Protein Kinase C Regulates Both Production and Secretion of Interleukin 2*

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Inhibiting protein kinase C (PKC) activity abrogated interleukin 2 (IL2) production by mitogen-stimulated human T lymphocytes. This effect was due partially to a 50% decrease in IL2 gene expression. However, when PKC inhibitors were added after IL2 gene transcription had already proceeded for 3–4 h, the IL2 in the culture supernatants was still reduced by 30–80%, and intracellular IL2 was increased by up to 50%. The inhibition of PKC affected the expression of IL2 receptors by these cells differently; it had little effect on gene expression or on the membrane-bound form of the receptor, but it decreased soluble receptors in the supernatants by 50–80%. These data indicate that in addition to its previously defined role in gene expression, PKC can also regulate extracellular secretion of proteins critical for T cell proliferation.

Activation of T cells by ligands that bind to the T cell antigen receptor complex (Ti-CD3) stimulates hydrolysis of phosphoinositides by phospholipase C within seconds to minutes (Imboden and Stobo, 1985; Imboden et al., 1987). This in turn results in mobilization of intracellular ionized calcium (Ca++), reviewed in Gardner, 1989) and activation of protein kinase C (PKC)† (Farrar and Ruscetti, 1986; Mire et al., 1986; and Nel et al., 1987). Protein kinase C and Ca++ contribute to a continuing cascade of biochemical events that lead to the expression of genes associated with cellular growth including interleukin (IL) 2 and IL2 receptors (reviewed in Waldmann, 1986, Smith, 1988, and Crabtree, 1989). The IL2 receptor α chain is further modified post-transcriptionally into a membrane bound form (p55/Tac; Uchiyama et al., 1981; Waldmann, 1986) that forms part of the high affinity IL2 receptor complex or a secreted form (p45, Rubin et al., 1985; Robb and Kutny, 1987), whose function is unclear. The subsequent interaction of soluble IL2 with high affinity surface IL2 receptors provides the signal that drives lymphocyte proliferation (reviewed in Smith, 1988).

The importance of Ca++ and PKC as "second messengers"

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The abbreviations used are: PKC, protein kinase C; IL, interleukin; PHA, phytohemagglutinin, TPA, 12-O-tetradecanoylphorbol 13-acetate; PBL, peripheral blood lymphocytes; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

is underscored by the fact that agonists which stimulate mobilization of intracellular Ca++ or activation of PKC can induce IL2 production, IL2 receptor expression, and proliferation of T cells directly (Truneh et al., 1988; Farrar and Ruscetti, 1986; Modiano et al., 1988), bypassing the requirement for binding of ligand to the Ti-CD3 complex. Specifically, the importance of PKC in IL2 production by human T cells has been inferred from the observations that agonists of PKC (including phorbol esters or sn-1,2-diC8) induce IL2 gene expression and production of biologically active IL2 (Kern et al., 1986; Isakov et al., 1987; Subramaniam et al., 1988). Furthermore, H7 (an inhibitor of PKC), but not dibutyryl cAMP, dibutyryl cGMP, prostaglandin E2, or polyanine inhibitors, was shown to diminish both IL2 mRNA expression and IL2 activity from human tonsilar T cells stimulated by PHA + TPA (Yamamoto et al., 1986).

The specific role of PKC(s) in IL2 production is still unclear. The transcription of the IL2 gene is dependent on signals mediated through the T cell antigen receptor, but is not optimal without an additional accessory signal that can be delivered by agonists of PKC (Kern et al., 1986; Crabtree, 1989). A phorbol response element (AP-1/Fos binding site) is present in the IL2 promoter, and this element can drive transcription following the activation of PKC; however, the deletion of this element from the promoter only inhibits the transcriptional activity of the promoter partially (Serfling et al., 1989; Muegge et al., 1989; Crabtree, 1989).

Once transcription of IL2 occurs, the protein appears to be only slightly modified post-transcriptionally (Robb et al., 1981; Robb et al., 1984; Taniguchi et al., 1986; Smith, 1988; Kung et al., 1989). There are no N-glycosylation sites on the IL2 protein (Taniguchi et al., 1983), it is variably O-glycosylated (Robb et al., 1984) or sialylated (Robb et al., 1981), and the biological activity of unglycosylated, recombinant IL2 produced in Escherichia coli is indistinguishable from that produced in mammalian cells (Taniguchi et al., 1986). Recently, human recombinant IL2 produced in E. coli or in Cos cells was shown to be phosphorylated by PKC at the serine on position 6 (Kung et al., 1989), but this phosphorylation did not affect the ability of the soluble protein to promote T cell proliferation.

Our aim was to examine further the role of PKC in mitogen-induced activation of human lymphocytes. Our results indicate that the chemical inhibition of PKC diminished both IL2 gene expression and IL2 secretion, as well as the secretion of soluble IL2 receptors in human T cells stimulated by PHA or by PHA in combination with phorbol esters. It had little effect, however, on the expression of the IL2 receptor α gene or of IL2 receptors on the cell membrane.

EXPERIMENTAL PROCEDURES

Cells—Peripheral blood lymphocytes (PBL) were separated by Ficoll-Hypaque (Histopaque 1.077, Sigma) density gradient centri-
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Purification of Protein Kinase C—PKC was partially purified from bovine brain utilizing a cation exchange chromatography and gel filtration. Cells were lysed in buffer A (300 mM sucrose, 20 mM Tris, pH 7.3, 2 mM EDTA, 0.5 mM EGTA, 0.1% Nonidet P-40, 1 mM N-ethylmaleimide, 0.03 μM aprotinin, 0.5 μM leupeptin), and the nonextractable material cleared by centrifugation (15,000 × g at 4°C for 5 min). The cleared lysates were then loaded on a 1.5-ml DEAE (Bio-Rad) column with a linear gradient of KCl (0–350 mM) and 0.5-ml fractions tested for activity. Active fractions were pooled and dialyzed extensively in buffer B (20 mM Tris, pH 8.0, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM dithiothreitol, 1 mM N-ethylmaleimide, 0.03 μM aprotinin, and 0.5 μM leupeptin). The samples were then loaded on 5 ml G50–150 Sephadex columns (Whatman) and eluted with buffer B. Active fractions were pooled and used to assay the inhibition by staurosporine.

Protein Kinase C Activity Assays—The specific activity of PKC was determined by the specific phosphorylation of histone I as described (Kikkawa et al., 1984). One unit/μg of PKC activity was defined as the incorporation of 1 pmol of phosphate from [32P]ATP into the substrate over 30 min at 30°C/μg of protein in the assay fraction. Assays for autophosphorylation were essentially identical to the activity assays, but no exogenous substrate (histone I) was added. The reaction was stopped after 30 min by the addition of sodium orthovanadate. After the samples were boiled, the proteins were separated by 7.5% SDS-polyacrylamide gels and electrophoresis as described (Laemmli, 1970). The gels were autoradiographed on XAR-5 film (Kodak) at ~70°C, and autoradiograms quantitated by laser densitometry using an Ultrascan XL (Pharmacia LKB Biotechnology Inc.).

Tyrosine Phosphorylation Assays—The activity of cellular tyrosine kinases was determined by analyzing the relative phosphorylation of tyrosine phosphoproteins with an anti-phosphotyrosine antibody (Boehringer Mannheim). This antibody was used both for immunoprecipitation and immunoblotting studies.

Western Blotting—Proteins were routinely cultured for 3–6 h in the presence or absence of mitogens and with or without staurosporine. Total RNA was isolated by the guanidine isothiocyanate/CaCl2 method as described (Kern et al., 1986). Cytosolic RNA was isolated by the method of Wilkinson (1988). Ten to 20 μg of RNA were separated electrophoretically over 1% agarose, 5% formaldehyde denaturing gels and transferred to Nylon 6 membranes (Genescreen Plus, Du Pont–New England Nuclear) by capillary blotting. Steady state levels of mRNA expression were assessed using a human IL2 cDNA (Holbrook et al., 1984) or a human IL2 receptor cDNA (Leonard et al., 1984). A cDNA encoding the human IL2 receptor has been described that also ensures that the amounts of RNA present in each sample were approximately equivalent. The cDNA probes were labeled by random primer extension and hybridized to the immobilized RNA as described (Kern et al., 1986). Blots were autoradiographed on XAR-5 film and autoradiograms quantitated densitometrically as described above.

Cell-free supernatants were separated from the cell pellets by centrifugation, and the pellets were lysed in buffer C (50 mM NaCl, 50 mM Tris, pH 7.6, 0.5% Triton X-100, 1 mM N-ethylmaleimide, 0.03 μM aprotinin, and 0.5 μM leupeptin). The protein content in the supernatants and in the cell lysates was determined by the bicinchoninic acid method (Pierce Chemical Co.).

There were minimal variability from sample to sample. Each sample was concentrated as necessary using a microfiltration unit (Ultrafiltr, Millipore, Bedford, MA). Subsequently, 800–1000 μg of supernatant protein or 20–100 μg of cellular protein were separated by SDS-polyacrylamide gel electrophoresis over 15% polyacrylamide mini-gels and transferred to nylon-reenforced nitrocellulose membranes (NEN, New England Nuclear). The blots were probed with either a rabbit anti-human recombinant IL2 antisera (Collaborative Research Inc., Bedford, MA) or a goat anti-human recombinant IL2 antisera (R & D, Minneapolis, MN) followed by secondary anti-rabbit or anti-goat antibodies conjugated to alkaline phosphatase (Promega, Madison, WI). The bands were visualized as described by the manufacturer. Quantity of the bands was performed by laser densitometry.

from Beckton Dickinson (Mountain View, CA). Fluorescein isothiocyanate-conjugated goat-anti mouse antibodies were from Tago (Bur-
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Reverse Transcriptase Assays—Reverse transcriptase activity in supernatants from MLA-144 cells were measured as described (Simonian et al., 1990). A murine Gross-murine leukemia virus was used as a positive control, and there was no significant difference between the amount of phosphate incorporated into polyadenosine primers from [32P]dTTP by the reverse transcriptase from the murine versus the ape viruses.

RESULTS

Staurosporine Inhibits Proliferation of Human PBL—When staurosporine, a relatively specific inhibitor of PKC, was added simultaneously with the mitogens to cultures of PBL stimulated by PHA or by anti-CD3 antibodies, proliferation decreased in a dose-dependent fashion (Fig. 1A) as measured by incorporation of [3H]thymidine into DNA. This effect was largely reversible if exogenous human recombinant IL2 was added at the onset of culture and proliferation measured after 48 or 72 h (Fig. 1B).

Staurosporine Inhibits PKC in Human Lymphocytes—We also analyzed the effects of staurosporine on the in vitro activity of PKC purified from human T cells, and on PKC function in intact cells, to determine whether it inhibited PKC from human lymphocytes in a similar fashion to PKC from nervous tissues (Tamaoki et al., 1986; Nakadate et al., 1988). Fig. 2 shows that PKC purified from H33 cells became autophosphorylated as an 82-kDa band in the presence of calcium, magnesium, phosphatidyserine, and diolein (A); calcium, magnesium, phosphatidyserine, diolein, and staurosporine (10 nM) (B); or EDTA (C). The molecular mass of autophosphorylated PKC was 82 kDa. The relative levels of autophosphorylation were quantitated by laser densitometry. Similar results were obtained in one (autophosphorylation) and two (PKC activity) additional experiments.

As another, albeit indirect, approach to this effect of staurosporine on PKC in human lymphocytes, we explored the fact that stimulation of T cells by agents that activate PKC induces the phosphorylation and subsequent modulation of CD3 complex molecules on the cell surface (Cantrell et al., 1985). Fig. 3A shows that stimulation of H33 cells by TPA + PHA for 30 min decreased the amount of CD3 on the cell surface by 50-60% as determined by indirect immunofluorescence. Staurosporine alone (at 10 nM) did not affect the

Fig. 1. Staurosporine (Stauro) inhibits proliferation of PHA-stimulated PBL. Unstimulated PBL or PBL stimulated by PHA (5 μg/ml) were cultured for 48-120 h. [3H]Thymidine uptake was measured for the last 8 h of culture. The relative responses were not significantly different among the various time points tested, but the absolute magnitude of the peak responses among the different donors ranged from 18,000 to 81,000 cpm. [3H]Thymidine uptake by unstimulated cells was 3-8% of the maximal stimulation. A, staurosporine was added to PBL stimulated by PHA at the onset of culture as indicated. The data shown are the means ± S.E. of measured after 72 h of culture of five experiments done. B, PBL were cultured as indicated for 72 h. Staurosporine was used at 5 nM and IL2 at 2 nM. The results shown are the means ± S.D. of two experiments done.

Fig. 2. Staurosporine inhibits PKC activity in vitro. Protein kinase C was purified from unstimulated H33 cells as described. PKC activity was assessed by autophosphorylation (top panel) or by phosphorylation of histone I (bottom panel) in the presence of calcium, magnesium, phosphatidyserine, and diolein (A); calcium, magnesium, phosphatidyserine, diolein, and staurosporine (10 nM) (B); or EDTA (C). The molecular mass of autophosphorylated PKC was 82 kDa. The relative levels of autophosphorylation were quantitated by laser densitometry. Similar results were obtained in one (autophosphorylation) and two (PKC activity) additional experiments.

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Stauroporine (Stauro) Inhibits PKC Activity

Staurosporine is an effective inhibitor of PKC purified from human T cells as well as of PKC in situ, and at concentrations between 5 and 10 nM, staurosporine does not inhibit the phosphorylation of major tyrosine phosphoproteins that may also be important signaling molecules in T cells.

Inhibitors of PKC Decrease IL2 Production by Human T Cells

Consistent with previous results from our laboratory and others, IL2 was detectable in culture supernatants from PBL or either cell line 3-4 h following the mitogenic stimulation as measured by the CTLL2 murine indicator line bioassay (Fig. 4). This response increased linearly for 10 to 15 h when it plateaued, presumably reflecting a steady state between production and utilization of IL2.

Either staurosporine or H7 reduced the IL2 levels in supernatants from J32 cells stimulated by PHA plus TPA up to 100% (Fig. 6). IL2 production by H33 cells stimulated with PHA plus TPA was also markedly inhibited by staurosporine (Fig. 4). The inhibitory effect of staurosporine on IL2 production by these two cell lines was dose dependent (Fig. 4B). Staurosporine also showed a dose-dependent inhibition of IL2 production by PBL stimulated by PHA alone or in combination with TPA (Fig. 4). The observed decrease in the levels of IL2 in these culture supernatants was directly related to an inhibitory effect on IL2 production, rather than a consequence of the inhibitory effects of staurosporine on cellular proliferation. The former effect was observable between 3 and 20 h after stimulation of PBL by mitogens, at which time staurosporine affected neither viability nor cell numbers. In contrast, the latter effect was not apparent for >36 h when PBL entered the S phase of the cell cycle.

Fig. 3. Stauroporine (Stauro) Inhibits PKC Activity in Situ. H33 cells were incubated for 30-60 min at 4 °C (unstimulated) or at 37 °C in the presence of PHA (5 μg/ml), TPA (20 nM), staurosporine (10 nM), α-Leu-4 (an α-CD3 antibody), or combinations thereof. Samples were analyzed using a FACS analyzer. A, cells were stained with OKT3 (an α-CD3 antibody which binds an epitope distinct from α-Leu-4) directly conjugated to phycoerythrin. FL2 indicates relative red fluorescence; the y axis represents cell numbers; MFC, mean fluorescence channel. B, cells were first stained with a goat α-mouse Ig antibody directly conjugated to fluorescein and then followed with OKT3 directly conjugated to phycoerythrin. FL1 indicates relative green fluorescence and represents cell surface expression of the α-Leu-4 epitope of CD3; FL2 represents relative red fluorescence and represents cell surface expression of the OKT3 epitope of CD3. One of four experiments done is shown.

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expression of surface CD3 on these cells. But when staurosporine was used in conjunction with TPA + PHA, there was only a 20–30% loss of surface CD3 after a 30 min stimulation, suggesting there was an inhibitory effect on the PKC-dependent modulation of the CD3 complex. Staurosporine also inhibited to a similar degree the modulation of surface CD3 from PBL stimulated by PHA or from J32 cells stimulated by PHA plus TPA. As a control, we stimulated T cells for 30 min with soluble anti-CD3 antibodies, which appear to induce modulation of the CD3 complex by capping and are not strong activators of PKC (Ledbetter et al., 1986; Manger et al., 1987). Under these circumstances, staurosporine had no effect on the internalization of CD3 on H33 cells (Fig. 3b) or on PBL or J32 cells.

To determine whether staurosporine was acting as a non-specific inhibitor of protein kinases, we examined its effect on another group of kinases which are specific tyrosine phosphotransferases. We assessed the accumulation of tyrosine phosphoproteins in mitogen-stimulated PBL or J32 cells by immunoprecipitation or by immunoblot analysis using an anti-phosphotyrosine monoclonal antibody and found that staurosporine had no significant effect (not shown).

Taken together, these observations suggest that staurosporine is an effective inhibitor of PKC purified from human T cells as well as of PKC in situ, and at concentrations between 5 and 10 nM, staurosporine does not inhibit the phosphorylation of major tyrosine phosphoproteins that may also be important signaling molecules in T cells.

Inhibitors of PKC Decrease IL2 Production by Human T Cells—To begin to dissect the mechanism by which PKC may be important in regulating proliferation of normal human T cells, we determined how staurosporine or another inhibitor of PKC, H7, affected IL2 production and IL2 receptor expression in PBL stimulated by PHA. We also analyzed the effect of these inhibitors on IL2 production by the J32 and H33 human T cell lines, which appear to use the same signaling pathways as normal PBL (Imboden and Stobo, 1985; Imboden et al., 1987) but yield larger amounts of IL2 following stimulation by mitogens.

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R. Kolp and J. F. Modiano, unpublished observations.
The absolute amount of IL2 (in units/ml) produced by the different cell types is defined under "Experimental Procedures." The data concentrations of staurosporine. IL2 production was measured using cells were stimulated by PHA to be a murine IL2-dependent indicator cell line (CTLL2). The range for 22 h as indicated.

Staurosporine could be through a transcriptional or a post-transcriptional effect. We used Northern blot analysis to determine how staurosporine affected IL2 gene expression in mitogen-stimulated human T cells. The decrease in IL2 production induced by staurosporine alone, 0%; PHA + TPA = staurosporine at 5 nM, 48%, at 10 nm 46%, and at 50 nM, 58%. Similar results were obtained in two additional experiments.

**Staurosporine Inhibits IL2 Gene Expression in Human T Cells**—The decrease in IL2 production induced by staurosporine could be through a transcriptional or a post-transcriptional effect. We used Northern blot analysis to determine how staurosporine affected IL2 gene expression in mitogen-stimulated human T cells.

IL2 mRNA was not detected in unstimulated J32 cells and was initially seen in cells stimulated by PHA plus TPA after 2–3 h, with a peak at 4–5 h. Staurosporine, at a dose of 5–50 nM, inhibited the accumulation of IL2 mRNA in J32 cells stimulated for 3–6 h (Fig. 5). After being normalized for loading differences, the levels of IL2 mRNA ranged between 48 and 58% of control in stimulated cells which received staurosporine at concentrations between 5 and 50 nM. Thus it appeared that unlike the IL2 activity present in culture supernatants, the inhibition of IL2 mRNA accumulation was not dose dependent.

**Staurosporine Inhibits Secretion of IL2 by Human T Cells**—The fact that staurosporine only reduced partially the accumulation of IL2 mRNA in human T cells presented an apparent paradox, since IL2 activity in culture supernatants could be completely abrogated by either of the two PKC inhibitors studied. This discrepancy might be explained by changes in either IL2 mRNA stability, IL2 protein synthesis, secretion of IL2, or any combination of the three. The observations that PKC appears to have only a partial role in the transcription of the IL2 gene (Crabtree, 1989) and that IL2 can be phosphorylated directly by PKC (Kung et al., 1989) led us to investigate the latter two possibilities. We examined how staurosporine or H7 affected IL2 production by J32 cells after maximal IL2 gene expression had already occurred (3–6 h following mitogenic stimulation).

Fig. 6 shows that both agents reduced IL2 activity in culture supernatants by ~80 and 50%, respectively, when added 4 h after the initial stimulation by mitogen. In contrast to this effect seen with the inhibitors of PKC, Ha1004 enhanced IL2 production in mitogen-stimulated J32 cells by >20% when it
was added at the onset of culture and had no effect when added 6 h later. This compound is chemically similar to H7, but it preferentially inhibits cyclic nucleotide-dependent kinases rather than PKC.

Staurosporine also reduced IL2 production in mitogen-stimulated PBL or H33 cells by >50% when it was added to the cells 4–8 h after the onset of culture (data not shown).

To determine whether these effects of staurosporine reflected an inhibition of IL2 synthesis versus secretion, we assessed directly the levels of IL2 protein in culture supernatants and whole cell lysates from stimulated T cells by Western immunoblotting. Fig. 7 shows that when staurosporine was added to the cells concurrently with the mitogens, there was a decrease of 65–80% in the amount of IL2 protein found in the culture supernatants at 6 h (top panel, lane 3), as well as 40–50% decrease in the IL2 found within the cells (bottom panel, lane 3) as compared with the stimulated controls (lane 2); and these changes were consistent with the concurrent ~50% decrease in IL2 gene expression (Fig. 8).

However, when staurosporine was added to the cells 4 h after the mitogens, and the culture continued for an additional 2 h, the IL2 in the supernatants again was reduced by approximately 40–50% (Fig. 7, top panel, lane 4), but there was a concurrent increase of 30–40% in the amount of IL2 found inside the cells (Fig. 7, bottom panel, lane 4) as compared with the stimulated controls (lane 2). The increase in the level of intracellular IL2 did not reflect a generalized accumulation of cytosolic proteins since the levels of the α and β isoforms of PKC were unchanged in these cells regardless of the stimuli used (not shown). Also, in these experiments, the changes in the amounts of IL2 protein, in both the supernatants and in the cell lysates, correlated well with the changes in IL2 activity detected by the CTLIL2 bioassay (Fig. 8). Similar results were obtained when H7, but not HA1004, was used as a PKC inhibitor.

One additional piece of evidence suggesting that PKC-mediated processes may be involved in IL2 secretion was obtained from the gibbon ape T cell line MLA-144, which produces IL2 constitutively. In the case of this cell line, which carries infective gibbon sarcoma virus, IL2 gene expression may be driven by the viral promoter (Rabin et al., 1981). The addition of staurosporine to MLA-144 cells for up to 20 h did not measurably affect IL2 mRNA levels. Under the same conditions, however, there was up to an 85% decrease in the amount of IL2 in culture supernatants (Fig. 9). This effect of staurosporine on IL2 production appeared to be specific, since budding of viral particles from the MLA-144 cells, as measured by reverse transcriptase activity in the culture supernatants, was not affected (Fig. 9).

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**Fig. 7. Staurosporine (Stauro) inhibits the secretion of IL2 protein by J32 cells.** J32 cells were stimulated as indicated for a total of 6 h, at which time culture supernatants (top panel) or cell lysates (bottom panel) were harvested and analyzed for the presence of IL2 protein by Western immunoblotting. PHA was used at 5 μg/ml, TPA at 20 nM, and staurosporine at 10 μM. 800 μg of protein from the culture supernatants and 35 μg of cell-lysate protein were loaded onto each lane in the gels. The blots were quantitated densitometrically and the value obtained for lane 2 (positive control) was considered 100% in each case. These data are representative of four experiments done.

**Fig. 8. Staurosporine inhibits the secretion of biologically active IL2 from J32 cells.** J32 cells were stimulated by PHA (5 μg/ml) + TPA (20 nM) and staurosporine added at the onset of culture (0 h) or after 4 h. After 6 h, the cells were separated from the cell-free supernatants. RNA or protein were isolated from cell lysates. Expression of IL2 mRNA was determined by Northern blotting as in Fig. 6. IL2 activity in culture supernatants (cell-free) and in cell lysates (cell-associated) was determined as in Fig. 4. The cell lysates were diluted by >100-fold to prevent lysis of the CTLIL2 cells by the detergent, and the cell-associated activity was normalized to the concentration of protein present initially in each sample. Similar results were obtained in two (mRNA) and three (activity) experiments.

**Fig. 9. Staurosporine (Stauro) inhibits IL2 secretion but not virus budding in MLA-144 cells.** MLA-144 cells were cultured in the presence (closed symbols) or absence (open symbols) of staurosporine for 16 h. The concentrations of IL2 present in the virus-free supernatants from these cultures were 56 units/ml and 340 units/ml, respectively, as determined using the CTLIL2 bioassay (solid lines). Reverse transcriptase activity was measured in serial dilutions from the culture supernatants (dashed lines). The data shown are representative of four experiments done.
**FIG. 10.** Stauorospoline (Stauro) inhibits secretion of soluble IL2 receptors but not expression of membrane bound IL2 receptors or of the IL2 receptor gene. A, expression of IL2 receptor α mRNA in unstimulated J32 cells, or in cells stimulated for 6 h by PHA (5 μg/ml) + TPA (20 nM) with staurosporine (10 nM) added as indicated, was determined by Northern blotting with a human IL2 receptor cDNA. The exposure of the autoradiogram was 72 h, and the results are representative of two experiments done. B, expression of membrane-bound IL2 receptor α (p55/Tac) by PHA-stimulated PBL, in the absence (circles) or presence (squares) of staurosporine (10 nM), was determined by indirect immunofluorescent analysis. The results shown were compiled from three independent experiments. Unstimulated PBL routinely exhibited <0.5% CD2+/IL2Rα+ cells. C, the concentration of soluble IL2 receptors in culture supernatants from PBL stimulated by PHA in the absence (circles) or presence of staurosporine which was added to the cells either at the onset of culture (triangles) or 4 h later (squares) were measured by a sandwich enzyme-linked immunosorbent assay. No soluble IL2 receptors were detected in the supernatants from unstimulated PBL. The results shown were compiled from two experiments; similar results were obtained in two additional experiments using J32 cells.

**Bound IL2 Receptors**—To begin to determine whether these multiple effects of PKC in the control of IL2 production were specific, we analyzed whether staurosporine also affected the expression or the secretion of IL2 receptors by human T cells stimulated by mitogen. The expression of IL2 receptor mRNAs (3.5- and 1.5-kilobase transcripts) was induced by stimulation of J32 cells with PHA+TPA (Fig. 10A, lane 2), and the steady state level of IL2 receptor mRNAs was only inhibited by staurosporine <25% in these cells (Fig. 10A, lane 3).

Similarly, stimulation of PBL by PHA induced the expression of IL2 receptor α (p55/Tac) on the cell surface, and this was maximal by 50–72 h as determined by indirect immunofluorescence (Fig. 10B). In these cells, staurosporine affected
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the expression of membrane-bound IL2 receptor α only minimally (<15%) at 50 h or not at all (Fig. 10B). Staurosporine also did not appear to affect the expression of surface IL2 receptors as measured by radiolabeled IL2. In one experiment, the specific binding of IL2 to PHA-stimulated PBL showed 2.6 × 10^7 molecules/cell versus 2.1 × 10^7 molecules/cell at a concentration of 5 pM IL2 and 2.7 × 10^6 molecules/cell versus 3.3 × 10^5 molecules/cell at a concentration of 20 nM IL2 without and with staurosporine, respectively.

PHA also induced secretion of soluble IL2 receptor α by PBL as measured by a sandwich enzyme-linked immunosorbent assay (Fig. 10C). In contrast to the effect of staurosporine on the expression of membrane-bound IL2 receptors, this PKC inhibitor reduced by 50–80% the amount of soluble IL2 receptor α in culture supernatants from PHA-stimulated PBL when added either at the onset of culture or 4 h after stimulation (Fig. 10C). The secretion of soluble IL2 receptor α by mitogen-stimulated J32 cells was inhibited to a similar extent (not shown).

**DISCUSSION**

To study the role of PKC in the activation of human T cells, we assessed the effect of two chemically distinct inhibitors of PKC on proliferation of normal human PBL and on IL2 production and IL2 receptor expression in PBL and in two human T cell lines.

Our results show that proliferation of PBL stimulated by PHA was inhibited by staurosporine in a dose-dependent fashion. This was associated with impaired function of PKC from these cells, as measured in vitro and in intact lymphocytes. Staurosporine did not inhibit tyrosine kinase function in these cells; thus, its effect on proliferation of PBL may be mediated at least in part by inhibiting PKC and not through a nonspecific inhibition of protein kinases. This is consistent with previous observations which suggested PKC was important in the proliferative response of PBL to mitogens (Manger et al., 1987; Neld et al., 1987).

Our data also indicate that this decrease in the response of PBL to PHA could reflect primarily an effect on IL2, since staurosporine inhibited profoundly the elaboration of IL2 by PHA-stimulated PBL and by both cell lines stimulated by PHA plus TPA. The observed inhibition of IL2 production by staurosporine was partially explained by a reduction of ~50% in IL2 gene expression in PBL or J32 cells. These data are compatible with the observations of Yamamoto et al. (1986), where the disruption of pathways related to the activation of PKC, but not to PKA, PKG, or prostaglandins, inhibited IL2 gene expression. Furthermore, in our experiments, the observed decrease in IL2 production appeared to be directly related to the inhibition of PKC. IL2 production was reduced both by staurosporine and by H7, a chemically distinct inhibitor of PKC, but not by H10004, a preferential inhibitor of cyclic nucleotide-dependent protein kinases.

An effect on IL2 receptor expression or function could also have accounted for the inhibition of the proliferative response of PBL to PHA. But in contrast to the decrease in IL2 production, the expression of IL2 receptor α mRNA and of both this protein and high affinity IL2 receptors on the cell surface were either not affected or decreased only slightly by staurosporine. The signaling function of these receptors did not appear to be significantly affected by staurosporine either, since recombinant IL2 largely reversed the staurosporine-induced inhibition of proliferation of PBL stimulated by PHA.

Perhaps most interesting was the fact that chemical inhibitors of PKC also appeared to interfere with secretion of IL2 and of soluble IL2 receptors. Specifically, staurosporine or H7 could essentially abrogate IL2 activity in culture supernatants of PBL or J32 cells stimulated by mitogens with only a partial reduction in the accumulation of IL2 mRNA. Particularly, stimulated J32 cells (as well as PBL or H33 cells), to which staurosporine or H7 were added after IL2 gene expression had already occurred still showed a 50% decrease in the amount of IL2 protein and activity in culture supernatants, concurrent with a 30–40% increase in the amount of intracellular IL2, but not of other cytosolic proteins. Also, whereas the absolute level of IL2 in the supernatants from stimulated J32 cells increased linearly between 6 and 15 h, the amount of IL2 in the supernatants from cells which received staurosporine 4 h after stimulation, reached a plateau 2 h later. This suggests that essentially all secretion of IL2 ceased within two hours after exposure to staurosporine.

Soluble IL2 receptors represent a modified form of the membrane-bound IL2 receptor α chain, and they appear to be associated with activated or rapidly dividing lymphocytes (Rubin et al., 1985; Nelson et al. 1986; Robb and Kutny, 1987). Staurosporine also reduced, by up to 80%, the secretion of soluble IL2 receptors by PBL or J32 cells stimulated by mitogen. As noted above, IL2 receptor gene expression and transport of the membrane-bound form of this protein were not significantly affected by staurosporine.

Taken together, these data suggest that PKC may importantly regulate secretion of IL2 by mitogen-stimulated human T cells both indirectly and directly, acting at the level of gene expression and on the release of the protein from the cell. Additionally, PKC may also control the secretion of soluble IL2 receptor α molecules by these cells. Furthermore, this effect may be limited to molecules that utilize secretory pathways, since the budding of virus from MLA-144 cells, and the expression of PKC itself and of cell surface proteins including CD2, CD3, and membrane-bound IL2 receptors in human T cells, were either not affected or decreased only minimally by staurosporine.

These conclusions are consistent with results from experimental systems using platelets (Kaibuchi et al., 1983; Kajikawa et al., 1983; Higashihara et al., 1985), neutrophils (Kajikawa et al., 1983; Pontremoli et al., 1986), or cells of the nervous system (Zungli and Zisapel, 1985; reviewed in Kikawa et al., 1986), in which PKC agonists induce the release of serotonin, serine proteinase, or neurotransmitters, respectively, and these secretory responses can be blocked by inhibitors of PKC. Thus, PKC may have a widespread role in the regulation of secretory pathways in a variety of cell types (reviewed in Nishizuka, 1986). At this point, however, we cannot rule out that chronic exposure to staurosporine may also affect synthesis of IL2 or the post-transcriptional modification(s) of the soluble IL2 receptor, directly or indirectly.

It should be noted that some studies have suggested another role for PKC in human lymphocytes that may not be related to the earliest steps in signaling: PBL stimulated by anti-CD3 antibodies, exhibited in addition to the immediate, transient translocation of PKC, a second prolonged phase of up to 4 h in which the PKC enzyme(s) were found to associate primarily with the particulate fraction from these cells (Szamel et al., 1989). The time course through which staurosporine diminished IL2 secretion in our experiments could be consistent with an inhibitory effect on this second phase of PKC function. Additionally, recombinant IL2 has been shown to be a natural substrate for PKC in bacterial and in mammalian cells (Kung et al., 1989). The physiological importance of this phosphorylation of IL2 at serine 6 is unclear; although it did
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not affect the biological activity of the protein, its ability to be exported to the extracellular space was not investigated.

It is interesting to speculate whether the two independent regulatory steps (gene expression and secretion) involved in IL2 production are mediated by different isoforms of PKC. Human lymphocytes contain the α and β isoforms of PKC in approximately equimolar concentrations (Beyers et al., 1988; Berry et al., 1989). Studies analyzing the intracellular distribution of different PKC isoforms in the central nervous system (Brandt et al., 1987; Huang et al., 1987; Mochyl-Rosen et al., 1987) suggest there may be a degree of functional heterogeneity associated with them, such that one (possibly β) may be important in signal transduction and another (possibly α?) may be important for neurotransmitter release. Results from our laboratory and others (Berry et al., 1989) have not yet demonstrated any major differences in the relative quantity or subcellular localization of either isoform with respect to the other in stimulated PBL. However, the functional importance of each isoform at different stages of T cell activation remains to be determined.

In general, the present data confirm and extend previous studies in human lymphocytes which indicated that PKC is important in the regulation of IL2 production. These reports showed a high positive correlation among the subcellular localization of the enzyme ("translocation"; Isakov and Altman, 1987; Nel et al., 1987), the relative rates of PKC substrate phosphorylation in situ (Cantrell et al., 1985; Shackelford and Trowbridge, 1986; Nel et al., 1987; Friedrich and Gullberg, 1988), and the level of IL2 produced by stimulated human T cells (Yamamoto et al., 1986; Friedrich and Gullberg, 1988; Subramaniam et al., 1988; Szamel et al., 1989). Limited studies in murine cell lines have been less consistent. In one study, pharmacologic depletion of PKC from a cell line abrogated the elaboration of IL2 (and the expression of IL2 mRNA) in response to stimulation by lectin (concanavalin A) or by the combination of calcium ionophore plus phorbol ester and also decreased significantly the expression of the IL2 receptor gene (Valge et al., 1988). In a study of a different murine cell line, however, inhibiting PKC by staurosporine or depleting it by chronic treatment with TPA did not affect IL2 production stimulated by lectin (PHA) or anti-CD3 antibodies in combination with IL1 (Mills et al., 1989). It remains to be determined whether these variable findings reflect inherent differences among the murine cell lines used for these experiments or whether different modes of stimulation may preferentially utilize different signalling pathways.

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