portion of the CD2 immobilization induced by pairs of anti-CD2 mAbs (Liu et al., 1995). The difference between the fractional mobility of CD2 with and without EGTA + ionomycin pretreatment is 20–25% in cells stimulated either by cross-linking of the TCR/CD3 complex or by pairs of anti-CD2 mAbs. Second, kinetic studies show that $[Ca^{2+}]_i$ increases 10 min before CD2 becomes immobilized, either by cross-linking of the TCR/CD3 complex (the present study) or by pairs of anti-CD2 mAbs (Liu et al., 1995). Third, for each experimental treatment the fraction of cells with elevated $[Ca^{2+}]_i$ is equal to the fraction of cells with decreased CD2 mobility. Fourth, inhibition of the activity of CaM or CaM kinase, which are dependent on increased $[Ca^{2+}]_i$, completely prevents CD2 immobilization in cells stimulated by cross-linking of the TCR/CD3 complex. Although these results strongly suggest that increased $[Ca^{2+}]_i$ is necessary for CD2 immobilization, it is possible that Ca^{2+}-independent signaling pathways triggered by T cell stimulation are also involved. The requirement, if any, for activation of such pathways remains to be elucidated.

CaM, an intracellular Ca^{2+} binding protein, binds to and regulates the function of enzymes such as kinases and phosphatases and of cytoskeletal proteins such as actin in T lymphocytes (Premack and Gardner, 1992). Among other activities, CaM binds to lymphocyte plasma membranes upon cell stimulation by PHA and concanavalin A; this binding is inhibited by TFP or W-7. The concentration of TFP that inhibits CaM binding to the membrane by 50% (IC_{50}) is 20–30 μM (Lee et al., 1987). Although the discrepancy between this value and the IC_{50} value found here for inhibition of Ca^{2+}/CaM-mediated CD2 immobilization remains to be explained, the ability of TFP to prevent CD2 immobilization is unlikely to be due to nonspecific effects such as those observed at high concentrations of the inhibitor (>10 μM) (Davis et al., 1983; Valverde et al., 1981). Another CaM inhibitor, W-7, causes inhibition of CaM binding to the membrane (Lee et al., 1987) and of CD2 immobilization induced by cross-linking of the TCR/CD3 complex at similar concentrations.

Ca^{2+}/CaM-dependent protein kinase II (CaM kinase) also plays an important role in T lymphocyte activation (Bland et al., 1993; Hanson and Schulman, 1992; Nghiem et al., 1993). CaM kinase is activated by CaM binding to the regulatory domain of the enzyme, which leads to deinhibition of kinase activity. Autophosphorylation of a conserved threonine residue in the regulatory domain slows CaM dissociation, thereby prolonging kinase activity. Autophosphorylation of the regulatory domain also changes the kinase from a Ca^{2+}-dependent to a Ca^{2+}-independent form, thus potentiating the response to transient Ca^{2+} signals (Schulman, 1993; Schulman et al., 1992). Eventually, dephosphorylation of CaM kinase renders the enzyme inactive. Here we find that $[Ca^{2+}]_i$ increases immediately after cell stimulation, whereas CD2 becomes immobilized 10 min later, and that the fraction of cells with elevated $[Ca^{2+}]_i$ declines 30 min after cell stimulation, whereas CD2 remains immobilized for 60 min. This sequence of events is consistent with the kinetics of CaM kinase activation. Ca^{2+}/CaM-induced autophosphorylation of CaM kinase could maintain the enzyme in an activated state and thereby prolong the immobilization of CD2. CaM kinase has broad substrate specificity; the enzyme phosphorylates caldesmon, intermediate filaments, microtubule-associated proteins, calcineurin, and other substrates (Hanson and Schulman, 1992). CaM kinase could regulate interactions between cy-

toskeletal proteins and the cytoplasmic domain of CD2 by phosphorylation of these or other protein targets.

Calcineurin, a Ca^{2+}-dependent protein phosphatase, dephosphorylates phosphoserine and phosphothreonine residues on many substrates of the Ca^{2+}-dependent kinases. The enzyme is therefore an important feedback regulator of Ca^{2+}-dependent phosphorylation (Klee, 1991; Premack and Gardner, 1992). Ca^{2+}/CaM activates calcineurin by binding to the A subunit of the enzyme (Hubbard and Klee, 1989). Calcineurin activity is also modulated by CaM kinase- or PKC-mediated phosphorylation of a serine residue within the CaM-binding domain, which prevents CaM binding (Hashimoto and Soderling, 1989). In T lymphocytes, both antigen-dependent (TCR-mediated) and -independent (CD2-mediated) pathways activate calcineurin, which in turn dephosphorylates the CD3 γ-chain and activates the transcription factor NF-AT. NF-AT then translocates from the cytoplasm to the nucleus and initiates transcription of interleukin-2 and other genes (Alexander et al., 1989; Bierer et al., 1991, 1993; Elliott et al., 1984; Emmel et al., 1989; Granelli-Piperno et al., 1986; Kino et al., 1987a,b; Kronke et al., 1984; Premack and Gardner, 1992; Thomson, 1989; Tocci et al., 1989). CsA inhibits these calcineurin-dependent activities by binding to its intracellular receptor cyclophilin, which then forms a complex with calcineurin and inhibits calcineurin phosphatase activity (Bierer et al., 1993; Clipstone and Crabtree, 1992; Dutz et al., 1993; Emmel et al., 1989; Flanagan et al., 1991; Fruman et al., 1992; Liu et al., 1991, 1992; Schreiber, 1991; Schreiber and Crabtree, 1992). Here we find that CsA specifically prevents the reversal of CD2 immobilization upon cross-linking of the TCR/CD3 complex; the concentration dependence of this effect agrees quantitatively with that for inhibition of calcineurin phosphatase activity in Jurkat cells activated by OKT3 and PMA (Fruman et al., 1992). It therefore appears that calcineurin phosphatase activity is involved in the reversal of CD2 immobilization induced by T cell stimulation. Calcineurin could act to reverse CD2 immobilization by dephosphorylating cytoskeleton-associated substrates such as MAP-2 and tau (Klee, 1991).

This work suggests that two Ca^{2+}/CaM-dependent enzymes, CaM kinase and calcineurin phosphatase, are involved in the CD2 immobilization and the subsequent reversal of CD2 immobilization, respectively, in T cells stimulated by cross-linking of the TCR/CD3 complex. The mechanism by which simultaneous activation of enzymes with opposing functions could result in sequential CD2 immobilization and reversal of immobilization is likely to involve differential Ca^{2+}/CaM affinities and kinetics of the two enzymes. CaM binds with 100-1000-fold greater affinity to calcineurin than to CaM kinase (Klee, 1991), and calcineurin activation is more rapid and requires lower concentrations of Ca^{2+} than does CaM kinase activation. Thus calcineurin activation is likely to occur immediately upon a TCR-mediated increase in $[Ca^{2+}]_i$, whereas the relatively low $[Ca^{2+}]_i$ is still insufficient to activate CaM kinase. This delay in CaM kinase activation could explain the 10-min delay between the increase in $[Ca^{2+}]_i$ and the immobilization of CD2. As $[Ca^{2+}]_i$ increases, sufficient Ca^{2+}/CaM could be generated to activate CaM kinase; the resulting autophosphorylation of CaM kinase could then prolong the activity of this enzyme even after the peak of $[Ca^{2+}]_i$ is reached. Activated CaM kinase could also phosphorylate a serine residue within the CaM-binding domain of calcineurin, thereby preventing stable CaM binding to calcineurin and deactivating the phosphatase (Hashimoto and Soderling, 1989). By these mechanisms CaM kinase activity could become dominant, such that CD2 immobilization is sustained for 60 min after TCR-mediated cell stimulation. As $[Ca^{2+}]_i$ then decreases, CaM kinase could become deactivated, both because of decreased Ca^{2+}/CaM binding and because of calcineurin-mediated dephosphorylation of the autoregulatory residues on CaM kinase that potentiate enzyme activity. The increased calcineurin activity and decreased CaM kinase activity could eventually result in dephosphorylation of CaM kinase substrate phosphorylation sites, leading to the reversal of CD2 immobilization. Studies similar to those reported here, on cells with mutations in CD2 and CD2-associated proteins, could further define these molecular mechanisms.

T cell activation regulates the strength of cell-cell adhesion mediated by the receptor-ligand pair CD2-CD58 (Hahn et al., 1993). Stimulation of cells by anti-CD3 mAb induces a transient increase in CD2 avidity for CD58, reaching a maximum at 30 min after cell stimulation and returning to basal levels within 90 min (Hahn et al., 1993). Here we find that TCR-mediated increases in $[Ca^{2+}]_i$ induce CD2 immobilization, and that the CD2 immobilization spontaneously reverses between 1 and 2 h after cell stimulation. Thus the kinetics of changes in CD2 mobility upon cell stimulation appear to correlate temporally with the effects of TCR-mediated regulation of CD2 avidity for CD58.

The ability of $[Ca^{2+}]_i$ to modulate CD2 mobility is likely to contribute to changes in T cell adhesivity to target membranes. T cell stimulation through the TCR/CD3 complex or through CD2 could induce rapid increases in $[Ca^{2+}]_i$, activating CaM and CaM kinase and leading both to phosphorylation of CD2 or a closely associated protein and to lateral immobilization of CD2. Because there is a 10-min delay between the TCR/CD3- or CD2-induced $[Ca^{2+}]_i$ increase and CD2 immobilization, CD2 is capable of anisotropic redistribution from noncontact regions of the T cell membrane into sites of contact with target membranes during this time period. Confocal fluorescence microscopy has recently shown that CD2 redistributes into sites of contact with model bilayers expressing CD58 on a time scale of ~10 min (Zhu et al., manuscript in preparation). After the 10-min delay, immobilization of CD2 could maintain this adhesion molecule at high density at the contact sites, thereby decreasing the chemical activity of the stabilized CD2-CD58 adhesion complexes and increasing cellular adhesion strength. Subsequently, activation of calcineurin could cause both dephosphorylation of the previously phosphorylated residues on CD2 or a closely associated protein, and

reversal of CD2 immobilization. This reversal of CD2 immobilization 1–2 h after cell stimulation could allow CD2 to redistribute laterally away from contact sites, thus destabilizing CD2-CD58 binding interactions and facilitating T cell "deadhesion" (detachment) from target membranes. This model could underlie the observations that an increase in $[Ca^{2+}]_i$ both stabilizes interactions between T cells and APCs, and reduces the ability of T cells to migrate (Donnadieu et al., 1994; Negulescu et al., 1996).

The topology of the contact site between apposing T lymphocyte and APC membranes could enhance cell-cell adhesion strength by augmenting the mechanisms that regulate CD2 mobility. It has recently been suggested that kinases associated with the inner surface of the T cell plasma membrane are included within such contact sites, whereas membrane-associated phosphatases are physically excluded from such sites (Shaw and Dustin, 1997). Prolongation of CaM kinase action, which appears to be involved in the immobilization of cell surface CD2, and prevention of calcineurin phosphatase action, which appears to mediate the reversal of CD2 immobilization, could stabilize immobilization of CD2 at the contact site and thereby promote adhesive cell-cell interactions. It has also been proposed that cross-linking of CD58 on APCs induces immobilization of class II MHC proteins in the membranes of these cells (Bierer et al., 1989). Because MHC proteins present antigen to the TCR and thereby stimulate and maintain T cell activation, this observation raises the intriguing possibility that the adhesion strength of interactions between T cells and APCs could be regulated not only by immobilization of receptors on the T cell membrane, but also by immobilization of ligands for T cell surface receptors on the APC membrane.

More generally, phosphorylation and dephosphorylation activities may be important in modulating adhesion mediated by a variety of receptor/ligand pairs. For example, chemoattractant (fMLP) stimulation of neutrophils induces repeated transient increases in $[Ca^{2+}]_i$, and inhibition of calcineurin retards release of neutrophils from sites of adhesion to vitronectin (Hendey et al., 1992). Transient increases in neutrophil $[Ca^{2+}]_i$ could regulate cellular adhesion strength by modulating the lateral mobility of vitronectin receptors, which are α-v and β-3-like integrins (Lawson and Maxfield, 1995). In one such model, increased $[Ca^{2+}]_i$ could immobilize cell surface integrins, leading to an increase in cellular adhesion strength, whereas subsequent calcineurin activation could reverse receptor immobilization and promote release of cells from adhesive surfaces by allowing lateral diffusion of adhesion molecules away from contact sites.

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