Activation of phospholipase D: a signaling system set in motion by perturbation of the T lymphocyte antigen receptor/CD3 complex

Stanford J. Stewart,*†‡ Gary R. Cunningham,*
John A. Strupp,* Frances S. House,*
Linda L. Kelley,*† Gregory Stephen Henderson,†
John H. Exton,§ and Stephen B. Bocckino§
Departments of *Medicine, †Pathology
and §Molecular Physiology and Biophysics
and the §Howard Hughes Medical Institute
Vanderbilt University School of Medicine
‡Department of Veterans Affairs Medical Center
Nashville, Tennessee 37212

A number of cellular signaling systems are called into play by interaction of the T lymphocyte antigen receptor/CD3 complex with its cognate antigen. Well-described signaling systems include phosphoinositide turnover, tyrosine phosphorylation, protein kinase C activation, and increased cytosolic calcium. We have explored the possibility that another recently described signaling system, activation of phospholipase D, may be operative. Data presented here demonstrate that stimulation of Jurkat T cells with anti-CD3 antibodies or phorbol esters resulted in activation of phospholipase D, as measured by production of phosphatidylethanol and phosphatidic acid. The combination of anti-CD3 antibody plus phorbol ester led to a greater than additive production of phosphatidylethanol and to the additive production of phosphatidic acid (in the absence of ethanol). Phorbol esters as a second stimulus with anti-CD3 antibody led to an additive increase in cellular diacylglycerol content but provided no increased production of inositol phosphates, suggesting that diacylglycerol production in these cells results from hydrolysis of noninositol containing lipids as well as from phosphoinositides. Exogenous addition of phosphatidic acid led to increases in cytosolic calcium that, depending on the concentration used, resulted from release of an intracellular store of calcium and influx of extracellular calcium. Changes in cytosolic calcium occurred in the absence of inositol phosphates production. These studies establish a role for increased phospholipase D activity in T lymphocyte activation.

Introduction

Physiological T lymphocyte activation (reviewed in Altman *et al.*, 1990) is set in motion by the interaction of processed antigen in the context of products of the Major Histocompatibility Complex with the T lymphocyte antigen receptor (TCR), which is in close physical association with the CD3 complex. The multicomponent CD3 complex is presumed to function in transmission of the activation signal from the TCR to the cellular interior. Antibodies directed against TCR or CD3 can serve as surrogate antigen and lead to changes consistent with the activated state. Among these changes are tyrosine phosphorylation of various substrates (Hsi *et al.*, 1989); production of inositol 1,4,5 trisphosphate (InsP_3) and consequent changes in cytosolic free calcium concentration ($[\text{Ca}^{++}]$) (Imboden and Stobo, 1985); increases in diacylglycerol (DAG) production and consequent activation of protein kinase C (Nel *et al.*, 1987); production of mRNA for certain proto-oncogenes (Granelli-Piperno *et al.*, 1986) lymphokines, and membrane receptors (Crabtree, 1989); and, ultimately, proliferation (van Wauwe *et al.*, 1980).

For several years it appeared that the early events of T lymphocyte activation paralleled those of several receptor-mediated, Ca^{++} -dependent, activation schemes that involve G-protein activation of a phospholipase C that catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers, InsP_3 and DAG (Berridge and Irvine, 1984). However, several recent studies have emphasized the role of tyrosine phosphorylation events in T lymphocyte activation (June *et al.*, 1990; Mustelin *et al.*, 1990; Samelson *et al.*, 1990), and two recent studies have cast doubt on the necessity of phosphoinositide hydrolysis for T lymphocyte activation. In a T-cell hybridoma carrying transfected TCR chains, interleukin 2 (IL-2) production in response to antigen and anti-CD3 monoclonal antibodies (MAb), comparable with that in the parent T-cell line, occurred despite much reduced production of inositol phosphates (Sussman *et al.*, 1988). In a

cytotoxic T-cell clone, abolition of detectable inositol phosphates production by chelation of external Ca^{++} failed to affect antigen-specific cytolysis (O'Rourke and Mescher, 1988). These findings suggested that a search for other signaling systems activated by perturbation of the TCR/CD3 complex would be appropriate.

Recently, several investigators have suggested that activation of phospholipase D resulting in phosphatidic acid (PA) production may be an important intracellular signaling pathway in cellular systems that also employ the phosphoinositide-dependent signaling pathways (reviewed in Exton, 1990). PA has long been considered a potential cellular regulator (Tyson *et al.*, 1976). Early work suggested that PA was a calcium ionophore. Recent work has suggested that exogenously added PA may serve as a mitogen and/or intracellular second messenger. Addition of PA to A431 carcinoma cells led to increases in $[\text{Ca}^{++}]$, due to release of an intracellular store of Ca^{++} and expression of *c-myc* and *c-fos* proto-oncogene products (Moolenaar *et al.*, 1986). Addition of PA to Rat1 fibroblasts led to DNA synthesis (Moolenaar *et al.*, 1986). PA has been shown to be mitogenic for 3T3 fibroblasts (Siegmann, 1987) and mammary epithelia (Imagawa *et al.*, 1989). Derivatives of PA, particularly lysophosphatidate, are also potent mitogens for fibroblasts (van Corven *et al.*, 1989). The notion that PA is a second messenger is supported by evidence that cellular PA production follows vasopressin stimulation of hepatocytes, apparently by the phospholipase D-mediated cleavage of phosphatidylcholine (Bocckino *et al.*, 1987a) and by evidence of a PA-specific protein kinase operating in rat liver cytosol (Bocckino *et al.*, 1991). PA also has been shown to activate inositol 1,2, cyclic phosphate 2 phosphohydrolase (lipocortin III) (Ross *et al.*, 1990), which may play an important role in regulation of the intracellular concentration of certain inositol phosphates.

We have explored the possibility that activation of phospholipase D is a consequence of perturbation of the TCR/CD3 complex. Data presented here demonstrate that anti-CD3 MAb or phorbol ester stimulation of the human T lymphocyte cell line, Jurkat, leads to the accumulation of products of phospholipase D activation that may have important roles in T lymphocyte activation.

Results

Anti-CD3-stimulated phospholipase D activity in T lymphocytes

A product of phospholipid breakdown by phospholipase D is PA. However, PA also can be

produced as an intermediate in phospholipid biosynthesis and from DAG via DAG kinase. Therefore, PA accumulation is not a definitive measure of phospholipase D activity. However, in the presence of primary alcohols, phospholipase D catalyzes a characteristic and specific transphosphatidyl transfer reaction resulting in production of the appropriate phosphatidyl alcohol (Dawson, 1967). This property of the enzyme has been used successfully by several investigators to examine phospholipase D activity in a variety of cell systems (Bocckino *et al.*, 1987a,b, 1989; Kinsky *et al.*, 1989; Liscovitch, 1989; Chalifa *et al.*, 1990; Huang and Cabot, 1990; Randall *et al.*, 1990; Reinhold *et al.*, 1990). We took advantage of this reaction to examine the role of phospholipase D in activation of ethanol-treated Jurkat T lymphocytes. Figure 1 demonstrates that perturbation of the TCR/CD3 complex led to an increase in phosphatidylethanol (PEth) production. The data in Figure 1 also suggest that phorbol myristate acetate (PMA) alone and the combined stimulation of cells with anti-CD3 MAb and PMA led to more profound

Figure 1. PEth and PA production in stimulated T lymphocytes. Jurkat cells were labeled with $^{32}\text{PO}_4$, then stimulated in the presence (lanes 1–4) or absence (lanes 5–8) of 0.5% ethanol. Cells were stimulated with anti-CD3 MAb, 1 $\mu\text{g}/\text{ml}$ (lanes 1 and 5); anti-CD3 plus PMA, 10 ng/ml (lanes 2 and 6); PMA (lanes 3 and 7); or PBS (control) (lanes 4 and 8) for 30 min. Lipids were extracted and separated by thin-layer chromatography. Film was exposed to the TLC plate. Experiment shown is representative of two.

Figure 2. Effect of other MAb on PLD activation. Jurkat cells were labeled with $^{32}\text{PO}_4$, then stimulated with the indicated MAb for 30 min. Lipids were extracted, isolated by TLC, and radioactivity incorporated was determined by liquid scintillation counting. Data were normalized to adjust for different levels of labeling. Data presented represent the mean $\pm$ SEM for triplicate experiments.

increases in PEth production. These results were examined in detail below.

Figure 1 also shows that anti-CD3, PMA, and anti-CD3 plus PMA caused increases in intracellular PA. Reduced production of PA in the presence of ethanol (compare lanes 1–3 with

lanes 5–7) suggests that competition occurred between the hydrolysis reaction and the transphosphatidylation reaction.

The effects of antibodies directed against other T lymphocyte cell surface antigens on phospholipase D (PLD) activity were examined. As shown in Figure 2, MAb directed against the pan T-cell antigens, CD2 and CD5, and against the common leukocyte antigen, CD45, failed to increase PEth production, whereas anti-CD3 significantly increased PEth production. These data confirm that the anti-CD3-stimulated increase in PLD activity results from specific activation of the enzyme via the TCR/CD3 complex and does not result from a non-specific membrane event.

Effects of concentration on anti-CD3 and PMA stimulation of PLD activity

The production of PEth in response to broad concentration ranges of the two agonists, anti-CD3 and PMA, was examined. Figure 3A demonstrates a concentration-dependent increase in PEth formation in response to the anti-CD3 MAb, with a maximal effect of the antibody occurring between 0.1 and 1.0 $\mu\text{g/ml}$. Figure 3B demonstrates a concentration-dependent increase in PEth formation in response to PMA. Maximal effect with PMA far exceeded that with anti-CD3 MAb (see again Figure 1 and compare vertical axes of Figure 3, A and B). The PMA data could be fit to a logistic equation yielding

Figure 3. Concentration-response relationships for anti-CD3 MAb- and phorbol ester-stimulated PLD activation. Jurkat cells were labeled with $^{32}\text{PO}_4$, then stimulated with anti-CD3 MAb (A) or PMA (B) at the concentrations indicated for 30 min. Lipids were extracted, isolated by TLC, and radioactivity incorporated was determined by liquid scintillation counting. Data presented represent mean $\pm$ SEM for five experiments (A) and for two experiments (B).

a 50% effective concentration (EC₅₀) of 26 ng/ml. This EC₅₀ is similar to those reported for other effects of phorbol esters—effects generally ascribed to activation of protein kinase C (Ashendel, 1985).

Dual control of PLD activity

The roles of anti-CD3 and PMA in activation of PLD were further examined in a series of experiments in which cells were stimulated with either anti-CD3, PMA, or the combination. Figure 4A demonstrates that across a concentration range of anti-CD3 MAb, the combination of anti-CD3 plus PMA led to an increase in PEth production that was equal to or greater than the sum of the increments produced by anti-CD3 and PMA alone. Thus, the combination of anti-CD3 and PMA was supra-additive for PLD activation. Of note, in experiments conducted in the presence of ethanol (see Figure 4B), PMA caused no net increase in cellular PA when used alone or in combination with the anti-CD3 MAb. However, in the absence of ethanol, PMA increased PA production over that caused by anti-CD3 alone, in an additive fashion, except at the maximally effective concentration of anti-CD3 (0.25 μ g/ml) (Figure 4C).

Production of DAG in response to anti-CD3 and PMA

PA can be converted to DAG through dephosphorylation by phosphatidate phosphohydrolase. Therefore, we examined production of DAG after stimulation of Jurkat cells with PMA, anti-CD3 MAb, or both. Figure 5A demonstrates

that both PMA and anti-CD3 MAb led to increases in DAG in these cells, and the combination of anti-CD3 MAb and PMA additively increased cellular DAG content. This pattern of responses differed from that seen when inositol phosphates production resulting from stimulation with these agents was examined (Figure 5B). Here, PMA alone failed to increase inositol phosphates production, and when used in combination with anti-CD3 MAb, failed to increase anti-CD3-stimulated inositol phosphates production. These data suggest that cellular DAG arises not only from metabolism of phosphoinositides but from other sources as well.

Time course of anti-CD3-stimulated PLD activity

Figure 6 demonstrates the time course of accumulation of PEth in response to anti-CD3 stimulation. Incorporation of radioactivity into PEth after anti-CD3 stimulation was transient during the first 10 min, accelerated from 10 to 30 min, then again slowed to near that seen with constitutive activity of the enzyme from 30 to 60 min. Figure 6 also demonstrates that in the absence of radioactive phosphate in the medium, labeling of the PA pool transiently increased and then declined, presumably due to further metabolism of PA.

Effects of exogenously added PA on $[Ca^{++}]_i$

To evaluate what impact enhanced PLD activity and hence PA production might have on the T lymphocyte, we examined the effect of exogenously added PA on $[Ca^{++}]_i$. Other investigators

Figure 4. Effects of dual stimulation with anti-CD3 and phorbol esters on PLD activity in Jurkat cells. Jurkat cells were labeled with $^{32}PO_4$, then stimulated in presence of 0.5% ethanol (A and B) or in the absence of ethanol (C). Cells were stimulated with various concentrations of anti-CD3 MAb with or without PMA (10 ng/ml), as indicated, for 30 min. Lipids were extracted and separated by thin-layer chromatography. PEth and PA were isolated by TLC and evaluated by liquid scintillation chromatography. Data in A and B are expressed as mean $\pm$ SEM for five such experiments. Data in C are expressed as mean $\pm$ SEM of normalized data from four such experiments. Statistical analysis employed an ANOVA and Fisher's LSD test of significance.

Figure 5. DAG and inositol phosphates production in stimulated T lymphocytes. (A) Jurkat cells were stimulated as indicated for 10 min. Lipids were extracted and DAG analyzed by DAG kinase assay. Data presented represent the mean $\pm$ SEM for triplicate determinations in two separate experiments. (B) Jurkat cells were stimulated as indicated for 10 min, then lysed in 10% TCA. Water soluble inositol containing compounds were analyzed by anion exchange chromatography and liquid scintillation counting. Data presented represent mean $\pm$ SEM of values from three experiments.

have reported changes in $[Ca^{++}]_i$ in response to PA administration in other cell systems (Tyson *et al.*, 1976; Moolenaar *et al.*, 1986). Figure 7 demonstrates that after PA addition, $[Ca^{++}]_i$ increased dramatically in the Jurkat cells. Changes in $[Ca^{++}]_i$ were dependent on the con-

centration of PA employed. Figure 7A shows that, at 10 μ g/ml, PA caused a rapid, substantial, but transient, increase in $[Ca^{++}]_i$. However, at 100 μ g/ml (Figure 7B), PA caused a rapid, and sustained, increase in $[Ca^{++}]_i$. In each case

Figure 6. Time course of anti-CD3 stimulated PLD activation. Jurkat cells were labeled with $^{32}PO_4$, then stimulated with anti-CD3 MAb 1 μ g/ml for the times indicated. Lipids were extracted and separated by TLC and incorporated radioactivity was determined by densitometric analysis of autoradiograms (PEth) (when TLC plate fracture occurred) or by liquid scintillation counting (PA). Data shown are representative of two experiments.

Figure 7. Effect of PA on $[Ca^{++}]_i$. Fura-2-loaded Jurkat cells were stimulated with PA 10 μ g/ml (A) or PA 100 μ g/ml (B) in the presence or absence of the calcium chelator, EGTA (as indicated). Fura-2 fluorescence was continuously monitored and converted to $[Ca^{++}]_i$.

chelation of extracellular Ca^{++} using ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid caused little difference in the initial increase in $[\text{Ca}^{++}]_i$, suggesting that this early change in $[\text{Ca}^{++}]_i$ resulted largely from release of intracellular stores of Ca^{++} . The sustained elevation in $[\text{Ca}^{++}]_i$ seen after treatment of the cells with the higher concentration of PA (Figure 7B) was eliminated by chelation of extracellular Ca^{++} , suggesting that at later times elevation of $[\text{Ca}^{++}]_i$ was dependent on an influx of extracellular Ca^{++} into the cells.

One mechanism by which intracellular stores of calcium are released is by production of the lipid-derived second messenger, InsP_3 (Berridge and Irvine, 1984). Some investigators have reported that PA treatment of cells leads to InsP_3 production (Moolenaar *et al.*, 1986; Murayama and Ui, 1987). We examined inositol phosphates production in the Jurkat cells after PA stimulation. Figure 8 demonstrates that no detectable increase in any inositol phosphate was observed in the Jurkat cells after PA stimulation, whereas inositol phosphates production was obvious after stimulation of the cells with the anti-CD3 MAb as previously reported (Imboden and Stobo, 1985; Kelley *et al.*, 1990). Anti-CD3 MAb concentrations were chosen on the basis of previously reported findings in this system (Kelley *et al.*, 1990). The higher concentration of 5

$\mu\text{g/ml}$ leads to an early peak in the calcium change around 60 s as compared with the 30 s required for PA to cause an increase in $[\text{Ca}^{++}]_i$. The lower concentration of 0.5 $\mu\text{g/ml}$ has been shown to lead to a change in $[\text{Ca}^{++}]_i$ of approximately the same magnitude as that produced by PA. Inositol phosphates production in response to this lower concentration of anti-CD3 MAb was examined at the time of peak calcium change. These data suggest that PA leads to release of an intracellular store of calcium without InsP_3 production.

Discussion

The data presented here demonstrate that a previously unrecognized signaling system is activated by perturbation of the TCR/CD3 complex. This signaling system employs phospholipase D to produce PA, a potential second messenger and precursor of the second messenger, DAG. Control of phospholipase D activity in this system is complex. In addition to agents that interact with the antigen receptor, agents that activate protein kinase C (i.e., phorbol ester) (Nishizuka, 1984) activate phospholipase D, as indicated by the stimulated production of PEth. Our results differ from those of Kinsky *et al.* (1989), who found no increase in PLD activity in Jurkat cells, several other T-

Figure 8. Effect of PA on inositol phosphates production. Jurkat cells were labeled with ^3H -myo-inositol for 3 h, then stimulated with anti-CD3 MAb (5 $\mu\text{g/ml}$ for 30 s or 0.5 $\mu\text{g/ml}$ for 3 min), PA in concentrations, for times, as indicated, or PBS (control). Water-soluble inositol-containing compounds were separated by anion exchange chromatography and radioactivity was determined by liquid scintillation counting. Data presented represent mean $\pm$ SEM of dpm observed normalized to incorporation of radioactivity into a 10 min InsP_2 control sample for four separate experiments. Controls were not carried out (n.d.) at 30 and 60 s because previous work (Stewart *et al.*, 1987) has shown them to be unchanged from 3 min values. Statistical analysis employed ANOVA and Fisher's LSD test of significance.

cell lines, or in normal peripheral blood lymphocytes in response to phorbol esters. The reason for this discrepancy is not apparent because similar assays and phorbol ester concentrations were used. PLD activity resulting from stimulation of the TCR/CD3 was not examined by those authors.

The combination of receptor stimulation and protein kinase C stimulation reproducibly caused increases in PEth production that exceeded the sum of increases in PEth produced by anti-CD3 MAb alone and PMA alone. The excess production was in most cases small and therefore not easily amenable to statistical analysis. We have chosen to use the term supra-additive to describe this increased production (avoiding the term synergistic with its attendant implications), believing that this term best describes the observed data. This supra-additive combination of phorbol ester and agents that interact with TCR/CD3 has been shown to result in IL-2 production in T lymphocytes (Weiss *et al.*, 1984), whereas neither agent alone was sufficient. This observation raises the intriguing possibility that some threshold of phospholipase D activity may be required for complete T lymphocyte activation.

The mechanism by which anti-CD3 MAb and PMA supra-additively interact is not immediately obvious. Table 1 indicates, using data presented throughout the manuscript, that the metabolism of various lipid-derived products resulting from phospholipase D activation is complex. Anti-CD3 stimulation of Jurkat cells led to increased production of PEth, PA, inositol phosphates, and DAG. PMA alone increased production of PEth, PA, and DAG. The combined stimulus of anti-CD3 MAb plus PMA led to 1) additive or supra-additive increases in PEth and DAG compared with those caused by either agent alone, 2) additive increases in PA production, but 3) increases in inositol phosphates production no greater than those produced by anti-CD3 MAb alone. The additive production of DAG resulting from combined stimulation with anti-CD3 MAb plus PMA, in the absence of any additive increase in inositol phosphates production suggests that DAG production in these cells results from metabolism of non-ino-

sitol-containing lipids as well as from phosphoinositides. Although activation of a non-PIP₂-specific PLC cannot be formally ruled out, the data presented here are consistent with the notion that anti-CD3 plus PMA stimulation increases phosphatidate phosphohydrolase activity and shunts PLD-generated PA to DAG. Thus, physiological protein kinase C activation, sufficient for T lymphocyte activation, may require DAG derived from activity of both the TCR/CD3-associated PIP₂-specific phospholipase C and TCR/CD3-associated phospholipase D.

Increases in PLD activity after anti-CD3 MAb and PMA stimulation suggest that anti-CD3 may activate PLD via protein kinase C. Preliminary experiments employing the protein kinase C inhibitors sphingosine (Hannun and Bell, 1989) and H-8 (n-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) (Hidaka *et al.*, 1984) have failed to demonstrate any effect of the inhibitors on anti-CD3-stimulated PEth production. The sphingosine observations are complicated by the fact that sphingosine increased cellular PEth and PA levels presumably through inhibition of phosphatidate phosphohydrolase (Lavie *et al.*, 1990) and activation of PLD (Lavie and Liscovitch, 1990). Whether anti-CD3 MAb increases PLD activity through protein kinase C or through some other mechanism remains a focus for further studies.

Physiological roles for PLD products in the T lymphocyte were investigated. Addition of PA to Jurkat cells resulted in changes in [Ca⁺⁺]_i. These changes were considerably smaller in magnitude than those seen after anti-CD3 stimulation of Jurkat cells (Kelley *et al.*, 1990). At lower concentrations of PA, these calcium changes were dependent on release of an intracellular pool of Ca⁺⁺, whereas at higher concentrations changes were dependent on both release of intracellular stores and influx of Ca⁺⁺ from the cellular exterior. Production of InsP₃ resulting from PA treatment did not accompany the changes in [Ca⁺⁺]_i in the T-cell line, although PA-stimulated inositol phosphates production has been reported in other systems. The mechanism of this Ca⁺⁺ release has not been elucidated. However, other InsP₃-independent Ca⁺⁺

Table 1. Effects of stimuli on lipid-derived products and second messengers

	PEth	PA	InsPs	DAG
anti-CD3	+	+	+	+
PMA	+	+	—	—(+)
anti-CD3 + PMA	+++	++(+)	+	++(+)

release phenomena have been described in this system (Kelley *et al.*, 1990).

In A431 squamous carcinoma cells, PA stimulation has been shown to be sufficient for increased *c-fos* and *c-myc* mRNA expression (Moolenaar *et al.*, 1986). Remote events of T lymphocyte activation such as proto-oncogene mRNA expression, lymphokine mRNA expression, and lymphokine production in response to PA also were examined. In Jurkat cells (under the conditions employed) no increases in proto-oncogene or lymphokine mRNA expression were seen in response to PA alone. Failure to detect changes in proto-oncogene expression in the T lymphocytes although it was previously seen in the A431 cells may represent phenotypic differences between cells of different lineages or be associated with differences in mechanisms of malignant transformation in the two cell lines.

The data presented in this paper establish a role for receptor-mediated phospholipase D activity in human T lymphocyte activation. These studies also underline the complexity of the signaling resulting from stimulation of the T-cell antigen receptor. Clearly many pathways intertwine, eventually resulting in phenotypic cellular changes and proliferation. The exact contribution of each pathway remains to be dissected.

Materials and methods

Cell lines

The human T lymphocyte cell line, Jurkat (Gillis and Watson, 1980), was used in this study. The cell line was maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) (pRPMI) supplemented with 15% fetal bovine serum (GIBCO) and penicillin-streptomycin (GIBCO) (cRPMI).

Analysis of phospholipase D products

Jurkat cells were suspended in a medium of phosphate-free Eagle's minimum essential medium (GIBCO) containing 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 0.5% dialyzed heat inactivated fetal calf serum (DAFCS), buffered with *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) 10 mM (pH 7.3) (GIBCO), and supplemented with glutamine and penicillin-streptomycin (GIBCO) at a density of 1×10^6 /ml. To the cell suspension, ^{32}P as orthophosphoric acid 100 $\mu\text{Ci}/15$ ml (New England Nuclear, Boston, MA) was added. The cells were placed in a 37°C, 5% CO_2 incubator for ~16 h. Cells were harvested by centrifugation, washed with pRPMI, and resuspended in pRPMI for stimulation. For stimulation, cells were placed at 37°C. Stimulants included PMA (Sigma); anti-CD3 MAb, JE6 [the generous gift of Dr. Ronald Levy, Stanford University Medical Center (Takahashi *et al.*, 1989)]; anti-CD2 MAb, OKT11 (American Type Culture Collection, Bethesda, MD); anti-CD5 MAb, anti-Leu-1 (Becton Dickinson, Mountain View, CA); and anti-CD45 MAb, anti-Leu-18 (Becton Dickinson). At the end of stimulation, acidic phospholipids were extracted as described previously (Stewart *et al.*, 1986) using the Bligh-Dyer method (Bligh and Dyer, 1959; Bocckino *et al.*, 1987b).

The organic layer was dried under N_2 , then taken up in 20 μl chloroform, and applied to silica gel K6 plates (Whatman Labsales, Inc., Hillsboro, OR) along with PA and PEth standards (Avanti, Pelham, AL). The plates were developed with a solvent system of ethyl acetate/iso-octane/acetic acid (9:5:2) (Bocckino *et al.*, 1987b). Locations of standards were ascertained by staining the plates with Coomassie Blue, and labeled compounds were visualized by autoradiography. Labeled products of interest were scraped from the plates and radioactivity content was determined by liquid scintillation counting.

Measurement of DAG

Cellular levels of DAG were measured by the DAG kinase assay as previously described (Bocckino *et al.*, 1989) using the assay described by Preiss *et al.* (1986) as modified by Wright *et al.* (1988). Lipids were extracted from unstimulated and stimulated Jurkat cells using the Bligh-Dyer method (Bligh and Dyer, 1959) substituting H_2O for the HCl used above. The resulting organic phase was filtered through glass wool and dried under N_2 . The residue was incubated with DAG kinase (Lipidex, Westfield, NJ) in the presence of ^{32}P - γ -ATP (New England Nuclear). The resulting radioactive PA was isolated by thin-layer chromatography and ^{32}P incorporation quantitated by liquid scintillation counting. Radioactivities from experimental samples were compared with those of a standard curve of samples with known amounts of 1,2 diolein (Avanti).

Calcium studies

Changes in $[\text{Ca}^{++}]_i$ were evaluated as previously described by this laboratory (Kelley *et al.*, 1990). In brief, Jurkat cells in HEPES-buffered pRPMI were incubated with 2 $\mu\text{g}/\text{ml}$ acetoxymethyl ester of fura-2 (Calbiochem, San Diego, CA) dissolved in 25% Pluronic F-127 (BASF-Wyandotte, Wyandotte, MI) in dimethyl sulfoxide (Sigma) for 45 min. Cells were washed and resuspended in HEPES-buffered pRPMI. Immediately before analysis, cells were suspended in a simplified stimulation buffer (Tsien *et al.*, 1985) containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 , 1 mM CaCl_2 , 0.5 mM MgSO_4 , 5 mM glucose, and 10 mM HEPES (pH 7.4). Cells were maintained at a constant temperature of 37°C and stirred. Fura-2 fluorescence was continuously monitored at 505 nm during alternate excitation by light of 340 and 380 nm. $[\text{Ca}^{++}]_i$ was calculated using the ratio of fluorescence excited by light of 340 and that excited by light of 380 nm as described by Grynkiewicz *et al.* (1985).

Inositol phosphates determinations

For examination of inositol phospholipid metabolism, Jurkat cells were suspended in Eagle's basal medium lacking myo-inositol (GIBCO) and containing 10% DAFCS. To this suspension $[\text{^3H}]\text{myo-inositol}$ (15 Ci/mmol) (American Radiolabeled Chemicals, St. Louis, MO) was added at 40 $\mu\text{Ci}/2 \times 10^7$ cells/ml. Cells were incubated at 37°C, 5% $\text{CO}_2 \times 3$ h. Labeled cells were washed and resuspended in simplified medium (see above) for stimulation. Inositol phosphates InsP_1 through InsP_6 were isolated from a 10% trichloroacetic acid soluble cellular fraction by anion exchange chromatography on AG1 $\times 8$ columns as previously described (Stewart *et al.*, 1987) using the methods of Berridge (1983). Radioactivity of each fraction was determined by liquid scintillation counting.

Acknowledgments

This work was supported by National Institutes of Health Grant GM-38610 and by the Department of Veterans Affairs.

Mr. Henderson was supported by NIH Medical Scientist Training Program Grant T32-GM07347. The authors thank Mangalika Aluthge, Pam Wilson, and Dewain Patterson for excellent technical assistance in support of the project.

Received: May 28, 1991.

Revised and accepted: July 23, 1991.

References

- Altman, A., Coggeshall, K.M., and Mustelin, T. (1990). Molecular events mediating T cell activation. *Adv. Immunol.* **48**, 227–360.
- Ashendel, C.L. (1985). The phorbol ester receptor: a phospholipid-regulated protein kinase. *Biochim. Biophys. Acta* **822**, 219–242.
- Berridge, M.J. (1983). Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.* **212**, 849–858.
- Berridge, M.J., and Irvine, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.)* **312**, 315–321.
- Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Bocckino, S.B., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987a). Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. *J. Biol. Chem.* **262**, 15309–15315.
- Bocckino, S.B., Wilson, P.B., and Exton, J.H. (1987b). Ca^{2+} -mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation. *FEBS Lett.* **225**, 201–204.
- Bocckino, S.B., Wilson, P.B., and Exton, J.H. (1989). An early elevation of diacylglycerol and phosphatidate in regenerating liver. *Biochem. Biophys. Res. Commun.* **164**, 290–294.
- Bocckino, S.B., Wilson, P.B., and Exton, J.H. (1991). Phosphatidate-dependent protein phosphorylation. *Proc. Natl. Acad. Sci. USA* **88**, 6210–6213.
- Chalifa, V., Mohn, H., and Liscovitch, M. (1990). A neutral phospholipase D activity from rat brain synaptic plasma membranes. *J. Biol. Chem.* **265**, 17512–17519.
- Crabtree, G.R. (1989). Contingent genetic regulatory events in T lymphocyte activation. *Science* **243**, 355–361.
- Dawson, R.M.C. (1967). The formation of phosphatidylglycerol and other phospholipids by the transferase activity of phospholipase D. *Biochem. J.* **102**, 205–210.
- Exton, J.H. (1990). Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265**, 1–4.
- Gillis, S., and Watson, J. (1980). Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin-2 producing human leukemia T cell line. *J. Exp. Med.* **152**, 1709–1719.
- Granelli-Piperno, A., Andrus, L., and Steinman, R.M. (1986). Lymphokine and non-lymphokine mRNA levels in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin A. *J. Exp. Med.* **163**, 922–937.
- Gryniewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca^{++} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440–3450.
- Hannun, Y.A., and Bell, R.M. (1989). Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* **243**, 500–507.
- Hidaka, H., Inagaki, M., Kawamoto, S., and Sakai, Y. (1984). Isopurinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochem. J.* **23**, 5036–5041.
- Hsi, E.D., Siegel, J.N., Minami, Y., Luong, E.T., Klausner, R.D., and Samelson, L.E. (1989). T cell activation induces rapid tyrosine phosphorylation of a limited number of cellular substrates. *J. Biol. Chem.* **264**, 10836–10842.
- Huang, C., and Cabot, M.C. (1990). Phorbol diesters stimulate the accumulation of phosphatidate, phosphatidylethanol, and diacylglycerol in three cell types. Evidence for the indirect formation of phosphatidylcholine-derived diacylglycerol by a phospholipase D pathway and direct formation of diacylglycerol by a phospholipase C pathway. *J. Biol. Chem.* **265**, 14858–14863.
- Imagawa, W., Bandyopadhyay, G.K., Wallace, D., and Nandi, S. (1989). Phospholipids containing polyunsaturated fatty acyl groups are mitogenic for normal mouse mammary epithelial cells in serum-free primary cell culture. *Proc. Natl. Acad. Sci. USA* **86**, 4122–4126.
- Imboden, J.B., and Stobo, J.D. (1985). Transmembrane signaling by the T cell antigen receptor: perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. Exp. Med.* **161**, 446–456.
- June, C.H., Fletcher, M.F., Ledbetter, J.A., and Samelson, L.E. (1990). Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* **144**, 1591–1599.
- Kelley, L.L., Blackmore, P.F., Graber, S.E., and Stewart, S.J. (1990). Agents that raise cAMP in human T lymphocytes release an intracellular pool of calcium in the absence of inositol phosphates production. *J. Biol. Chem.* **265**, 17657–17664.
- Kinsky, S.C., Loader, J.E., and Benedict, S.H. (1989). Phorbol ester activation of phospholipase D in human monocytes but not peripheral blood lymphocytes. *Biochem. Biophys. Res. Commun.* **162**, 788–793.
- Lavie, Y., and Liscovitch, M. (1990). Activation of phospholipase D by sphingoid bases in NG108-15 neural-derived cells. *J. Biol. Chem.* **265**, 3868–3872.
- Lavie, Y., Piterman, O., and Liscovitch, M. (1990). Inhibition of phosphatidic acid phosphohydrolase activity by sphingosine: dual action of sphingosine in diacylglycerol signal termination. *FEBS Lett.* **277**, 7–10.
- Liscovitch, M. (1989). Phosphatidylethanol biosynthesis in ethanol-exposed NG108-15 neuroblastoma X glioma hybrid cells. Evidence for activation of a phospholipase D phosphatidyl transferase activity by protein kinase C. *J. Biol. Chem.* **264**, 1450–1456.
- Moolenaar, W.H., Kruijer, W., Tilly, B.C., Verlaan, I., Bierman, A.J., and de Laat, S.W. (1986). Growth factor-like action of phosphatidic acid. *Nature (Lond.)* **323**, 171–173.
- Murayama, T., and Ui, M. (1987). Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipid breakdown in 3T3 fibroblasts. *J. Biol. Chem.* **262**, 5522–5529.
- Mustelin, T., Coggeshall, K.M., Isakov, N., and Altman, A. (1990). T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* **247**, 1584–1587.

- Nel, A.E., Bouic, P., Latanze, G.R., Stevenson, H.C., Miller, P., Dirienzo, W., Stefanini, G.F., and Galbraith, R.M. (1987). Reaction of T lymphocytes with anti-T3 induces translocation of C-kinase activity to the membrane and specific substrate phosphorylation. *J. Immunol.* **138**, 3519–3524.
- Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.)* **308**, 693–698.
- O'Rourke, A.M., and Mescher, M.F. (1988). T cell receptor-mediated signaling occurs in the absence of inositol phosphates production. *J. Biol. Chem.* **263**, 18594–18597.
- Preiss, J., Loomis, C.R., Bishop, R.W., Stein, R., Niedel, J.E., and Bell, R.M. (1986). Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells. *J. Biol. Chem.* **261**, 8597–8600.
- Randall, R.W., Bonser, R.W., Thompson, N.T., and Garland, L.G. (1990). A novel and sensitive assay for phospholipase D in intact cells. *FEBS Lett.* **264**, 87–90.
- Reinhold, S.L., Prescott, S.M., Zimmerman, G.A., and McIntyre, T.M. (1990). Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J.* **4**, 208–214.
- Ross, T.S., Tait, J.F., and Majerus, P.W. (1990). Identity of inositol 1,2-cyclic phosphate 2-phosphohydrolase with lipocortin III. *Science* **248**, 605–607.
- Samelson, L.E., Phillips, A.F., Luong, E.T., and Klausner, R.D. (1990). Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc. Natl. Acad. Sci. USA* **87**, 4358–4362.
- Siegmann, D.W. (1987). Stimulation of quiescent 3T3 cells by phosphatidic acid-containing liposomes. *Biochem. Biophys. Res. Commun.* **145**, 228–233.
- Stewart, S.J., Prpic, V., Powers, F.S., Bocckino, S.B., Isaacks, R.E., and Exton, J.H. (1986). Perturbation of the human T cell antigen receptor-T3 complex leads to the production of inositol tetrakisphosphate. Evidence for conversion from inositol trisphosphate. *Proc. Natl. Acad. Sci. USA* **83**, 6098–6102.
- Stewart, S.J., Kelley, L.L., and Powers, F.S. (1987). Production of inositol pentakisphosphate by a human T lymphocyte cell line. *Biochem. Biophys. Res. Commun.* **145**, 895–902.
- Sussman, J.J., Mercep, M., Saito, T., Germain, R.N., Bonvini, E., and Ashwell, J.D. (1988). Dissociation of phosphoinositide hydrolysis and Ca^{2+} fluxes from the biological responses of a T-cell hybridoma. *Nature (Lond.)* **334**, 625–628.
- Takahashi, S., Maecker, H.T., and Levy, R. (1989). DNA fragmentation and cell death mediated by T cell antigen receptor/CD3 complex on a leukemia T cell line. *Eur. J. Immunol.* **19**, 1911–1919.
- Tsien, R.Y., Rink, T.J., and Poenie, M. (1985). Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium* **6**, 325–334.
- Tyson, C.A., vandeZande, H., and Green, D.E. (1976). Phospholipids as ionophores. *J. Biol. Chem.* **251**, 1326–1332.
- van Corven, E.J., Groenink, A., Jalink, H., Eichholtz, T., and Moolenaar, W.H. (1989). Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell* **59**, 45–54.
- van Wauwe, J.P., deMey, J.R., and Goossens, J.G. (1980). OKT3: a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. *J. Immunol.* **124**, 2708–2713.
- Weiss, A., Wiskocil, R.L., and Stobo, J.D. (1984). The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pretranslational level. *J. Immunol.* **133**, 123–128.
- Wright, R.M., Rangan, L.A., Shin, H.S., and Raben, D.M. (1988). Kinetic analysis of 1,2-diacylglycerol mass levels in cultured fibroblast: comparison of stimulation by α -thrombin and epidermal growth factor. *J. Biol. Chem.* **263**, 9374–9380.