Characterization of T Cell Mutants with Defects in Capacitative Calcium Entry: Genetic Evidence for the Physiological Roles of CRAC Channels

Christopher M. Fanger,* Markus Hoth,* Gerald R. Crabtree,‡ and Richard S. Lewis*

*Department of Molecular and Cellular Physiology and ‡Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94305-5426

Abstract. Prolonged Ca^{2+} influx is an essential signal for the activation of T lymphocytes by antigen. This influx is thought to occur through highly selective Ca^{2+} release-activated Ca^{2+} (CRAC) channels that are activated by the depletion of intracellular Ca^{2+} stores. We have isolated mutants of the Jurkat human T cell line NZdipA to explore the molecular mechanisms that underlie capacitative Ca^{2+} entry and to allow a genetic test of the functions of CRAC channels in T cells. Five mutant cell lines (CJ-1 through CJ-5) were selected based on their failure to express a lethal diphtheria toxin A chain gene and a *lacZ* reporter gene driven by NF-AT, a Ca^{2+}- and protein kinase C-dependent transcription factor. The rate of Ca^{2+} influx evoked by thapsigargin was reduced to varying degrees in the mutant cells whereas the dependence of NF-AT/*lacZ* gene transcription on $[Ca^{2+}]_i$ was unaltered, suggesting that the transcriptional defect in these cells is caused by a reduced level of capacitative Ca^{2+} entry. We examined several factors that determine the rate of Ca^{2+} entry, including CRAC channel activity, K^+-channel activity, and Ca^{2+} clearance mechanisms. The only parameter found to be dramatically altered in most of the mutant lines was the amplitude of the Ca^{2+} current (I_{CRAC}), which ranged from 1 to 41% of that seen in parental control cells. In each case, the severity of the I_{CRAC} defect was closely correlated with deficits in Ca^{2+} influx rate and Ca^{2+}-dependent gene transcription. Behavior of the mutant cells provides genetic evidence for several roles of I_{CRAC} in T cells. First, mitogenic doses of ionomycin appear to elevate $[Ca^{2+}]_i$ primarily by activating CRAC channels. Second, I_{CRAC} promotes the refilling of empty Ca^{2+} stores. Finally, CRAC channels are solely responsible for the Ca^{2+} influx that underlies antigen-mediated T cell activation. These mutant cell lines may provide a useful system for isolating, expressing, and exploring the functions of genes involved in capacitative Ca^{2+} entry.

T HE activation of T lymphocytes encompasses a highly coordinated sequence of cellular events, beginning with antigen binding to the T cell receptor (TCR)[1] and culminating in clonal T cell proliferation and the acquisition of immune function. The earliest events include tyrosine kinase activation, which triggers the activation of phospholipase Cγ and the consequent generation of diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃; Weiss and Littman, 1994). DG activates protein kinase C (PKC), whereas IP₃ leads to a biphasic rise in $[Ca^{2+}]_i$; both of these signals appear to be required to promote T cell activation, largely by ensuring the production of interleukin-2 (IL-2), which drives the progression of T cells through the cell cycle in a Ca^{2+}- and PKC-independent fashion (Truneh et al., 1985; Crabtree and Clipstone, 1994).

$[Ca^{2+}]_i$ must be elevated for tens of minutes to enable IL-2 production and the commitment of T cells to the activation pathway, yet release of Ca^{2+} from intracellular stores by IP₃ produces only a transient rise and is unable by itself to support activation (Goldsmith and Weiss, 1988; Negulescu et al., 1994). Several studies in T cells (Gouy et al., 1990; Mason et al., 1991; Sarkadi et al., 1991) have shown that the Ca^{2+} signal is sustained by a process termed capacitative Ca^{2+} entry (Putney, 1990), by which the maintained depletion of intracellular stores by IP₃ activates Ca^{2+} influx across the plasma membrane. The channels underlying capacitative Ca^{2+} entry have been characterized in detail in several nonexcitable cells, including T cells and mast cells (Hoth and Penner, 1993; Zweifach and Lewis, 1993; Premack et al., 1994; for reviews see Penner

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1. *Abbreviations used in this paper:* β-gal, β-galactosidase; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CPA, cyclopiazonic acid; dipA, diphtheria toxin A chain; EF-2, elongation factor 2; FDG, fluorescein di-β-D-galactopyranoside; I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current; IL-2, interleukin-2; IP₃, inositol 1,4,5-trisphosphate; MUG, 4-methylumbelliferyl β-D-galactoside; NF-AT, nuclear factor of activated T cells; PdBU, phorbol 12,13-dibutyrate; PKC, protein kinase C; TCR, T cell receptor; TG, thapsigargin.

et al., 1993; Fasolato et al., 1994; Lewis and Cahalan, 1995). The corresponding current has been termed Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}; Hoth and Penner, 1992), and it is thought to be responsible for $[Ca^{2+}]_i$ oscillations in T cells (Lewis and Cahalan, 1989; Donnadieu et al., 1992; Dolmetsch and Lewis, 1994), as well as for prolonged Ca^{2+} elevation in activated mast cells (Fasolato et al., 1993). The capacitative Ca^{2+} entry mechanism appears to be extremely widespread (for review see Putney and Bird, 1993) and may involve multiple types of Ca^{2+}-permeable, store-operated channels (SOCs; Lückhoff and Clapham, 1994; Vaca et al., 1994) of which the CRAC channels described above are one subtype.

Little is known about the capacitative Ca^{2+} entry mechanism at a molecular level. The sensor for the content of Ca^{2+} stores, as well as the signaling pathway that communicates store depletion to the CRAC channels, has not yet been identified. Multiple signaling mechanisms have been proposed, including a novel diffusible activator, GTP-binding proteins, tyrosine kinases, cGMP and direct coupling between the proteins in the ER and the plasma membrane (for review see Putney and Bird, 1993; Fasolato et al., 1994). A novel approach may be needed to resolve the mechanism of CRAC channel activation.

Isolation and cloning of the CRAC channel would aid greatly in studies of the activation mechanism, but several factors make this a challenging goal. First, a lack of specific, high-affinity I_{CRAC} inhibitors precludes channel purification based on ligand binding. CRAC channels are insensitive to a variety of classical Ca^{2+} channel antagonists but are inhibited by imidazole antimycotic compounds (e.g., econazole and SK&F 96365) with $K_{1/2}$ values in the micromolar range (Chung et al., 1994; Franzius et al., 1994). However, at these concentrations, the compounds also block other types of channels (Villalobos et al., 1992; Franzius et al., 1994), making their use problematic in studies of the functional roles of CRAC channels in intact cells. Second, CRAC channels differ in their fundamental properties of ion selectivity, gating, and unitary conductance from all previously cloned voltage-gated Ca^{2+} channels, lessening the likelihood of a successful cloning approach based on homology. However, recent evidence that the trp gene of Drosophila encodes a depletion-activated channel similar but not identical to the CRAC channel (Vaca et al., 1994) raises the possibility that homologous genes may encode channels underlying capacitative Ca^{2+} entry in vertebrates. Finally, the ubiquitous nature of capacitative Ca^{2+} entry complicates cloning attempts based on heterologous expression in oocytes and other cells, and will hinder functional studies of cloned and mutated CRAC channel genes.

Mutant cell lines defective for capacitative Ca^{2+} entry may provide powerful tools to address these problems. A recent study by Partiseti et al. described a patient with a severe T cell immunodeficiency due to a defect in CRAC channel activity (Partiseti et al., 1994). In addition, a specific method for selecting Ca^{2+} influx mutants has been described (Serafini et al., 1995), employing a strategy based on activation of the nuclear factor of activated T cells (NF-AT), a ras/PKC- and Ca^{2+}-dependent transcription factor (Clipstone and Crabtree, 1992). NF-AT integrates the ras/PKC- and Ca^{2+}-signaling pathways in the following way. A

nuclear-targeted subunit (NF-AT$_n$) is synthesized de novo in response to PKC activation, while a cytosolic subunit (NF-AT$_c$ or NF-AT$_p$) translocates to the nucleus in response to the activation of the phosphatase calcineurin by Ca^{2+}/calmodulin (Crabtree and Clipstone, 1994; Rao, 1994). Association of the nuclear and cytosolic subunits within the nucleus creates active NF-AT, which in conjunction with other transcription factors promotes the synthesis of IL-2 and other T cell activation proteins (Durand et al., 1988; Flanagan et al., 1991). Serafini et al. (1995) exploited the extremely high induction ratios of NF-AT to construct a Ca^{2+}-dependent "suicide" gene in the Jurkat human T cell line, consisting of a trimer of NF-AT binding sites driving the expression of the diphtheria toxin A chain (dipA) gene. Mutagenized cells that survived in the presence of ionomycin and phorbol ester included two mutant clones, M101 and M108, that displayed a significant defect in capacitative Ca^{2+} entry.

Here we report the isolation of five additional Jurkat mutants (CJ-1 through CJ-5) defective for capacitative Ca^{2+} entry. We have characterized the physiological basis of the deficits in NF-AT–dependent gene transcription and Ca^{2+} influx in these and the previously derived mutants. In all cases, the severity of the defects in gene transcription and capacitative Ca^{2+} entry are closely linked. Furthermore, the reduced level of Ca^{2+} influx in the mutants appears to be due to a selective defect in either the CRAC channel, its expression, or its activation, rather than to other factors like K^+ channel expression or Ca^{2+} clearance mechanisms that indirectly influence net Ca^{2+} influx. These mutants provide compelling genetic evidence for the roles of I_{CRAC} in refilling stores and in mediating the sustained $[Ca^{2+}]_i$ rise necessary for the activation of T lymphocytes by antigen. The mutant cell lines may also present a tractable system for the isolation and expression of genes encoding elements of the capacitative Ca^{2+} entry pathway.

Materials and Methods

Cell Lines and Culture

The Jurkat clones J.NFATZ.1 (Fiering et al., 1990) and NZDipA.1.5.22 (Serafini et al., 1995) have been described previously. J.NFATZ.1 cells carry a construct consisting of the hygromycin resistance gene and a trimer of NF-AT binding domains linked to a minimal IL-2 promoter driving expression of lacZ. The parental line (NZDipA 1.5.22) was derived from J.NFATZ.1 by stable transfection with a similar construct including the neomycin resistance gene and in which NF-AT controls the expression of dipA, the gene encoding the membrane-impermeant catalytic A chain of diphtheria toxin. Cells were grown in culture medium consisting of RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FCS (Gemini Bio-Products, Inc., Calabasas, CA), 2 mM glutamine (Mediatech), and penicillin-streptomycin (50 U/ml and 50 μg/ml; Mediatech). Cells were continuously maintained in log-phase growth at 37°C with 6% CO_2. Cells were counted in the presence of acridine orange/ethidium bromide stain to distinguish live from dead cells. Cells were cycled bimonthly through three passages in 300 μg/ml hygromycin (Calbiochem-Novabiochem International, San Diego, CA) and 1 mg/ml geneticin (Sigma Chemical Co., St. Louis, MO) to ensure the stability of the NFATZ and NFAT-DipA constructs, respectively. OKT3 mAb (Ortho Pharmaceuticals, Raritan, NJ) and goat anti–mouse IgG (Southern Biotechnologies, Birmingham, AL) were generously provided by Dr. P. Katsikis (Stanford Univ., Stanford, CA). Anti-integrin–associated protein mAb was the kind gift of Dr. F. Lindberg (Washington Univ., St. Louis, MO), and anti-CD5, CD11a, and CD45 mAbs were provided by Dr. M. Roederer (Stanford Univ., Stanford, CA). All other antibodies were purchased from Caltag (So. San Francisco, CA).

Mutagenesis

Mutants were generated following a protocol modified from Serafini et al. (1995). 2×10^8 NZDipA cells were mutagenized by 200 rads of γ radiation from a ^{137}Cs source (<30% lethality). The irradiated cells were divided equally into 13 flasks within 1 h and were thereafter passaged independently. After 6 d, cells ($\sim 5 \times 10^5$/ml) were treated for 24 h at 37°C in stimulation medium (culture medium containing 2 μM ionomycin [Calbiochem-Novabiochem] and 50 nM phorbol 12,13-dibutyrate [PdBU; Sigma]), washed twice and returned to culture medium at a density of $0.5-1 \times 10^6$/ml. The parental (NZDipA) cell line, in which NF-AT controls expression of dipA, dies in response to stimulation with the NF-AT activators ionomycin and PdBU within 3–4 d. The cells were stimulated again after they resumed a normal growth rate, and this protocol was repeated 3–6 times until the cells no longer died significantly after stimulation. Surviving cells include the desired trans mutants with defects in NF-AT–dependent gene transcription as well as cis mutants. Cis mutants include cells in which the NFAT-dipA construct has been modified or lost, and cells that have acquired dipA tolerance. Many of the undesired cis mutants were eliminated by FACS-sorting 10^6 cells from each flask and retaining those with low $[Ca^{2+}]_i$ after treatment with 1 μM thapsigargin (TG; LC Services, Woburn, MA), or those with low β-galactosidase (β-gal) expression (see below) after an 8-h incubation in stimulation medium. Each flask was sorted 3–6 times in this way, allowing 4–6 d recovery between sorts, and cloned into 96-well plates in culture medium containing 20% HL-1 (Hycor, Portland, ME), yielding a total of 956 clones. Of these, 239 clones tested negative for β-gal in a 4-methylumbelliferyl β-D-galactoside (MUG) screening assay (see below) and were grown in hygromycin and geneticin for three passages to eliminate any remaining cis mutants. MUG assays were repeated and more strictly scored on the surviving 82 clones, reducing the total number to 36. We selected for further analysis three clones derived from different original flasks (CJ-2 sorted on Ca^{2+}; CJ-4 and CJ-5 sorted on β-gal) and two clones derived from the same flask but displaying distinctly different Ca^{2+} influx phenotypes (CJ-1 and CJ-3, sorted on both β-gal and Ca^{2+}). One additional clone, CJ-6, had a defect in β-gal induction but no detectable alteration in Ca^{2+} entry. This clone was not further characterized. The remaining 29 clones were omitted from further consideration because they were derived from the same flasks as those described above and showed similar Ca^{2+} signaling phenotypes, increasing the likelihood that they represent sibling clones. In this way, we maximized the likelihood that clones CJ-1 through CJ-5 arose through separate mutagenic events. All clones died rapidly in diphtheria toxin (2 μg/ml), indicating that their derivation was in no way due to the acquisition of dipA resistance.

Construction of Diphtheria Toxin-resistant Cell Lines

10^7 Mutant and parental (NZDipA) cells were transfected with 50 μg of uncut pgHED7-1 cDNA plasmid by electroporation (0.25 V, 960 μF; Bio-Rad) in 300 μl culture medium. pgHED7-1 contains a mutant, ADP-ribosylation-resistant form of elongation factor 2 (EF-2) that confers resistance to protein synthesis inhibition by diphtheria toxin (Nakanishi et al., 1988). Cells were then seeded at a density of 10^5/ml into 24-well culture plates. After 48 h, diphtheria toxin (Calbiochem-Novabiochem) was added to a final concentration of 2 μg/ml (sufficient to kill 100% of control cells within 3 d) and was maintained at this level by periodic replacement of the medium for three weeks. Subclones of surviving cells were selected using the criterion that their acute Ca^{2+} response to TG closely matched that of the original (dipA-sensitive) clone. Diphtheria toxin did not alter the growth rate of any of the mutant EF-2-transfected clones.

Stimulation and β-Galactosidase Assays

Cells (5×10^5/ml) were treated for 8 h at 37°C in stimulation medium. Expression of lacZ was assayed by the FACS-Gal technique (Roederer et al., 1991), in which cells were loaded with fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR), incubated on ice to allow β-gal to cleave FDG and generate fluorescein, and analyzed or sorted by FACS. Cells with fluorescence equivalent to unstimulated controls were considered β-gal–negative. MUG assays were also used for quantitation of LacZ gene activation (Roederer et al., 1991). Briefly, 10^5 cells were placed in each well of a 96-well plate and stimulated as described above. Cells were then lysed, MUG solution was added to a final concentration of 3 mM, and fluorescence was measured using a Fluoroskan II platereader (Titertek, Elfab Oy, Finland). For experiments correlating β-gal

production with $[Ca^{2+}]_i$, cells were loaded with fura-2 (see below) and stimulated for MUG assays but with phenol red-deficient medium containing varying amounts of Ca^{2+}. Free $[Ca^{2+}]$ was adjusted by addition of $CaCl_2$ or EGTA, based on a concentration of 0.7 mM Ca^{2+} in normal culture medium calculated from the composition of RPMI and lot analyses of FCS obtained from the manufacturers.

FACS Sorting for Low $[Ca^{2+}]_i$

Cells were loaded with 1 μM indo-1/AM (Molecular Probes) for 30 min at 22–25°C, washed, and resuspended for analysis in staining medium (RPMI-1640 deficient in phenol red, 4% FCS, 10 mM Hepes, pH 7.4). Loaded cells were stimulated with 1 μM TG, and the ratio of fluorescence emissions at 405 and 515 nm were measured with a FACStar Plus (Becton-Dickinson, Los Angeles, CA) as an indication of $[Ca^{2+}]_i$ (Chused et al., 1987). In control Jurkat cells, the 405/515 emission ratio reached an elevated plateau within 5–7 min after TG treatment that was relatively stable for >30 min. Mutagenized cells with emission ratios in the lowest 5% of the population were collected during a period of 8–25 min after TG addition.

Video Microscopic Measurements of $[Ca^{2+}]_i$

Cells were loaded at 22–25°C for 30 min with 1 μM fura-2/AM (Molecular Probes) and attached to poly-L-lysine-coated glass coverslip chambers on the stage of a Zeiss Axiovert 35 microscope equipped with a Zeiss Achrostigmat objective (NA 1.3). Imaging experiments were performed as previously described (Dolmetsch and Lewis, 1994). Cells were alternately illuminated at 350 ± 5 nm and 380 ± 6 nm, and the fluorescence emission at λ >480 nm was captured with an intensified CCD camera (Hamamatsu Corp., Bridgewater, NJ) and was digitized and analyzed using a VideoProbe imaging system (ETM Systems, Irvine, CA). Ratio images were recorded at intervals of 3–5 s. $[Ca^{2+}]_i$ was estimated from the relation $[Ca^{2+}]_i = K^* (R - R_{min})/(R_{max} - R)$, where the values of K^*, R_{min}, and R_{max} were determined from an in situ calibration of fura-2 in Jurkat T cells loaded by intracellular dialysis as described previously (Dolmetsch and Lewis, 1994). Ringer's solution contained (in mM) 155 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 5 Hepes (pH 7.4 with NaOH). Ca^{2+}-free Ringer's solution was prepared by substituting $MgCl_2$ for $CaCl_2$, and EGTA Ringer's was made by the further addition of 1 mM EGTA (pH 7.4 with NaOH). All experiments were conducted at 22–25°C unless otherwise noted; for experiments at 37°C, Ringer's solutions were further supplemented with 5% FCS to maintain the cells in good health.

The Ca^{2+} clearance rate was measured in fura-2 imaging experiments by stimulating cells with ionomycin in Ringer's solution, and after steady-state $[Ca^{2+}]_i$ was reached, perfusing with EGTA-Ringer's. At steady-state, the net Ca^{2+} flux across both organellar membranes and the plasma membrane are zero; thus, sudden elimination of Ca^{2+} influx by extracellular EGTA causes a decline in $[Ca^{2+}]_i$ that reflects the rate of ongoing Ca^{2+} clearance from the cytosol. The perfusion chamber had a 90% exchange time of <1 s; therefore, the rate of decline $(d[Ca^{2+}]_i/dt)$ was measured from the steepest slope, which occurred within 3 s of the solution change. High concentrations of ionomycin (2–4 μM) and Ca^{2+} (5–10 mM) were necessary to elevate $[Ca^{2+}]_i$ in M101, M108, and CJ-1 to levels comparable to those attained by the parental cells and less severe mutants after stimulation with 0.5–1 μM ionomycin and 2 mM Ca^{2+}. The different sensitivities to ionomycin are presumably due to the activation by ionomycin of capacitative Ca^{2+} entry in cells with functional CRAC channels (see text). It should also be noted that FCS in the medium greatly attenuates the effect of ionomycin on $[Ca^{2+}]_i$, presumably by binding to the ionophore.

Heterokaryon Fusions

Before fusion, groups of partner cells were treated for 30 min at 22–25°C with 20 nM calcein/AM (Molecular Probes) to label the cytoplasm or with 25 ng/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (diI, stock solution at 2.5 mg/ml in 100% ethanol; Molecular Probes) to label the plasma membrane. After three washes in RPMI 1640, cells were fused at 22–25°C essentially as described previously (Goldsmith et al., 1988), but in the presence of (by volume) 54% polyethylene glycol (PEG-1000; Electron Microscopy Sciences, Fort Washington, PA), 46% RPMI 1640, and 25 μl/ml of 7.5% sodium bicarbonate. After fusion, cells were returned to the 6% CO_2 incubator at 37°C for 1 h to permit recovery, after which they were loaded with fura-2/AM for Ca^{2+} imaging as described above. After each Ca^{2+} imaging experiment, cells were observed

using fluorescein and rhodamine filter sets (Chroma Technology Corp., Brattleboro, VT) to determine which cells clearly contained both calcein and diI, indicating a fused pair. Neither dye interfered with fura-2 measurements under the conditions used. Typically, ~5% of the cells appeared to be unambiguously double-labeled, and their $[Ca^{2+}]_i$ values were selectively averaged using Igor Pro software (WaveMetrics, Lake Oswego, OR).

Patch-Clamp Recording

Patch-clamp experiments were performed at 22–25°C in the whole-cell configuration (Hamill et al., 1981). Patch pipettes were pulled from 100-μl capillaries (VWR Scientific Corp., South Plainfield, NJ), coated with Sylgard™ (Dow Corning Corp., Midland, MI), and fire-polished to resistances of 2–8 MΩ. Uncompensated series resistance ranged from 5–20 MΩ. Membrane currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA), filtered at 1.5–2 kHz, and digitized at a sampling rate of 5 kHz using an ITC-16 interface (Instrutech Corp., Great Neck, NY). Patch-clamp software consisted of extensions to Igor Pro generously provided by R. Bookman and J. Harrington (University of Miami, FL). All voltages were corrected for a liquid junction potential of -12 mV between internal solutions and the bath solution. Pipette and cell capacitance were measured and electronically canceled at the beginning of each experiment. All data were corrected for leak currents collected before activation of I_{CRAC}, $I_{K(V)}$, or $I_{K(Ca)}$ unless otherwise noted.

The external solutions described above were also used in patch-clamp experiments with the following additions and changes. 22 mM Ca^{2+}-Ringer's was prepared by the addition of 20 mM $CaCl_2$ to Ringer's. K^+ Ringer's, prepared by replacing NaCl in Ringer's with KCl, was used to maximize the size of Ca^{2+}-activated K^+ currents (Grissmer et al., 1992). For measurements of I_{CRAC} the pipette solution contained (in mM) 140 Cs aspartate, 3.01 $MgCl_2$, 0.66 $CaCl_2$, 11.68 EGTA ($[Ca^{2+}]_{free}$ ≈10 nM), and 10 Hepes (pH 7.2 with CsOH). For measuring voltage-gated K^+ current the pipette contained (in mM) 140 K aspartate, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA ($[Ca^{2+}]_{free}$ ≈17 nM), and 10 Hepes (pH 7.2 with KOH). For studies of Ca^{2+}-activated K^+ channels the pipette contained (in mM) 140 K aspartate, 2 $MgCl_2$, 1.15 $CaCl_2$, 1.1 EGTA ($[Ca^{2+}]_{free}$ ≈54 μM), and 10 Hepes (pH 7.2 with KOH).

Results

Generation of Mutants Defective for NF-AT Directed Transcription

T cell signaling mutants were selected from a population of Jurkat NZDipA cells after mutagenesis by γ-irradiation, as first described by Serafini et al. (1995). Using this strategy (see Methods) we have isolated five mutant cell lines, CJ-1 to CJ-5 (for Ca^{2+} Jurkat mutants). The mutant cell lines proliferate at rates comparable to that of J.NFATZ.1 before, during, and after stimulation with ionomycin and PdBU. A dipA-resistant subclone of each mutant line and of parental (NZDipA) cells was selected after stable transfection with a plasmid encoding a mutant EF-2 protein (Nakanishi et al., 1988; see Methods), and these cells were used in all experiments involving protein synthesis. As illustrated in Fig. 1, stimulation of the mutants elicited between 0 and 40% of the level of β-gal produced by control cells. The mutant phenotypes were maintained throughout more than 8 wk (35 generations) of continuous culture in the absence of any intentional selection pressure, thus demonstrating the genetic stability of the defects. Taken together with the original (dip-A sensitive) mutants' ability to survive stimulation with ionomycin and phorbol ester, the reduced production of β-gal shows that all five mutants bear defects in NF-AT–mediated gene transcription.

Ca^{2+} Dependence of Transcription in the Mutants

Deficient generation of β-gal could result from mutations
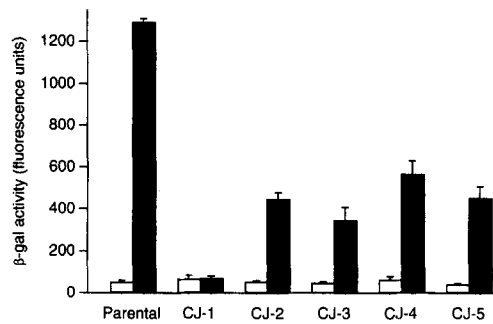
Figure 1. Somatic cell mutants CJ-1 through CJ-5 exhibit defects in NFAT-dependent expression of β-gal. β-gal levels were measured in a MUG assay in diphtheria toxin-resistant subclones of the mutant cell lines. Responses of unstimulated (*open bars*) and stimulated (8 h in culture medium with 2 μM ionomycin and 50 nM PdBU at 37°C, *solid bars*) cells are shown as mean ± SEM of triplicate wells from the combined results of at least three experiments. A diphtheria toxin-resistant subclone of the NZDipA parental cell line is shown here for comparison and is used as a control throughout this study.

at various points in the pathway linking ionomycin and PdBU to the expression of *lacZ*. As an initial attempt to determine the site at which NF-AT–mediated transcription was disrupted, we examined the Ca^{2+} dependence of β-gal induction in the mutant cells. Under standard stimulation conditions, ionomycin elevates $[Ca^{2+}]_i$ to a lower level in mutant cells than in control cells. After 45-min stimulation in complete medium with 2 μM ionomycin and 50 nM PdBU at 37°C, $[Ca^{2+}]_i$ was 638 ± 44 nM in parental cells, but only 318 ± 35 nM in CJ-1 and 393 ± 10 nM in CJ-4 (~600 cells/experiment, mean ± SEM, $n = 2$). Among the mutants, the degree of $[Ca^{2+}]_i$ elevation was well correlated with the severity of the transcriptional defect. To explore further the Ca^{2+} dependence of transcription, cells were treated with 2 μM ionomycin + 50 nM PdBU in the presence of varying levels of extracellular Ca^{2+} ($[Ca^{2+}]_o$). As shown in Fig. 2 *A*, transcription of *lacZ* in the mutants was restored by elevation of $[Ca^{2+}]_o$. Maximal β-gal expression levels ranged from ~0.6–1.1 times the levels observed in control cells. These maximal levels are within the range we observed from subclones derived from parental Jurkat cells (data not shown). Among the different mutants, the amount of additional Ca^{2+} needed to raise expression to normal levels is correlated with the severity of the defect in β-gal production.

If a smaller than normal $[Ca^{2+}]_i$ rise is in fact responsible for the mutants' transcriptional defect, then the rescuing effect of high $[Ca^{2+}]_o$ may reflect the restoration of normal $[Ca^{2+}]_i$. Alternatively, high $[Ca^{2+}]_o$ may be needed to boost $[Ca^{2+}]_i$ to supernormal levels if the NF-AT–transduction pathway has been altered to have a reduced Ca^{2+} sensitivity. To distinguish between these possibilities, we measured $[Ca^{2+}]_i$ in each of the mutants 45 min after stimulation under the conditions shown in Fig. 2 *A*. β-gal expression (normalized to maximal expression for each clone) was then replotted as a function of $[Ca^{2+}]_i$ in Fig. 2 *B* to illustrate the Ca^{2+} dependence of NF-AT–dependent transcription. The dependence of *lacZ* expression on $[Ca^{2+}]_i$ appears to be normal in all of the mutants, being initiated at $[Ca^{2+}]_i$ above

A

B
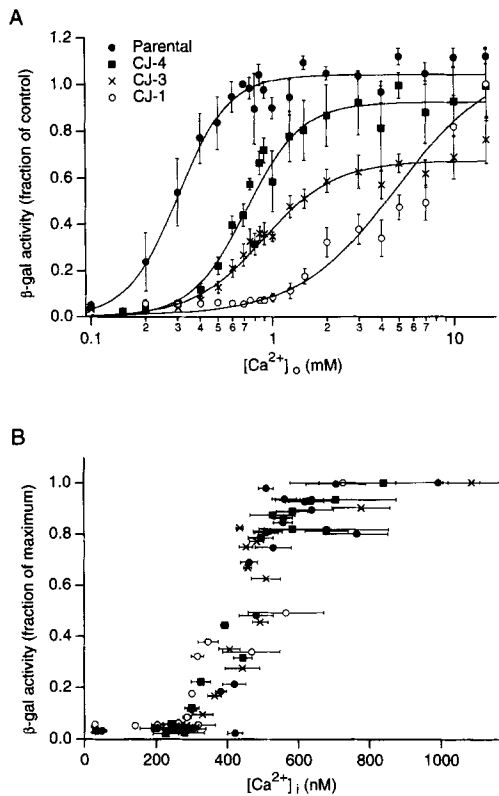
Figure 2. Dependence of β-gal expression on extracellular and intracellular Ca^{2+}. (*A*) β-gal expression in the indicated clones, stimulated as in Fig. 1 but with medium containing various levels of Ca^{2+}. Mean fluorescence values (± SEM) of background-corrected triplicate samples from three to four different experiments are expressed as a fraction of the control for each experiment. CJ-2 and CJ-5 were omitted for clarity but fall within the range of responses shown here. M101 and M108 have lower maximal responses than the other clones. (*B*) β-gal responses from *A*, plotted against $[Ca^{2+}]_i$. Each $[Ca^{2+}]_i$ value represents triplicate measurements from 200–300 fura-2–loaded cells in two independent experiments. β-gal levels are normalized to the maximal expression level for each clone to illustrate the Ca^{2+} sensitivity of transcription.

~300 nM and saturating at a $[Ca^{2+}]_i$ of ~600 nM. Therefore, the transcriptional pathway downstream of the $[Ca^{2+}]_i$ rise appears to function normally, and the transcriptional defect in the mutant cells can be attributed to a subnormal elevation of $[Ca^{2+}]_i$ by ionomycin.

Jurkat Mutants Are Defective in Capacitative Ca^{2+} Entry

Ionomycin has been shown to activate capacitative Ca^{2+} entry and I_{CRAC} in a variety of cells (Mason et al., 1991; Hoth and Penner, 1993; Morgan and Jacob, 1994; Premack et al., 1994), presumably by releasing Ca^{2+} from intracellular stores. Thus, the subnormal ionomycin-evoked $[Ca^{2+}]_i$ elevation in the mutants might reflect an underlying defect in the capacitative Ca^{2+} entry mechanism. However, the results could also be explained by a multidrug resistance phenotype by which the cells rapidly expel ionomycin. We therefore treated the mutants with thapsigargin (TG), an inhibitor of SERCA Ca^{2+}-ATPases that depletes intracellular Ca^{2+} stores and elicits capacitative Ca^{2+} entry (Gouy

et al., 1990; Thastrup et al., 1990; Mason et al., 1991; Sarkadi et al., 1991). In the absence of extracellular Ca^{2+}, TG causes a transient Ca^{2+} rise resulting from the unopposed leakage of Ca^{2+} from internal stores followed by Ca^{2+} extrusion across the plasma membrane. As illustrated in Fig. 3, a maximal dose of TG (1 μM) evoked a significant Ca^{2+} release transient in every cell line, demonstrating the presence of TG-sensitive Ca^{2+} stores. After $[Ca^{2+}]_i$ returned to baseline, the stores appeared to be completely depleted in both mutants and parental cells, as a high concentration of ionomycin (5 μM) released intracellular Ca^{2+} at only ~5% of the rate observed in untreated cells (data not shown). After store depletion was complete, 2 mM Ca^{2+} was reapplied to measure the degree of depletion-activated Ca^{2+} influx. The rate of Ca^{2+} entry varied among the mutants, as indicated by the maximal rate of $[Ca^{2+}]_i$ rise as well as the subsequent peak and plateau $[Ca^{2+}]_i$ values. The kinetics and amplitude of the responses appear to fall into three categories: a nearly complete absence of response (CJ-1, M101, and M108), small (CJ-2 and CJ-3), and intermediate

A

B

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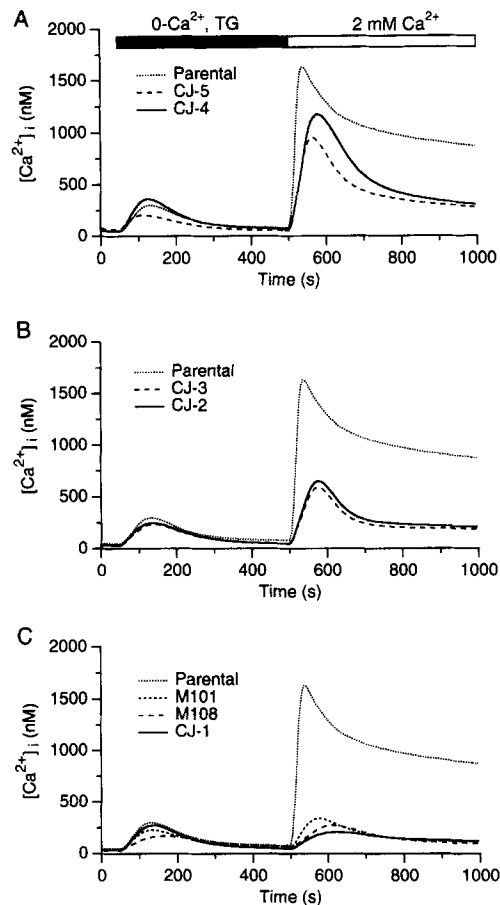
Figure 3. Mutants exhibit a range of defects in capacitative Ca^{2+} entry. In *A–C*, fura-2–loaded cells were stimulated with 1 μM TG in Ca^{2+}-free Ringer's solution to deplete internal stores (*filled bar*). Capacitative Ca^{2+} entry is indicated by the $[Ca^{2+}]_i$ rise that occurs after subsequent readdition of 2 mM Ca^{2+} (*open bar*). Each trace represents the average response of ~250 cells in four to eight experiments for each clone, totaling between 950 and 2200 individual cells. The parental cell trace (*dotted line*) appears in all three panels for comparison. (*A*) CJ-5 and CJ-4. (*B*) CJ-3 and CJ-2. (*C*) CJ-1, M101, and M108 responses.

(CJ-4 and CJ-5) responses. These defects in TG-triggered Ca^{2+} influx suggest that a deficit in the capacitative Ca^{2+} entry mechanism underlies the mutant phenotypes. The fact that intracellular Ca^{2+} release induced by TG and ionomycin appears to be normal precludes alternative explanations such as a multidrug resistance phenotype or aberrant expression of TG-insensitive pumps in the ER. The rate of ionomycin-evoked intracellular Ca^{2+} release in EGTA-Ringer's solution was 176 ± 65 nM/s for parental cells, 127 $\pm$ 44 nM/s for CJ-1, and 162 ± 71 nM/s in M108 (results of two to three experiments, total of >700 cells). Taken together, these results support the notion that mitogenic doses of ionomycin (1–2 μM in the presence of 10% FCS) elevate $[Ca^{2+}]_i$ in normal T cells primarily by depleting stores and promoting capacitative Ca^{2+} influx rather than by directly transporting a significant amount of Ca^{2+} across the plasma membrane (Morgan and Jacob, 1994; Wilson et al., 1994; Serafini et al., 1995).

Like the transcriptional defects described above, the mutant Ca^{2+} influx phenotypes were unchanged over 35 generations in culture (8 weeks), indicating that they too are genetically stable. Interestingly, TG fails to evoke Ca^{2+} entry in ~5% of parental Jurkat cells. However, subclones of unmutagenized parental cells selected by FACS for low Ca^{2+} responses generated populations that responded to TG in a manner indistinguishable from the original parental cells (data not shown). It is possible that the nonresponsive cells in the parental population correspond to cells in M phase, a period in which capacitative Ca^{2+} entry may be suppressed (Preston et al., 1991). Thus, mutagenesis appears to be necessary to derive genetically stable Ca^{2+} signaling mutants of the type we describe.

Heterokaryon Cell Fusions

Transient heterokaryon fusions between mutant and parental control cells were made to determine whether the mutant phenotypes result from dominant inhibitory effects at the protein level. Each donor population of cells was stained with either diI or calcein to permit microscopic identification of fused pairs, and $[Ca^{2+}]_i$ was measured using the protocol described in Fig. 3 in cell heterokaryons within several h of their formation. As illustrated in Fig. 4 A, the response of CJ-2–parental cell heterokaryons was intermediate between that of mutant–mutant and parent–parent responses. In a similar fashion, fusion of all other mutant cell lines with parental cells yielded intermediate responses. Thus, the mutant phenotypes are not completely dominant, and it is unlikely that the mutant cells produce an inhibitor of Ca^{2+} entry that cannot be overcome by sufficient quantities of coexpressed wild-type proteins.

Because the mutant phenotypes are not completely dominant, it is possible to test for complementation by inter-mutant fusion. Significant complementation, as manifested by a Ca^{2+} response larger than that of either mutant, would indicate that the normal gene products of different mutants are able to combine to restore normal Ca^{2+} influx. For these experiments we selected CJ-1, CJ-2, and CJ-4 as representative of the three different categories of mutant Ca^{2+} phenotype (see Fig. 3), and fused them in every possible pair-wise combination with each other. A typical ex-
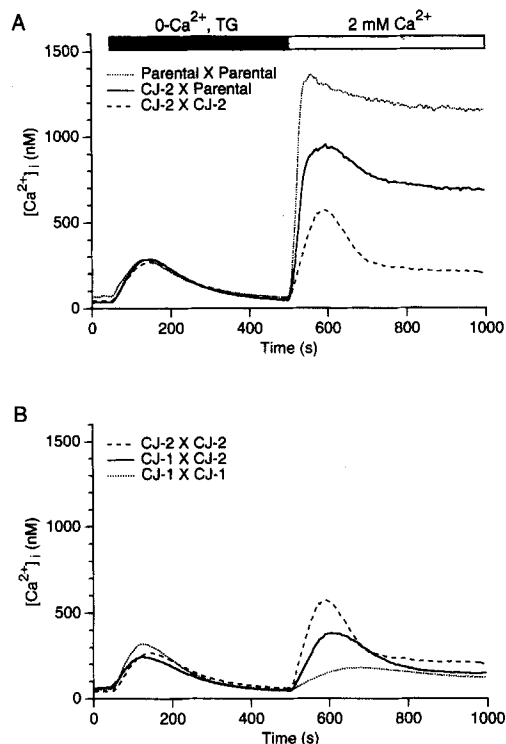
Figure 4. The mutant phenotypes are not completely dominant and are non-complementing. (*A*) The CJ-2 Ca^{2+} response phenotype is non-dominant. The average responses of CJ-2 cells fused to parental cells (30 heterokaryons) falls between those of self-fused parental cells (42 homokaryons) and self-fused CJ-2 cells (22 homokaryons). (*B*) CJ-1 and CJ-2 do not complement each other. The average response of CJ-1 cells fused to CJ-2 cells (25 heterokaryons) lies between those of self-fused CJ-1 cells (25 homokaryons) and self-fused CJ-2 cells (22 homokaryons), with no evidence of complementation of either mutant defect.

periment in which CJ-1 was fused to CJ-2 is shown in Fig. 4 *B*. In each case, heterokaryon fusions yielded Ca^{2+} influx responses intermediate between those of the two partners indicating a failure of preexisting proteins to complement the mutant phenotypes.

Characterizing the Defect in Capacitative Ca^{2+} Entry

Several factors are known to influence the net rate of capacitative Ca^{2+} entry in T cells, including CRAC channel activity, K^+ channel activity and the rate of Ca^{2+} clearance via membrane Ca^{2+}-ATPases (Donnadieu et al., 1992; Lewis and Cahalan, 1995). We systematically analyzed each of these parameters in the mutant cells to localize further the site of the Ca^{2+} influx defect.

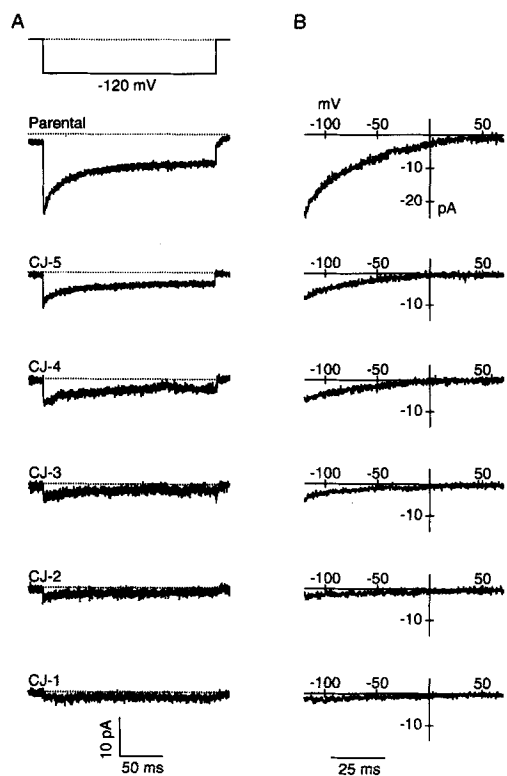*Measurements of I_{CRAC} in Mutant Cells.* CRAC channel activity was determined from the maximal amplitude of I_{CRAC} observed under voltage-clamp conditions. Internal stores were depleted by incubation with TG in Ca^{2+}-free Ringer's solution, and I_{CRAC} was measured after the subsequent addition of 22 mM external Ca^{2+}, a concentration that saturates the channel's conduction pathway (Hoth and Penner, 1993; Premack et al., 1994). I_{CRAC} was measured in response to hyperpolarizing voltage steps (0 to -120 mV, Fig. 5 *A*) or to voltage ramps (-120 to $+70$ mV, Fig. 5 *B*). The resulting currents were identified as I_{CRAC}

on the basis of several characteristic properties: a dependence on store depletion and extracellular Ca^{2+}, voltage-independent gating, rapid inactivation during hyperpolarizing voltage steps (Fig. 5 A), an inwardly rectifying current-voltage relation lacking a clearly defined reversal potential (Fig. 5 B), and the absence of visually detectable current noise (Penner et al., 1993; Fasolato et al., 1994; Lewis and Cahalan, 1995). The results of these experiments (summarized in Fig. 8) illustrate that maximal depletion of Ca^{2+} stores in the mutants activates I_{CRAC} to varying extents ranging from almost no current (e.g., CJ-1 or M101) to 41% current (CJ-5) relative to parental control cells. Because TG is able to fully deplete the stores in the mutants (Fig. 3, above), the diminished level of I_{CRAC} is likely to reflect abnormal CRAC channel function or expression, or a defect in the channel activation mechanism (see Discussion).
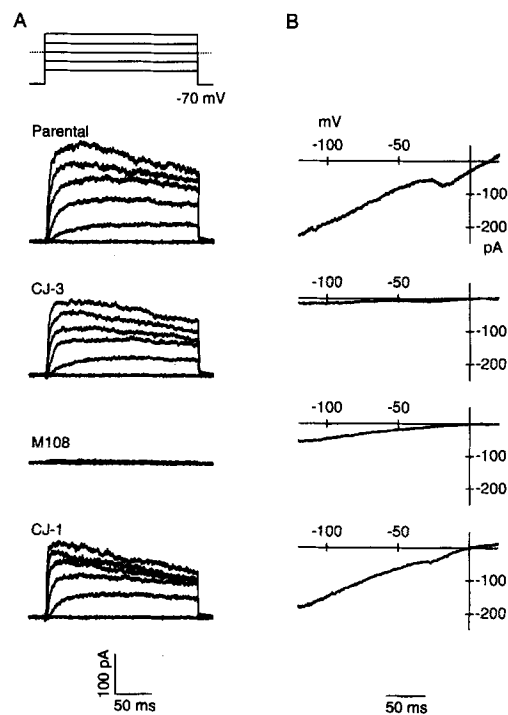
K^+ *Channel Function in Mutant Cells.* Jurkat T cells express both voltage-gated and Ca^{2+}-activated K^+ channels, and indirect evidence suggests that these channels can influence Ca^{2+} signaling by controlling the electrical driving force for Ca^{2+} entry (reviewed by Lewis and Cahalan, 1995). Voltage-gated K^+ currents ($I_{K(V)}$) were observed in response to depolarizing voltage steps more positive than ~-50 mV; representative examples from control cells and

three mutant cells are shown in Fig. 6 A. These currents were identified as type n K^+ currents (Lewis and Cahalan, 1995) on the basis of their voltage dependence, kinetics, and inactivation properties (Cahalan et al., 1985). Most of the mutants exhibited normal levels of $I_{K(V)}$; the sole exception was M108 (Fig. 6 A), which displayed only 5% of the control current level.

The expression of Ca^{2+}-activated K^+ channels was measured with pipette solutions containing a maximally activating concentration of Ca^{2+} (>10 μM, see Grissmer et al., 1992) and with extracellular K^+ Ringer's solution to enhance the size of the current ($I_{K(Ca)}$). Under these conditions, activation of Ca^{2+}-activated K^+ channels was complete within several minutes of intracellular dialysis. Currents were evoked by voltage ramps from -120 to $+20$ mV and were corrected for leak currents measured before activation of $I_{K(Ca)}$; representative results are shown in Fig. 6 B. Only CJ-3 expressed functional K(Ca) channels at a level significantly lower than control (18%); all other mutants expressed $I_{K(Ca)}$ at between 50 and 100% of the control level. The amplitudes of both voltage-gated and Ca^{2+}-activated
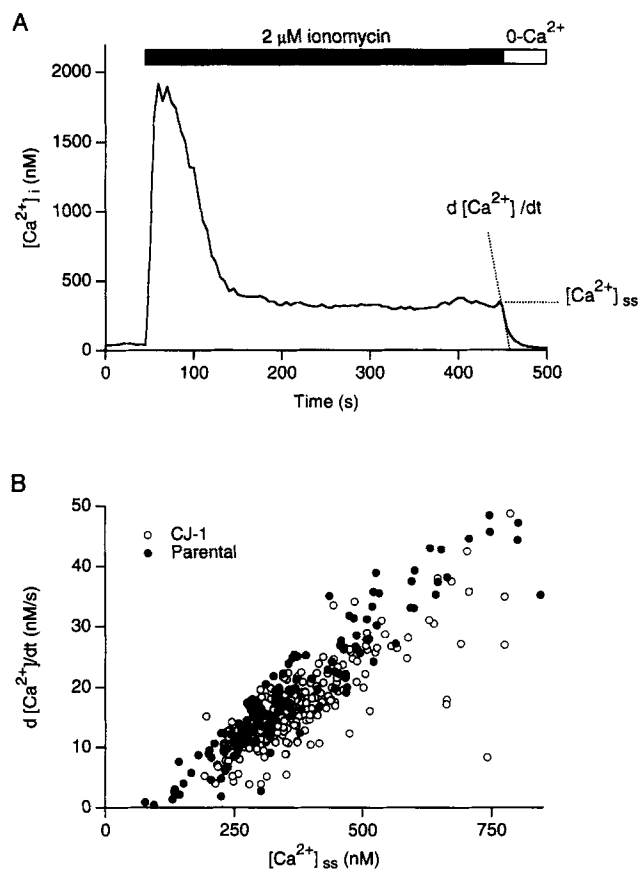
Figure 6. Functional expression of K^+ channels in mutant cells. (A) Voltage-gated type n K^+ currents in parental and selected mutant cells. $I_{K(V)}$ was activated by pulses from -40 to $+60$ mV in 20-mV increments (*top*), delivered every 30 s from a holding potential of -70 mV. (B) Ca^{2+}-activated K^+ currents in parental and mutant cells. $I_{K(Ca)}$ was activated by dialysis with >10 μM free Ca^{2+} in the presence of extracellular K^+ Ringer's; averaged currents in response to voltage ramps from -120 to $+20$ mV are shown. Each trace was obtained from a cell of the corresponding clone indicated in A. The inward inflection seen at voltages more positive than -40 mV results from slow changes in $I_{K(V)}$ during whole-cell recording (Cahalan et al., 1985), such that subtraction of the "leak" current collected just after break-in does not fully remove the contribution of $I_{K(V)}$ at later times. The slope conductance of $I_{K(Ca)}$ was measured between -100 and -50 mV, below the activation range of $I_{K(V)}$.

Figure 5. I_{CRAC} is reduced in the mutant cells. I_{CRAC} was recorded in the presence of 22 mM Ca^{2+} after store depletion with 1 μM TG. (A) I_{CRAC} elicited in single cells by hyperpolarizing voltage pulses from 0 to -120 mV. The currents decay due to rapid inactivation by intracellular Ca^{2+}. (B) I_{CRAC} evoked by 95-ms voltage ramps from -120 to $+70$ mV applied from a holding potential of 0 mV. Each trace was obtained from the corresponding cell shown in A. Responses in A and B have been corrected for leak current using traces collected in Ca^{2+}-free Ringer's solution before and after activation of I_{CRAC}.

K^+ currents in the entire series of mutants is summarized in Fig. 8.

The Rate of Ca^{2+} Clearance in Mutant Cells. Hyperactivity of Ca^{2+} clearance mechanisms such as plasma membrane Ca^{2+}-ATPases could also contribute to the apparent reduction in capacitative Ca^{2+} entry observed in the mutant cells. We therefore compared the Ca^{2+} clearance rates of mutant and control Jurkat cells. As illustrated by the example in Fig. 7 *A*, cells were stimulated with ionomycin to achieve a steady-state $[Ca^{2+}]_i$, whereupon extracellular Ca^{2+} was removed by perfusion with EGTA-Ringer's, resulting in a rapid decline of $[Ca^{2+}]_i$. For each cell in the population, the steady-state $[Ca^{2+}]_i$ was measured just before Ca^{2+} removal, and the slope $(d[Ca^{2+}]_i/dt)$ after Ca^{2+} removal was used to estimate the overall rate of Ca^{2+} clearance, a product of the combined activity of Ca^{2+}-ATPases, exchangers, and Ca^{2+} buffers in the cell. As depicted in Fig. 7 *B*, the Ca^{2+} clearance rates in CJ-1 and parental cells were similar, both increasing with $[Ca^{2+}]_i$. All other mutants showed the same relationship (data not
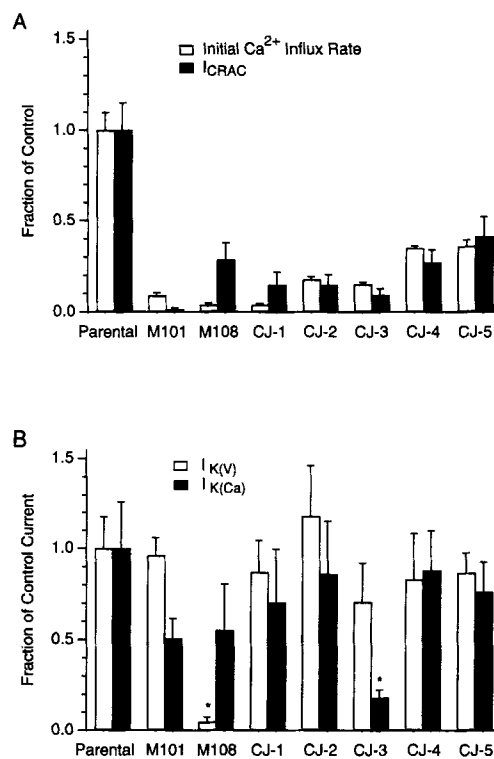
shown), demonstrating that Ca^{2+} clearance mechanisms in the mutant cells are normal.

Ion Channel Defects Account for Diminished Capacitative Ca^{2+} Entry

Of the several factors known to influence capacitative Ca^{2+} entry, only the activity of CRAC channels and K^+ channels were found to be altered in the mutant cells. Fig. 8 *A* compares the amplitudes of I_{CRAC} in these cells with the rate of capacitative Ca^{2+} entry (measured in Fig. 3). I_{CRAC} is significantly smaller than control (unpaired Student's *t*-test, $p < 0.02$) in all the mutants. In all but M108, the magnitude of I_{CRAC} is roughly correlated with the rate of capacitative Ca^{2+} entry, supporting the conclusion that subnormal activity of CRAC channels is responsible for


Figure 7. The Ca^{2+} clearance rate in mutant cells is normal. (*A*) Measurement of the clearance rate in a single CJ-3 cell. 2 µM ionomycin was applied in Ringer's solution to increase $[Ca^{2+}]_i$ to a steady-state value $([Ca^{2+}]_{ss})$. Subsequently, Ca^{2+}-free EGTA-Ringer's solution was applied and the steepest rate of $[Ca^{2+}]_i$ decline was determined $(d[Ca^{2+}]_i/dt)$. (*B*) Ca^{2+} clearance rates in parental (●) and CJ-1 (○) cells. Each point represents measurements of clearance rate $(d[Ca^{2+}]_i/dt)$ and $[Ca^{2+}]_{ss}$ from a single cell. The behavior of other mutant clones was indistinguishable from that of the CJ-1 and parental cells shown here.

Figure 8. Comparison of CRAC and K^+ channel activity with the rate of capacitative Ca^{2+} entry. (*A*) The maximal rates of capacitative Ca^{2+} influx (*open bars*) and the maximal amplitudes of I_{CRAC} (*filled bars*; normalized for cell capacitance) for the mutants are plotted relative to those of parental cells (influx rate: 65 ± 6 nM/s; I_{CRAC} amplitude: 0.61 ± 0.09 pA/pF). Influx rate was determined from the rate at which $[Ca^{2+}]_i$ increased immediately after readdition of Ca^{2+} to depleted cells (Fig. 3). I_{CRAC} was measured during the last 20 ms of current responses to hyperpolarizing pulses (Fig. 5 *A*). Bars show mean values $\pm$ SEM of four to eight experiments on ~250 cells each (influx rates) or of 5–12 cells (I_{CRAC}). (*B*) K^+ channel expression in the mutant cells. The maximal conductance of K(V) (*open bars*) and K(Ca) channels (*filled bars*) in the mutants are shown relative to levels in parental cells $(g_{K(V)}: 0.33 \pm 0.06$ nS/pF; $g_{K(Ca)}: 0.18 \pm 0.05$ nS/pF). The maximum current after a saturating pulse to $+20$ mV (Fig. 6 *A*) was used to calculate $g_{K(V)}$, based on a reversal potential of -80 mV. The slope conductance between -100 and -50 mV was used to measure $g_{K(Ca)}$ (Fig. 6 *B*). Bars show means $\pm$ SEM of 5–10 cells. Asterisks indicate values significantly different from parental cells ($p < 0.05$; unpaired Student's *t*-test).

the defects found in these cells. In most but not all of the mutants, the activity of voltage-gated K^+ channels and Ca^{2+}-activated K^+ channels does not differ significantly from that of parental cells (Fig. 8 B). K(V) channel activity was significantly lower than control ($p < 0.02$) only in M108, and Ca^{2+}-activated K^+ channel expression was significantly below control only in CJ-3. The near absence of functional voltage-gated K^+ channels in M108 may explain the discrepancy between I_{CRAC} amplitude and the Ca^{2+} influx rate in those cells, in light of evidence that blockade of K(V) channels inhibits the mitogen-evoked $[Ca^{2+}]_i$ rise in Jurkat cells (Lin et al., 1993).

The correlation between I_{CRAC} amplitude and the rate of capacitative Ca^{2+} entry in most of the mutants suggests that the mutant phenotypes result from a variable reduction in CRAC channel activity, such as might result from defective channel expression or activation. However, the kinetics of each mutant's response to Ca^{2+} readdition after store depletion is complex and is not simply a scaled version of the parental response (Fig. 9 A). For example, $[Ca^{2+}]_i$ in CJ-4 reaches a peak value approaching that of parental cells, but subsequently falls within 400 s to only a fraction of the parental level. Such kinetic behavior could reflect several possibilities, including enhanced slow inactivation of CRAC channels (Zweifach and Lewis, 1995b)
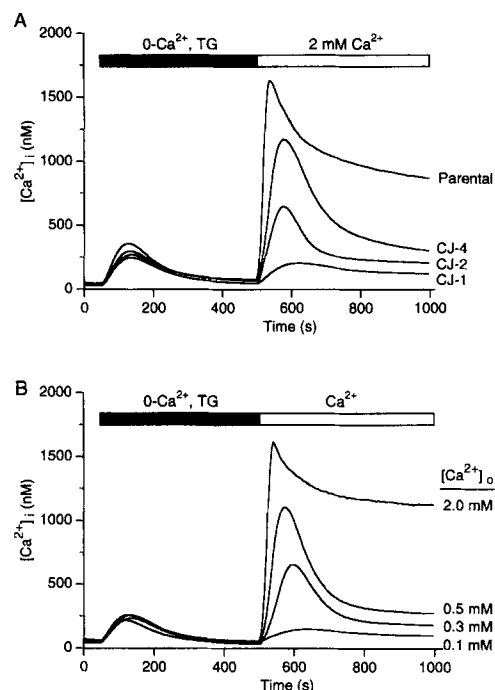

Figure 9. Mutant phenotypes can be mimicked in parental cells by a reduction in Ca^{2+} influx. (A) Overlay of parental and selected mutant cell responses to Ca^{2+} readdition after depletion of stores by TG. Data are replotted from Fig. 3. CJ-1, CJ-2, and CJ-4 each display a unique Ca^{2+} "signature" that encompasses the full range of capacitative Ca^{2+} entry defects found in the complete set of mutant clones. (B) Responses of store-depleted parental cells to the readdition of varying levels of Ca^{2+}. Each trace is the average response of >500 cells in two to four experiments. Under conditions of reduced Ca^{2+} influx, capacitative Ca^{2+} entry in wild-type cells closely resembles the characteristic responses of the mutant clones in A.

or the absence of a protein needed to maintain channel activity. To address this issue, we asked whether the range of mutant Ca^{2+} responses could be mimicked by a simple reduction of Ca^{2+} influx in parental cells. In the experiment shown in Fig. 9 B, the stores of parental cells were depleted with TG in Ca^{2+}-free Ringer's, and varying amounts of Ca^{2+} (0.2–2 mM) were added to produce a range of Ca^{2+} influx rates. The kinetics and amplitudes of the resulting Ca^{2+} responses are strikingly similar to those of mutants CJ-1, CJ-2, and CJ-4 shown under standard conditions (2 mM Ca^{2+}) in Fig. 9 A. In addition, the response of CJ-4 can be made to resemble that of parental cells by elevating extracellular Ca^{2+} to 22 mM (data not shown). Thus, the mutant phenotypes can be accounted for by a simple reduction in the activity of CRAC channels, without a need to invoke additional changes in their kinetic behavior.

Genetic Evidence for Functions of CRAC Channels

In many cells, the refilling of Ca^{2+} stores after depletion by IP_3 is dependent on extracellular Ca^{2+}, supporting the notion that depletion-activated Ca^{2+} entry provides the Ca^{2+} needed for repletion; however, the lack of specific blockers of capacitative Ca^{2+} entry has thus far prevented a direct test of this idea. In fact, the resting content of Ca^{2+} stores is not related to the maximal magnitude of I_{CRAC} in the mutant cells we have isolated, which appears to argue against this hypothesis (see Fig. 3). We therefore compared the refilling process in parental cells and in CJ-1 after store depletion by cyclopiazonic acid (CPA), a reversible inhibitor of SERCA Ca^{2+}-ATPases (Low et al., 1992). As shown in Fig. 10 A, stores were depleted with 20 μM CPA (a maximal dose) in EGTA-Ringer's, after which the CPA was washed out in the presence of FCS. Stores were then allowed to refill partially during a 200-s incubation in 0.2 mM Ca^{2+}, followed by removal of extracellular Ca^{2+} to permit $[Ca^{2+}]_i$ to return to its resting level. The store content was assessed from the maximal rate of Ca^{2+} release or from the peak $[Ca^{2+}]_i$ observed after the subsequent application of 1 μM TG. In the experiment shown in Fig. 10 A, stores in CJ-1 cells appear to refill more slowly than those in parental cells, which correlates with the reduced CRAC channel activity in CJ-1. The results from all experiments are summarized in Fig. 10 B. The degree of refilling after a 200-s exposure to 0.2 mM Ca^{2+} was calculated by dividing the store content after partial refilling (corrected for the residual store content after CPA treatment) by the steady-state store content in resting cells; CJ-1 cells refill 22 ± 4% of their store content under these conditions compared to 42 ± 8% for parental cells. Similar results were obtained by estimating store content from the peak $[Ca^{2+}]_i$ evoked by TG; in this case the degree of refilling was 14 ± 2% for CJ-1 and 39 ± 8% for parental cells. Thus, reduced CRAC channel activity correlates with a reduced rate of refilling, providing genetic evidence in support of a role for CRAC channels in replenishing Ca^{2+} stores.

The mutants also provide a means to address whether CRAC channels are the only source of Ca^{2+} influx during T cell activation. Because our mutant selection strategy based on ionomycin and phorbol ester stimulation favors defects in capacitative Ca^{2+} entry, other Ca^{2+} entry pathways are likely to be normal in the mutant cell lines. Thus,

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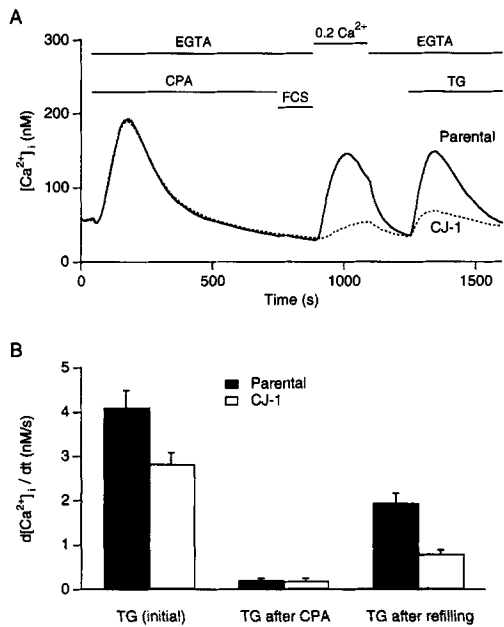
Figure 10. Refilling of Ca^{2+} stores in I_{CRAC}-deficient mutant cells is slowed. (A) Protocol to measure store refilling in parental and CJ-1 cells. As indicated by the bars, 20 μM CPA was applied in EGTA Ringer's to empty the stores, after which CPA was washed out for 100 s with EGTA Ringer's + 5% FCS. Subsequently, 0.2 mM Ca^{2+} Ringer's was added for 200 s to allow refilling. After perfusion of EGTA Ringer's for 150 s, the store content was assessed by adding 1 μM TG. Traces show the average responses of 263 parental and 188 CJ-1 cells. (B) Quantification of store depletion and refilling in parental cells (filled bars) and CJ-1 cells (open bars). Store content was estimated from the maximal rate of $[Ca^{2+}]_i$ rise induced by 1 μM TG in EGTA Ringer's at three times: after 150 s in EGTA Ringer's (left; initial store content, not shown in A), after CPA treatment and washing (middle; at 900 s in the experiment shown in A), and after halting the refilling process (right; at 1250 s in A). Bars reflect the average response of three experiments each with 165–285 cells. The last two bars are significantly different from each other (unpaired Student's t-test, $p < 0.01$).

if depletion-independent channels contribute to Ca^{2+} signaling in T cells, a more physiological stimulus (TCR cross-linking) should evoke Ca^{2+} influx in mutants lacking CRAC channel activity. To examine this possibility, we cross-linked the TCR with OKT3, a murine mAb against CD3, followed by a goat anti–mouse polyclonal antibody. In individual parental, CJ-1, and CJ-4 cells, TCR cross-linking in EGTA Ringer's solution evoked one to several small $[Ca^{2+}]_i$ transients due to intracellular Ca^{2+} release (Fig. 11, A and B). 2 mM Ca^{2+} was reintroduced to assess the degree of Ca^{2+} influx. Fewer mutants than parental cells displayed intracellular release transients in response to antibody treatment. Therefore, in order to compare the Ca^{2+} influx responses of the different cell lines we averaged the responses of only those cells displaying release transients that exceeded a level of 300 nM within the first 100 s of stimulation. These criteria equalized cells of the CJ-1, CJ-4, and parental clones for the level of store release and hence the strength of signaling through CD3. In parallel experiments, addition of 5 μM ionomycin instead of 2 mM Ca^{2+} released remaining Ca^{2+} stores in mutants and parental cells at similar rates, confirming that stores in

the selected populations were similarly depleted. The average responses of cells selected in this manner are shown in Fig. 11 C. Both CJ-1 and CJ-4 cells show greatly diminished Ca^{2+} influx in response to TCR cross-linking relative to the parental control cells. In fact, the CD3-stimulated $[Ca^{2+}]_i$ rise appears to be more severely reduced than the TG-stimulated rise (compare Figs. 3 A and 9 A). This difference may be due to the higher temperature of the anti-CD3 experiment (37°C instead of 22–25° C as in the TG experiments), or to I_{CRAC} inactivation via partial store refilling (Zweifach and Lewis, 1995b), which is prevented by thapsigargin but not by stimulation through CD3. These results support the conclusion that CRAC channels are the sole route by which TCR stimulation triggers Ca^{2+} entry.

Discussion

We have applied a selection strategy based on the activation of NF-AT-dependent genes to isolate T cell mutants with defects in capacitative Ca^{2+} entry. The set of mutants M101, M108, and CJ-1 through CJ-5 express from 4 to 36% of the level of capacitative Ca^{2+} influx found in parental Jurkat cells. Several results indicate that the deficits in ionomycin- and phorbol ester-induced transcription in the mutant cells can be attributed to the defect in capacitative Ca^{2+} entry. First, 2 μM ionomycin in normal culture medium elicits a subnormal $[Ca^{2+}]_i$ rise in the mutants that parallels β-gal production. Second, elevation of $[Ca^{2+}]_o$ succeeds in restoring NF-AT–dependent β-gal expression, and a close comparison of $[Ca^{2+}]_i$ with the degree of expression under these conditions reveals that the Ca^{2+} sensitivity of the transcriptional pathway is normal in the mutant cells (Fig. 2). These results establish a threshold of $\sim$300 nM Ca^{2+} for activation of NF-AT in Jurkat cells, similar to that reported to activate NF-AT–dependent transcription in a murine T cell hybridoma (Negulescu et al., 1994). This value also agrees well with the level of Ca^{2+} needed to activate purified calcineurin/calmodulin in vitro (Stemmer and Klee, 1994), thus supporting the critical role of calcineurin in NF-AT–dependent transcription, and providing further evidence that the signal transduction pathway downstream of Ca^{2+} is intact in the mutant cell lines.

The Nature of the Defect in the Mutants

Examination of several factors that contribute to the net rate of Ca^{2+} influx reveals a specific defect in CRAC channel activity as the source of the mutant Ca^{2+} signaling phenotype. Ca^{2+} store content (assessed by TG or ionomycin) and clearance mechanisms appear normal, as does K^+ channel expression in most of the mutant lines. In contrast, direct measurement of I_{CRAC} under voltage-clamp conditions revealed subnormal CRAC channel activity ranging from 1 to 41% of control in M101, M108, and CJ-1 through CJ-5. In all mutants except M108, the level of I_{CRAC} was roughly correlated with the initial rate of capacitative Ca^{2+} entry (4–36%) measured after Ca^{2+} readdition to depleted, intact (unclamped) cells. Because gene expression was also in this range (0–40%), there is a clear link between I_{CRAC}, $[Ca^{2+}]_i$, and NF-AT–dependent gene expression. Our failure to isolate a true null mutant for I_{CRAC} is intriguing; it is
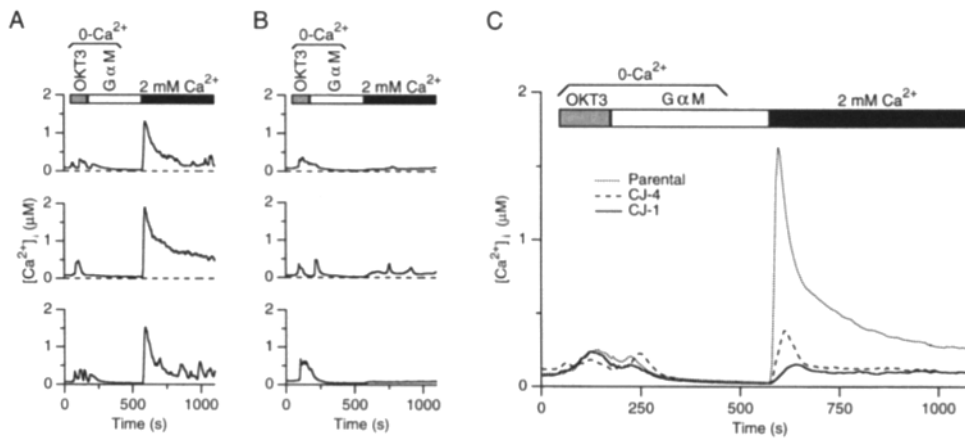
Figure 11. I_{CRAC} mutants are deficient for Ca^{2+} influx triggered via the T cell receptor. (*A*) Ca^{2+} release and influx in three parental cells. As indicated by the bar, cells were stimulated in EGTA-Ringer's containing 1 μg/ml OKT3 mAb followed by secondary cross-linking using 5 μg/ml goat anti–mouse antibody. Stimulation triggered Ca^{2+} release transients in single cells. Readdition of 2 mM Ca^{2+} caused a large $[Ca^{2+}]_i$ increase due to influx. (*B*) Responses of single CJ-1 cells to TCR cross-linking. In response to the same stimulation conditions as in *A*, release of Ca^{2+} stores is not accompanied by significant Ca^{2+} influx in the mutants. (*C*) Comparison of the average responses of parental cells, CJ-1, and CJ-4 mutants to TCR cross-linking. Cells were stimulated as described in *A* and *B*, and averages were made from cells showing a Ca^{2+} release transient exceeding 300 nM and beginning within 100 s of stimulation; 54% of parental cells, 25% of CJ-1 cells, and 16% of CJ-4 cells met this response criterion. Data were obtained from a total of 478 parental cells, 147 CJ-1 cells, and 92 CJ-4 cells in two experiments, performed at 37°C using solutions supplemented with 5% FCS.

possible that the CRAC channel gene is not functionally haploid in the parental cells and therefore is not susceptible to a complete knockout by mutagenesis. Alternatively, a minimal amount of capacitative Ca^{2+} entry may be needed to support cell viability or growth. One possibility is that the complete absence of I_{CRAC} might promote prolonged periods of store depletion, a condition that has been shown to inhibit cell proliferation (Ghosh et al., 1991).

What is the molecular locus of the I_{CRAC} defect? Although mutant proteins apparently do not prevent Ca^{2+} influx as shown in parental/mutant cell fusions, CJ-1, CJ-2 and CJ-4 did not display an enhanced response when fused to one another. One interpretation of this result is that the same gene product may be affected to different extents in the different mutants; for example, the expression level of the CRAC channel or some rate-limiting protein in its activation pathway may be reduced to varying degrees. Alternatively, different genes may be affected in different mutants, but the proteins they encode may need to interact during or shortly after synthesis, or organelles from the fused cells may need to intermix to reconstitute the Ca^{2+} entry mechanism. Because gamma ray mutagenesis frequently results in large chromosomal deletions (Kao and Puck, 1969), loss-of-function mutations are more likely than subtle alterations in protein sequence. Consistent with this prediction, several characteristic properties of the CRAC channels expressed in the mutants appeared unchanged. For example, the rapid Ca^{2+}-dependent inactivation (Fig. 5 *A*) and the selectivity of CRAC channels (as shown by the current-voltage relation; Fig. 5 *B*) were normal. Because rapid inactivation has been shown to be a function of single-channel current independent of the number of open CRAC channels (Zweifach and Lewis, 1995a), we also conclude that the single-channel conductance of CRAC channels is not altered. Finally, the unique time course of the capacitative Ca^{2+} rise in each of the mutants could be mimicked in parental cells simply by reducing ex-

tracellular $[Ca^{2+}]$ (Fig. 9), indicating that the range of mutant phenotypes can be explained by a variable reduction in the activity of otherwise normal CRAC channels. The mutant phenotypes are clearly not due to a general defect in ion channels or membrane proteins, because the activity of K^+ channels (in all but M108 and CJ-3), Ca^{2+}-ATPases, and the expression of a variety of surface markers (CD2, CD4, CD5, CD45, and integrin-associated protein, data not shown) was indistinguishable from that of parental cells. Based on these results, we surmise that the mutants bear defects in either the expression of CRAC channels or proteins involved in their activation.

The Physiological Functions of I_{CRAC}

The specificity of the I_{CRAC} defects in the mutant cells allows genetic tests of the physiological functions of CRAC channels. One example is the mechanism of elevation of $[Ca^{2+}]_i$ by ionomycin, which, in combination with phorbol esters, is often used as a surrogate in T cell stimulation (Truneh et al., 1985). The success of our approach in isolating mutants in capacitative Ca^{2+} entry derives in part from the failure of ionomycin by itself to transport enough Ca^{2+} across the plasma membrane to stimulate NF-AT–dependent transcription. Instead, the parallel defects in *lacZ* expression and the $[Ca^{2+}]_i$ rise ionomycin in the mutants suggests that mitogenic doses of ionomycin (1–2 μM in FCS-containing medium; Truneh et al., 1985) activate T cells primarily by depleting stores and opening the cells' endogenous CRAC channels. This conclusion is consistent with the ability of ionomycin to activate I_{CRAC} (Hoth and Penner, 1993; Premack et al., 1994) and with the ability of SK&F 96365 (a relatively nonspecific blocker of I_{CRAC}) to inhibit the $[Ca^{2+}]_i$ rise induced by ionomycin (Mason and Grinstein, 1993; Morgan and Jacob, 1994). It is important to note that micromolar concentrations of ionomycin in the absence of FCS effectively transport Ca^{2+} into the cell across the plasma membrane (Fig. 7), presumably because

of a higher concentration of free ionophore in the absence of added protein.

A widely assumed function of CRAC channels involves the refilling of depleted Ca^{2+} stores. Indirect evidence supports this function: depletion of stores activates Ca^{2+} influx, refilling of stores is dependent on extracellular Ca^{2+}, and store refilling terminates Ca^{2+} influx (Jacob, 1990; Putney, 1990; Montero et al., 1992; Zweifach and Lewis, 1995b). Nevertheless, it is possible that store refilling occurs through a mechanism distinct from CRAC channels; indeed, in resting CJ-1 cells, which display only ~15% of the normal level of I_{CRAC}, the resting content of intracellular Ca^{2+} stores is approximately normal. However, the stores in CJ-1 refill more slowly than those in parental cells, thus providing strong evidence for a role of CRAC channels in replenishing stores. The apparent discrepancy between the level of I_{CRAC} (~15%) and the rate of refilling (35–50%) relative to parental cells has two possible interpretations. First, additional pathways not detectable with patch-clamp methods may contribute to store refilling. Second, the number of CRAC channels in wild-type Jurkat cells may exceed that needed for store replenishment; thus, they may carry out additional functions such as the generation of the prolonged increase in $[Ca^{2+}]_i$ that underlies T cell activation, as discussed below.

A multitude of Ca^{2+} influx mechanisms have been proposed to mediate TCR-induced Ca^{2+} influx, including CRAC channels (Zweifach and Lewis, 1993; Partiseti et al., 1994; Premack et al., 1994), voltage-dependent Ca^{2+} channels (Dupuis et al., 1989; Densmore et al., 1992), IP_3-dependent Ca^{2+} channels in the plasma membrane (Kuno and Gardner, 1987; Khan et al., 1992), Na^+/Ca^{2+} exchange (Balasubramanyam et al., 1994), and other mechanisms independent of store depletion (Chow et al., 1993; Sei et al., 1995). Several types of evidence favor CRAC channels as the major Ca^{2+} influx pathway. First, the sustained $[Ca^{2+}]_i$ rise evoked by TCR stimulation is strongly inhibited by depolarization (Lewis and Cahalan, 1989; Donnadieu et al., 1992; Hess et al., 1993) and by SK&F 96365 (Chung et al., 1994). Second, in patch-clamp studies the Ca^{2+} current activated through TCR stimulation is indistinguishable from I_{CRAC} elicited by TG (Zweifach and Lewis, 1993; Partiseti et al., 1994; Premack et al., 1994). Finally, a severe immunodeficiency in humans has recently been linked to a defect in Ca^{2+} signaling and CRAC channel activity (Partiseti et al., 1994). The hyporesponsiveness of primary T cells from these patients strongly supports the role of CRAC channels in T cell Ca^{2+} signaling. One slight caveat, however, is that because these patients were "selected" for immunodeficiency, their T cells may have lost the function of multiple redundant Ca^{2+} entry mechanisms, should they exist. It is unlikely that the mutants we have isolated would have a defect in both I_{CRAC} and another Ca^{2+} influx pathway, since our ionomycin-based selection protocol confers no selective advantage to cells harboring additional defects in a non-capacitative Ca^{2+} entry mechanism. Thus, the failure of TCR cross-linking to induce significant Ca^{2+} influx in mutants CJ-1 and CJ-4 (Fig. 11) indicates that other Ca^{2+} influx pathways do not contribute significantly after stimulation with anti-CD3. Moreover, the correlation between activation of I_{CRAC}, Ca^{2+} influx, and gene expression emphasizes the causal links among these three processes, and leads to the conclusion that Ca^{2+} entry through CRAC channels is necessary for T cell activation.

The current lack of cells bearing specific defects in I_{CRAC} hampers attempts at identifying the genes encoding the CRAC channel and the pathway underlying its activation. Since their signaling defect appears to be restricted to capacitative Ca^{2+} entry, mutants M101 and CJ-1, CJ-2, CJ-4 and CJ-5 may serve as useful cloning systems for isolating these genes by complementation. The recoverable genes may not be limited to those that have been affected in the mutants; because cells such as CJ-4 can be rescued by an incremental increase in $[Ca^{2+}]_i$, any gene whose overexpression enhances the operation of the existing pathway may be identified. In addition, the extreme I_{CRAC} mutants (M101 and CJ-1) may provide a suitable null background in which to express and study the function of cloned and mutated genes involved in capacitative Ca^{2+} entry.

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