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Stimulation of T Cells through the CD3/T-Cell Receptor Complex: Role of Cytoplasmic Calcium, Protein Kinase C Translocation, and Phosphorylation of pp60$^{c\text{-}src}$ in the Activation Pathway

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Stimulation of T cells or the Jurkat T-cell line with soluble antibodies to the CD3/T-cell receptor complex causes mobilization of cytoplasmic Ca^{2+}, which is blocked by pertussis toxin but not by ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and translocation of protein kinase C activity from the cytoplasm to the membrane. Such stimulation also causes phosphorylation of pp60$^{c\text{-}src}$ at an amino-terminal serine residue. These activities are consistent with induction of phosphatidylinositol metabolism after antibody binding. Anti-CD3 stimulation with antibody in solution, however, does not cause Jurkat cells to release interleukin 2 and blocks rather than induces proliferation of T cells. Induction of interleukin 2 production by Jurkat cells and proliferation by normal T cells requires anti-CD3 stimulation with antibody on a solid support, such as Sepharose beads or a plastic dish. Thus, we examined phosphorylation of pp60$^{c\text{-}src}$ after stimulation of Jurkat cells with anti-CD3 in solution or on solid phase. Both of these caused serine phosphorylation of pp60$^{c\text{-}src}$ that was indistinguishable even after 4 h of stimulation. These results indicate that the mode of anti-CD3 stimulation (in solution or on solid phase) controls a cellular function that modifies the consequences of signal transduction through phosphatidylinositol turnover.

It has been observed recently that treatment of Rous sarcoma virus-transformed cells with the tumor-promoting phorbol ester 12-O-tetradecanoyl-13-acetate (PMA) results in the phosphorylation of pp60$^{v\text{-}src}$, the product of the RSV *src* gene, at the novel amino-terminal serine residue (15, 42). Treatment of uninfected cells with PMA resulted in the phosphorylation of pp60$^{c\text{-}src}$, the normal cellular homolog of the viral-encoded protein, at the same amino-terminal serine residue as is phosphorylated on pp60$^{v\text{-}src}$ (11, 15). This serine residue has been identified as the serine at amino acid 12 (serine-12) and lies only four amino acids away from the cyclic AMP-dependent serine phosphorylation site at serine-17.

Protein kinase C (PKC) is thought to be the mediator of the phosphorylation events that occur after treatment of cells with PMA (for a review, see reference 36). PKC has been shown to phosphorylate pp60$^{v\text{-}src}$ in vitro at the same serine residue observed in pp60$^{v\text{-}src}$ when isolated from cells treated with PMA (15, 41).

Stimulation of T cells with antibodies to the CD3/T-cell receptor complex (23, 32, 33, 47) causes turnover of phosphatidylinositol to form inositol trisphosphate (IP3) (20), which can mobilize calcium from cytoplasmic stores. Diacylglycerol is also formed, leading to the activation of PKC (1). This signal transduction pathway (for reviews, see references 1 and 2) can lead to T-cell activation when other required signals are provided by accessory cells, interleukin 1, or anti-Tp44 or anti-CD5 monoclonal antibodies (MAbs) (4, 27, 28, 48, 51).

We report here that treatment of Jurkat cells with anti-CD3 MAb resulted in the translocation of PKC and phosphorylation of pp60$^{c\text{-}src}$ at the same amino-terminal serine residue observed when pp60$^{c\text{-}src}$ was isolated from PMA-treated cells. The increase in cytoplasmic Ca^{2+} in normal T cells after anti-CD3 stimulation was sensitive to inhibition by petussis toxin, supporting the conclusion that this activation pathway relies on phospholipase C-dependent formulation of IP3 and diacylglycerol. In addition, we correlated the functional consequences of pp60$^{c\text{-}src}$ phosphorylation with production of interleukin 2 (IL2) by Jurkat cells and with expression of IL2 receptors and proliferation by normal T cells after we stimulated the CD3/T-cell receptor complex either in solution or on solid phase.

MATERIALS AND METHODS

Monoclonal antibodies. Anti-Tp44 MAb 9.3 [an immunoglobulin G2a (IgG2a) produced in (BALB/c × C57BL/6)F_1] has been described previously (7, 8, 17, 30). This antibody was purified from ascites fluids on protein A-Sepharose columns. Prior to functional assays, antibodies were dialyzed extensively against phosphate-buffered saline (PBS) and filtered through sterile filters (pore size, 0.45 μm). Anti-CD3 MAb G19-4 (BALB/c IgG1) (27) was purified from ascites fluids by salt precipitation and DEAE chromatography. This antibody was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and used in some experiments to activate T cells.

$F(ab')_2$ fragments of G19-4 were prepared by digestion of purified antibody (1 mg/ml) with pepsin (Sigma Chemical Co., St. Louis, Mo.) at an enzyme/antibody ratio of 10 μg/mg in 0.1 M citrate buffer (pH 3.8) for 75 min at 37°C, as described previously (39). $F(ab')_2$ fragments were separated from intact antibody on a Sephacryl S200 column (2 m; Pharmacia).

Proliferation assays. Mononuclear cells were isolated from heparinized, fresh peripheral blood by centrifugation on lymphocyte separation medium (Litton Bionetics, Kensington, Md.). Cells were stimulated with previously titrated

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G19-4–Sepharose beads or with G19-4 F(ab')$_2$ fragments at 1 μg/ml. Anti-Tp44 was used at 1 μg/ml. Cells were cultured in quadruplicate samples in flat-bottom, 96-well microtiter plates at 5×10^4 cells per well in RPMI 1640 medium containing 15% human AB serum (Pel Freez, Brown Deer, Wis.). Cell proliferation was measured by uptake of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) after a 6-h pulse. Uptake of [^{3}H]thymidine was measured by harvesting cells onto glass fiber filters with a cell harvester, and radioactivity was measured in a liquid scintillation counter. For some experiments, recombinant IL2 (rIL2) (Genzyme, Boston, Mass.) was used at 100 U/ml. Each of the functional experiments presented here was repeated at least three times.

Measurement of PKC activity. Phosphatidylserine; diolein; ATP; EDTA; ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA); dithiothreitol (DTT); phenylmethylsulfonyl fluoride (PMSF), PMA, and histone III-S were obtained from Sigma. [γ-^{32}P]ATP was obtained from New England Nuclear.

PKC activity was measured by modification of previously described methods (25, 29). Jurkat cells were grown in RPMI 1640 medium–12% fetal calf serum. The cells were washed three times and suspended in 25×10^6/ml in minimal essential medium. Portions of 1 ml were mixed with either PMA at 100 ng/ml or MAbs at 50 to 100 μg/ml. The cells were briefly centrifuged, the supernatant was aspirated, and the cells were lysed with buffer A (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 10 mM EGTA, 2 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, 2 mM PMSF [pH 7.5]). The reported assay duration began with the addition of the stimulant to the cell and ended with the lysis of the cells. Thirty seconds was the shortest period that could be assayed. The cells were homogenized at 4°C with 20 strokes of a tight-fitting Dounce glass homogenizer. Membrane (pellet) and cytosol (supernatant) preparations were collected by centrifugation at $100,000 \times g$ for 60 min at 4°C. A solubilized membrane fraction was prepared from the pellet by suspension in buffer A containing 1% Nonidet P-40 followed by a further homogenization and centrifugation ($45,000 \times g$ for 15 min) at 4°C.

The cytosol fractions and the supernatants from the solubilized membrane fractions were applied to a separate DEAE-Sephacryl column (1.5-ml bed volume) previously equilibrated with buffer B (20 mM HEPES, 2 mM EGTA, 2 mM EDTA, 2 mM DTT, 2 mM PMSF [pH 7.5]). Each column was washed with 10 bed volumes of buffer B, and the PKC activity was eluted with 4 ml of buffer B containing 0.1 M NaCl.

Enzyme activity was assayed in a reaction mixture containing 26 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.6 mM EGTA, 0.6 mM EDTA, 30 mM NaCl, 60 μg of phosphatidylserine per ml, 6 μg of diolein per ml, 5.6 mM DTT, 1 mg/ml histone III-S, and 50 μm [γ-^{32}P]ATP (1,000 to 2,000 cpm/pmol). The reaction was initiated by the addition of 15 μl of the DEAE-Sephacryl column eluate to yield a final reaction volume of 50 μl. Background activity was determined in samples in which phosphatidylserine, diolein, and $CaCl_2$ were omitted and an additional 0.5 mM EGTA was added. After 5 min of incubation at 30°C, the assays were terminated by spotting 40 μl of the reaction mixture onto squares (2 by 2 cm) of phosphocellulose paper (P-81; Whatman, Inc., Clifton, N.J.). After washing, the bound ^{32}P was measured by liquid scintillation spectrophotometry. PKC activity was determined by subtracting the amount of ^{32}P that was measured by liquid scintillation spectrophotometry. PKC activity was determined by subtracting the amount of ^{32}P incorporated into histone under background conditions from the amount incorporated in the presence of Ca^{2+}, phosphatidylserine, and diolein. PKC activity was expressed as picomoles of ^{32}P incorporated in 5 min per 15-μl fraction from 10^7 cells. All samples were assayed in triplicate.

Stimulation of Jurkat cells for IL2 production. Jurkat cells (13) were harvested during the logarithmic phase of growth and were cultured at 10^6/ml for 24 h in the presence of purified MAbs or antibody on Sepharose beads (2 μl of beads per 10^6 cells). Culture supernatants were harvested, filtered, and assayed for IL2 activity.

IL2 assay. Murine CTLL cells (12) (kindly provided by Thomas Stanton, University of Washington) were used as indicator cells to quantitate IL2 secretion. Fractions (25 μl) of twofold serially diluted culture supernatants were added to 75-μl fractions containing 10^4 indicator cells and cultured in flat-bottom half-area microwells (Costar, Cambridge, Mass.) for 20 h. Cultures were then pulsed for 4 h with [^{3}H]thymidine, harvested, and counted as described above. The relative IL2 activity was calculated by probit analysis (12) with the use of recombinant human IL2 (Genzyme) as a standard. The threshold sensitivity of the assay was 0.06 U/25 μl.

Treatment of Jurkat cells with MAb G19-4 bound on solid phase. MAb G19-4 was absorbed onto the surface of tissue culture dishes by treatment with 20 μg of MAb G19-4 per ml in PBS for 1 h at room temperature. Dishes were subsequently blocked with 5% bovine serum albumin (Calbiochem-Behring, San Diego, Calif.) and extensively washed in phosphate-free Dulbecco modified Eagle medium. Control dishes received only the blocking solution.

Jurkat cells labeled with $^{32}P_i$ for 4 h in phosphate-free Dulbecco modified Eagle medium containing 5% dialyzed fetal calf serum and $^{32}P_i$ (1 mCi/ml) were overlayered onto control or MAb G19-4-absorbed tissue culture dishes in labeling media. Cells layered onto MAb G19-4 dishes were clearly attached after 1 h of treatment. The media was carefully removed, and the attached cells were rinsed in cold Tris saline prior to detergent lysis. Between 60 and 90% of the cells remained on the dish. Cells that were layered onto control dishes were not attached and were collected by centrifugation.

Treatment of Jurkat cells with MAb G19-4 in solution. Jurkat cells labeled with $^{32}P_i$ as described above were treated with MAb G19-4 (100 μg/ml), PMA (100 ng/ml), MAb 9.3 (100 μg/ml), or PBS for 5 min at 37°C. Cells were then washed once and then lysed for immunoprecipitation of pp60$^{c\text{-}src}$ as described below.

Immunoprecipitation analysis. Cells were lysed in a detergent containing buffer (20 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.5% sodium deoxycholate, 1.0% Nonidet P-40, 1 mM EDTA), and insoluble debris was removed by centrifugation at $100,000 \times g$ at 4°C. pp60$^{c\text{-}src}$ was immunoprecipitated from the detergent lysate with MAb 327 (a generous gift of J. Brugge) as described previously (3). Immunoprecipitates were fractionated on 10% polyacrylamide-sodium dodecyl sulfate gels, and the phosphoproteins were localized by autoradiography with x-ray film (Cronex 4) and Lightning-Plus (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) intensifying screens.

Peptide mapping. Two-dimensional tryptic phosphopeptide maps were performed on proteins isolated from polyacrylamide gel slices, as described previously (6). One-dimensional typtic mapping was accomplished by elec-

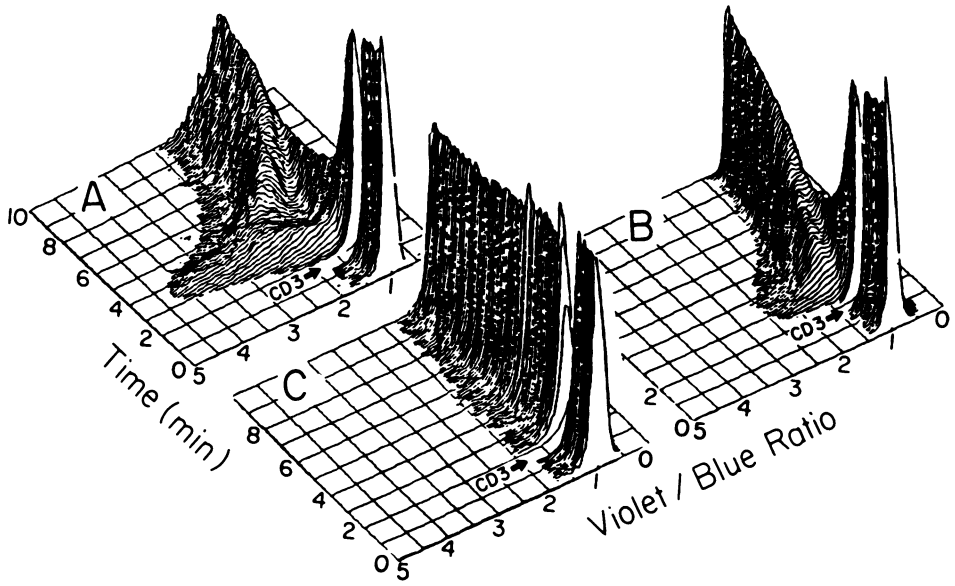

FIG. 1. Effect of EGTA and pertussis toxin on calcium levels in cytoplasm in normal resting cells after stimulation with anti-CD3 MAb G19-4. The histograms show time versus cytoplasmic-free calcium (violet/blue indo-1 ratio) with 10 μg of anti-CD3 per ml introduced at 1.5 to 2.0 min. (A) Control response in medium containing 1 mM calcium. (B) Response in the presence of EGTA to chelate extracellular calcium. (C) Response after a 1-h pretreatment with 10 μg of pertussis toxin per ml at 37°C. Indo-1 violet/blue ratios of 1, 2, 3, and 4 correspond to cytoplasmic calcium concentrations of 131, 338, 679, and 1,346 nM, respectively.

trophoresis in pyridine-acetate buffer (pH 6.5) with cellulose thin-layer sheets. Phosphopeptides were identified by autoradiography.

Measurement of cytoplasmic-free calcium by using indo-1. Our procedure for using indo-1 with a cell sorter for measuring cytoplasmic-free calcium has been described in detail elsewhere (44). Briefly, cells were loaded with the acetoxymethyl ester of indo-1 (Molecular Probes, Eugene, Oreg.) by using an initial concentration of 8 μM. This resulted in an intracellular indo-1 concentration of approximately 50 μM. Viability of indo-1-loaded cells exceeded 98%, as determined by propidium iodide exclusion. After the loading procedure, the cell were washed, placed in fresh medium at 2.5×10^6 ml, and stored in the dark at room temperature until analysis. For each assay indo-1-loaded cells were diluted to 10^6/ml with medium and equilibrated at 37°C. The cells were analyzed by flow cytometry with a cell sorter (Cytofluorograph 50 HH; Ortho Diagnostics System, Inc., Westwood, Mass.). UV excitation was from an argon ion laser (Spectra Physics, Mountain View, Calif.) by using 80 mW at 351 to 364 nm. Blue (480 to 520 nm; Corion Optics, Holliston, Mass.) and violet (383 to 407 nm; Omega Optical Co., Brattleboro, Mass.) band pass filters were used to collect indo-1 fluorescence emission after separation with a 420-nm dichroic mirror (Ortho). Forward, narrow-angle light scatter was used to gate on lymphocytes. The indo-1 ratio of violet to blue fluorescence was directly related to the Ca^{2+} ion concentration and was digitally calculated in real time for each individual cell by using a linear scale. At the beginning of each experiment, the blue and violet photomultiplier settings were routinely adjusted so that the basal ratio was 1.0. The ratio subsequently did not vary by more than 4% during the course of a 4-h experiment.

The Ca^{2+} ion concentration can be calculated from the indo-1 violet/blue ratio by using the formula derived by Grynkiewicz et al. (16):

$$[Ca^{2+}]_i = K_d(R - R_{min})f_2/(R_{max} - R)b_2$$

where $[Ca^{2+}]_i$ is the intracellular ionized calcium concentration (in nanomolar), K_d is 250 nM for the intracellular dye; R is indo-1 violet/blue ratio; R_{min} is the indo-1 violet/blue ratio of calcium-free dye; R_{max} is the indo-1 violet/blue ratio of calcium-saturated dye; f_2 is the blue fluorescence intensity of calcium-free dye, and b_2 is the blue fluorescence intensity of calcium-saturated dye. Calibration of this method is described elsewhere, by which we found the basal $[Ca^{2+}]_i$ of T cells to be 131 ± 8 nM (mean ± standard deviation) (44). This compares favorably with the value obtained by the quin2 technique.

Data were collected on a 2150 computer time mode in which the y axis of a histogram represents the indo-1 violet/blue fluorescence ratio, the x axis represents units of time, and the z axis represents cell number. For isometric displays, small variations in the cell analysis rate were arithmetically normalized, and the data were smoothed before display.

Pertussis toxin (Islet-activating protein) was from List Biological Laboratory (Campbell, Calif.) and was used at 10 μg/ml with 10^6 lymphocytes for 45 min at 37°C prior to anti-CD3 stimulation.

RESULTS

Stimulation of CD3 causes mobilization of cytoplasmic Ca^{2+}. There is evidence that anti-CD3 causes cytoplasmic calcium levels to increase through two mechanisms: anti-CD3 is thought to control a membrane potentially sensitive calcium gate (37) and to cause mobilization of cytoplasmic calcium through phospholipase C-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate) (20). The calcium increase after CD3 stimulation was only partially inhibited by EGTA but was totally inhibited by pertussis toxin (Fig. 1).

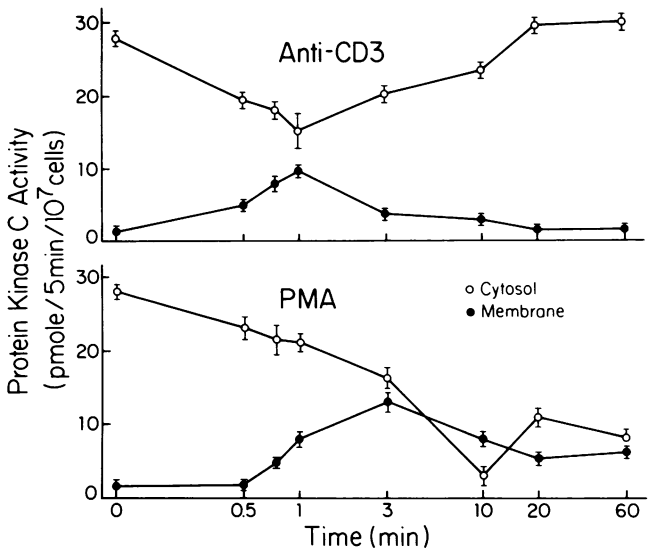

FIG. 2. PKC activity in membrane and cytosol of Jurkat cells after stimulation with 100 μg of anti-CD3 (G19-4) antibody per ml or with 75 ng of PMA per ml. Error bars at each point represent one standard deviation calculated from triplicate determinations of PKC activity.

Pertussis toxin ribosylates and thus inactivates Gi and other GTP-binding proteins (G proteins) that regulate signal transduction, including phospholipase C activation in neutrophils (45). Thus, our result suggests that although extracellular calcium is required for a portion of the CD3 response, the initiation of the Ca^{2+} response is dependent on a pertussis toxin-sensitive G protein for IP3 formation and subsequent calcium mobilization in the cytoplasm. This interpretation is supported by results of a recent study in which it was shown that IP3 formation in the Jurkat cell line is dependent on a G protein that is inactivated by cholera toxin (19).

PKC translocation in Jurkat cells. In addition to the mobilization of cytoplasmic calcium, hydrolysis of phosphatidylinositol-4,5-biphosphate should lead to the translocation of PKC from the cytoplasm to the membrane of the cell. The kinetics of this translocation are shown in Jurkat cells after stimulation with anti-CD3 or PMA (Fig. 2). Maximal membrane-associated activity was observed at 30 s after stimulation, the earliest time point that could be measured. The stimulation by anti-CD3 in solution caused a transient translocation of PKC activity that quickly returned to the cytoplasm. The PKC response to PMA was more prolonged than the PKC response to anti-CD3 stimulation.

Treatment of Jurkat cells with anti-CD3 results in the phosphorylation of pp60$^{c\text{-}src}$ at serine-12. The results of two-dimensional tryptic phosphopeptide analysis of pp60$^{c\text{-}src}$ immunoprecipitated from Jurkat cells treated with PBS, PMA, MAb G19-4, and MAb 9.3 are shown in Fig. 3. As described previously (11, 15), treatment of normal cells with PMA resulted in the hyperphosphorylation of pp60$^{c\text{-}src}$ at a novel residue (c in fig. 3D). Treatment of cells with the anti-CD3 MAb G19-4 resulted in the phosphorylation of pp60$^{c\text{-}src}$ at this same site (Fig. 3C). Cells treated with either PBS or MAb 9.3, directed against a non-CD3-related surface antigen (Tp44) did not contain pp60$^{c\text{-}src}$ phosphorylated at this site (Fig. 3A and B). Peptide a was the major phosphoserine, and peptide b was the major phosphotyrosine residue phosphorylated on pp60$^{c\text{-}src}$ in untreated cells.

Production of IL2 by Jurkat cells. Jurkat cells can produce IL2 in response to appropriate stimulation (13). When Jurkat cells (10^6/ml) were stimulated with anti-CD3 MAb G19-4 in solution, no production of IL2 above that of control, non-stimulated cells was detected (less than 2.4 U/ml; the lower limit of sensitivity with recombinant IL2 as a standard for probit analysis) (12). Although PMA has been shown to act synergistically under these conditions for IL2 production (49), anti-Tp44 was unable to act synergistically with anti-CD3 in solution (<2.4 U of IL2 per ml). When anti-CD3 was immobilized on Sepharose beads, however, Jurkat cells produced IL2 (33.8 U/ml), and this production was further augmented by the addition of the functional anti-Tp44 antibody (102 U/ml). We have shown previously (27) that the CD3 complex is rapidly internalized when anti-CD3 is used in solution but is not internalized when anti-CD3 is attached to a solid support. Scanning electron microscope studies with anti-CD3 in solution have shown that under these conditions CD3 is rapidly internalized into endocytic vesicles and lysosomes rather than shed from the cell surface (O. Press et al., unpublished data). Therefore, internalization of the CD3 complex appears to control an important aspect of T-cell activation.

We also examined the proliferative response of normal resting T cells after stimulation with anti-CD3 in solution or on Sepharose beads. In this experiment we used F(ab')$_2$ fragments of anti-CD3 to avoid interaction of the antibody Fc with Fc receptors on accessory cells because Fc interaction can prevent complete internalization of anti-CD3 bound to the T-cell surface (4, 24, 27, 46). Experiments 1 and 2 in Table 1 indicate that only T cells stimulated with anti-CD3 on a solid support such as Sepharose beads can proliferate in response to rIL2 or the signal from anti-Tp44. The T cells stimulated with anti-CD3 F(ab')$_2$ in solution do not proliferate in response to rIL2, anti-Tp44, or PHA but do respond to PMA. The response to anti-CD3 on solid phase is specific for MAbs to the T-cell receptor complex, because MAbs to other T-cell antigens such as CD2, CD4, CD5, CD6, CD7, or CD8 do not stimulate T cells when the antibodies are on a solid support (J. A. Ledbetter et al., unpublished data).

The results with Jurkat cells and normal T cells suggest that the difference between CD3 stimulation in solution versus on solid phase is of critical importance in determining the functional outcome. Therefore, we examined pp60$^{c\text{-}src}$ when Jurkat cells were stimulated with anti-CD3 in solution or attached to a solid support. Tryptic phosphopeptide analysis of pp60$^{c\text{-}src}$ indicates that solution or solid-phase stimulation of Jurkat cells with anti-CD3 results in the phosphorylation of serine-12 that is indistinguishable even after 4 h of stimulation (data not shown). Therefore, it appears that the transient translocation of PKC can cause a sustained phosphorylation of pp60$^{c\text{-}src}$ for at least 4 h.

DISCUSSION

Results of previous studies (11, 15, 42) have indicated that treatment of Rous sarcoma virus-transformed cells and normal cells results in the phosphorylation of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ at a novel serine residue located in the amino-terminal end of the molecule. In vitro experiments with purified components have demonstrated that PKC can phosphorylate pp60$^{v\text{-}src}$ at the same site observed on pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ when isolated from cells treated with PMA (15, 41).

Treatment of Jurkat cells with the anti-CD3 MAb G19-4 resulted in the phosphorylation of pp60$^{c\text{-}src}$ at a single site which, when analyzed by two-dimensional tryptic phospho-

peptide mapping, appeared identical to that seen on $pp60^{c\text{-}src}$ obtained from cells treated with PMA. Other substrates of PKC include the epidermal growth factor receptor (5, 9, 21). Phosphorylation of the epidermal growth factor receptor by PKC affected both its tyrosine-specific kinase activity as well as epidermal growth factor binding (5, 9, 21). PKC has also been shown to modulate the activity of smooth muscle myosin light-chain kinase. The rate of the kinase activity of the enzyme decreased when it was phosphorylated by PKC in the absence of calmodulin (35).

The exact function of the $pp60^{c\text{-}src}$ tyrosine-specific kinase activity is unknown. In vitro, the specificity of $pp60^{c\text{-}src}$ has been shown to be similar to that of $pp60^{v\text{-}src}$ with respect to at least one target, a 37,000-dalton protein (40).

Stimulation of T cells with MAb that binds to the CD3/T-cell receptor complex can cause T-cell activation or can block T-cell activation (27). This critical difference may be controlled by the internalization of the T-cell receptor complex after antibody or antigen binding (26, 29, 52). Stimulation with antibody or antigen in solution leads to CD3 internalization and prevents T-cell responses, whereas stimulation with antibody on a solid support or antigen on an antigen-presenting cell induces T-cell responses. Thus, control of internalization of the CD3/T-cell receptor complex is thought to be a biochemical model for induction of T-cell tolerance (26, 27, 52).

The pathway for T-cell signaling through the T-cell receptor complex involves activation of phospholipase C to produce IP3 and diacylglycerol second messages (2, 3, 11, 21). We examined this biochemical pathway by measuring changes in cytoplasmic calcium, translocation of PKC, and

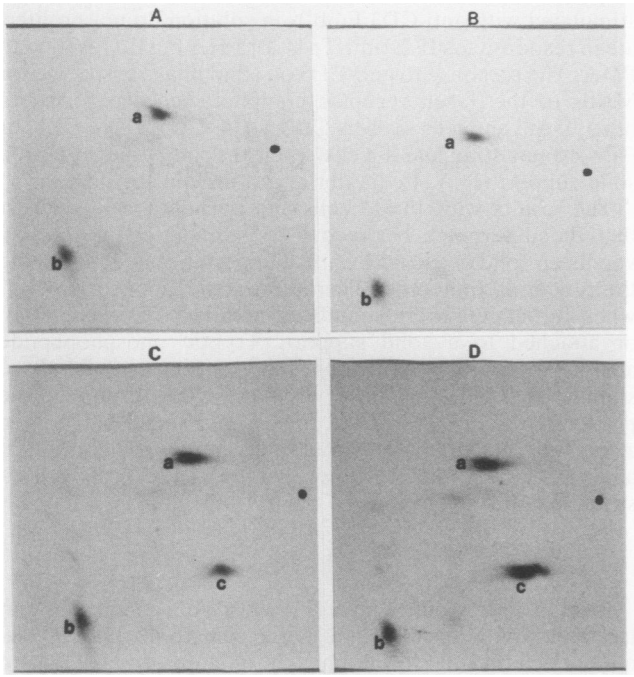

FIG. 3. Two-dimensional tryptic phosphopeptide analysis of $pp60^{c\text{-}src}$ Jurkat cells that were labeled with $^{32}P_i$ and treated with PBS (A), MAb 9.3 (anti-Tp44) at 100 μg/ml (B), mAb G19-4 (anti-CD3) at 100 μg/ml (C), or PMA at 100 ng/ml (D) for 10 min at 37°C. $pp60^{c\text{-}src}$ was immunoprecipitated from the cell lysates and analyzed by two-dimensional tryptic mapping, as described in the text. The black spot marks the origin. Chromatography was from right to left, and electrophoresis was from top to bottom, with the anode at the bottom.

TABLE 1. Proliferation of T cells after CD3 stimulation[a]

Expt no. and anti-CD3 signal	Proliferation ([^{3}H]thymidine incorporation) with the following additional signals:				
	0	PMA	PHA	rIL2	Anti-Tp44
Expt 1					
0	277	3,145		388	350
$F(ab')_2$	326	161,030		475	578
$F(ab')_2$-Sepharose	6,866	162,662		96,437	123,702
Expt 2					
0	219	1,611	105,381		
$F(ab')_2$	162	113,510	1,417		
$F(ab')_2$-Sepharose	37,015	91,620	135,903		

[a] Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.). A $F(ab')_2$ fragment of anti-CD3 antibody G19-4 was used at 1 μg/ml, and anti-CD3 antibody G19-4 $F(ab')_2$ conjugated to Sepharose was used at its optimal mitogenic concentration (0.2 μl of beads per well). PMA was used at 75 ng/ml, and PHA was used at 1 μg/ml. Anti-Tp44 antibody 9.3 was used at 1 μg/ml, and rIL2 (Genzyne) was used at 100 U/ml. Proliferation was measured by [^{3}H]thymidine uptake during a 6-h pulse on day 3 after stimulation. Proliferation was measured in quadruplicate, and the mean values are shown. Standard errors were less than 15% of the mean at each point.

phosphorylation of $pp60^{c\text{-}src}$ after stimulation of T cells or the Jurkat T-cell line with anti-CD3 MAb. The calcium response and PKC translocation response occurred very quickly after stimulation with anti-CD3 antibody in solution. Two-dimensional tryptic phosphopeptide analysis of $pp60^{c\text{-}src}$, a known substrate for PKC, indicates that the amino-terminal serine-12 residue is phosphorylated in response to treatment with Jurkat cells with anti-CD3. Although the consequences of anti-CD3 stimulation with antibody in solution or on solid phase are different, we did not detect a difference in the effect of anti-CD3 stimulation on $pp60^{c\text{-}src}$ phosphorylation. Thus, the consequences of T-cell stimulation through the IP3 $\rightarrow Ca^{2+}$ and DG $\rightarrow$ PKC $\rightarrow$ $pp60^{c\text{-}src}$ pathway appear to be controlled by the internalization of the CD3/T-cell receptor (27) through biochemical signals that have not yet been identified. In addition, there are likely to be calcium-regulated enzymes other than PKC that are important for T-cell responses.

T cells are activated to proliferate and Jurkat cells to produce IL2 only when anti-CD3 is used on a solid support. Anti-CD3 in solution actively blocks T-cell responses to all signals except for PMA, in which an active synergistic effect is still present. Because both anti-CD3 in solution and PMA causes PKC translocation, it is difficult to understand the basis of this synergistic effect. One possibility is the kinetics of the PKC translocation; unlike diacylglycerol, PMA is not actively metabolized (1), and PMA causes a more prolonged translocation of PKC than does anti-CD3 (Fig. 2) (10). Therefore, a PKC substrate, probably other than $pp60^{c\text{-}src}$, may be critically regulated by the kinetics of PKC translocation to the membrane. It is thus possible that anti-CD3 stimulation in solution or on solid phase differs primarily in the kinetics of PKC translocation. It is also possible, however, that regulatory molecules such as cyclic AMP or other cellular oncogene products are differentially regulated by the mode of CD3 stimulation.

Antibody binding to the Tp44 homodimer on human T cells gives a functional signal that acts synergistically with activation through the CD3/T-cell receptor (14, 18, 28, 34). Anti-Tp44 is thought to function by regulating IL2 production (31). We have shown here that anti-Tp44 stimulates IL2 production by Jurkat T cells but does not affect phosphory-

lation of pp60$^{c\text{-}src}$. Thus, the biochemical pathway stimulated by anti-Tp44 does not appear to involve direct effects on inositol metabolism.

Several studies have shown that cytoplasmic-free calcium increases after anti-CD3 stimulation of T cell or the Jurkat T-cell line (20, 22, 37, 38, 44, 49, 50). The function and contribution of IP3 in mobilizing cytoplasmic calcium compared with a transmembrane flux through calcium channels is somewhat controversial. A recent model by Putney (43) hypothesizes that IP3 first mobilizes cytoplasmic calcium, and then calcium channels are opened in response to the depletion of cytoplasic calcium stores. Results of our studies, with pertussis toxin used to block cytoplasmic calcium mobilization and EGTA used to chelate extracellular calcium, agree very well with this hypothesis. In the presence of EGTA, the early calcium response to CD3 stimulation of T cells is still present, but this response is not sustained and returns more quickly to the base line response. In the presence of pertussis toxin, which is used to block cytoplasmic calcium mobilization, there is no calcium response to CD3 stimulation. Thus, in agreement with the model described by Putney (43), mobilization of cytoplasmic calcium must occur first before extracellular calcium can enter the cell.

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