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INHIBITION OF ADENYLATE CYCLASE BY IL 2 IN HUMAN T LYMPHOCYTES IS MEDIATED BY PROTEIN KINASE C

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SUMMARY: Interleukin 2 (IL 2) inhibited basal as well as PGE_2, isoproterenol and forskolin stimulated cAMP production in human T lymphocytes. Although the stimulation of adenylate cyclase by activators of the enzyme was evident in lymphocyte membrane preparations, the inhibitory effect of IL 2 was observed only if cells were pretreated with IL 2 and the membranes activated with Ca^{++} and ATP. Additionally, when purified protein kinase C was reconstituted into untreated membranes and activated with Ca^{++} and ATP, both receptor and non-receptor stimulated adenylate cyclase was inhibited. These results suggest that the inhibition of adenylate cyclase in human T lymphocytes by IL 2 is mediated by protein kinase C.

Interleukin 2 (IL 2) is a growth factor produced by antigen- or lectin-stimulated T lymphocytes that stimulates the clonal expansion of antigen-sensitized lymphocytes (1-2). Additionally, IL 2 stimulates the differentiation of peripheral blood leukocytes into lymphokine activated killer (LAK) cells, capable of lysing a broad spectrum of fresh tumor cells and transformed cell lines (3). Recently, therapy with LAK cells and IL 2 has been shown to cause marked tumor regression in patients with advanced metastatic cancer (4).

Although IL 2 receptor interaction has been well studied (5-6), information concerning the biochemical transmembrane signalling events which mediate the biological actions of IL 2 are poorly understood (7). Unlike other growth factors, such as insulin (8) and EGF (9), the IL 2 receptor does not have a sufficient cytoplasmic domain to have intrinsic tyrosine kinase activity (10). However, IL 2 does stimulate the rapid translocation and activation of protein kinase C (11) and inhibits cAMP production in intact human T lymphocytes (12).

Our previous studies with human cells demonstrated that PGE_2 inhibits both the lymphoproliferative response to IL 2 as well as LAK cell differentiation (12), suggesting a regulatory role for adenylate cyclase in

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both of these process. The present studies were undertaken to examine the biochemical basis for the antagonistic effects of IL 2 and PGE_2 on human lymphocyte proliferation and differentiation.

METHODS

IL 2 was a generous gift of the Cetus Corporation (Emeryville, CA) and has a specific activity of 3-5 x 10^6 U/mg.

Human peripheral blood lymphocytes (PBL) were obtained from healthy adult donors as leukopacks at the National Institutes of Health platelet-pheresis laboratory. Purified human T lymphocytes were prepared as described (11). Briefly, PBL were isolated by Ficoll-Hypaque gradient centrifugation. The collected cells were washed 3 times with Hanks balanced salt solution and resuspended in complete medium. Nonadherent cells were obtained by recovering cells nonadherent to plastic flasks and passing them through a nylon wool column for additional depletion of monocytes and B cells. T lymphocytes were separated by Percoll discontinuous density gradient centrifugation as described (13). The high density cells from Percoll gradient were purified preparations of T lymphocytes that were greater than 95% positive for $T3^+$ expression and negative for large granular and B lymphocytes, as determined by cytofluorometry. However, this cell population always contained 0.5 to 1% monocytes/macrophages as judged by specific monoclonal antibody and nonspecific esterase staining.

T lymphocytes at a concentration of 10^6 cells/ml were activated by the addition of phytohemagglutinin (PHA) (µg/ml) in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics for 72 hours to insure maximal receptor expression (14). IL 2-dependent growth was maintained by the addition of either partially purified IL 2 (Cellular Products, Buffalo, NY) or recombinant purified IL 2 (a gift of Biogen, Geneva, Switzerland) every 3-5 days. Cells were rested in the absence of IL 2 for 24-48 hours to arrest the cell population at G_0/G_1.

For the measurement of cAMP production, cells were washed twice with RPMI 1640 medium and incubated for 30 min at room temperature in RPMI 1640, 20 mM HEPES, pH 7.4, 20 µM Ro 20-1724 (4-3-butoxy-4-methoxy-benzyl)2-imidazolidinone, Hoffman-LaRoche), an inhibitor of cAMP phosphodiesterase. A small aliquot of concentrated hormone (100X) was added and, after 5 min, the reaction was terminated by the addition of boiling water. Protein was removed by centrifugation and cAMP was determined by radioimmunoassay, utilizing a double antibody procedure as described (15).

Adenylate cyclase activity was measured in crude membrane preparations. Cells were washed twice with RPMI 1640, homogenized in 15 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride. This procedure inhibits protein kinase C activity and prevents proteolysis of the enzyme. Just prior to assay, membranes were centrifuged at 50,000 x g and resuspended in 20 mM Tris, pH 7.5. Adenylate cyclase activity was determined in a final volume of 100 µl containing 40 µg of membrane and final concentrations of 3 mM ATP, 1 µCi of [α-^{32}P]ATP (760 Ci/mmol, New England Nuclear), 1 mM cAMP, 2.5 U/ml adenosine deaminase, 10 mM $MgCl_2$, 30 mM Tris, 0.04% bovine serum albumin, 10 µM GTP, 80 mM NaCl, and an ATP-regenerating system consisting of 5 mM creatine phosphate and 25 U/ml creatine phosphokinase for 10 min at 31°C. Cyclic AMP was separated from ATP by the method of Salomon (16).

In some experiments, membranes were incubated under conditions previously shown to stimulate protein kinase C activity (17), prior to the

measurement of adenylate cyclase activity. Membranes were prepared as above and incubated for 5 min at 31°C in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 25 mM ATP, 100μ M $CaCl_2$. Samples were then diluted 10-fold directly into the adenylate cyclase reaction and activity was measured as described.

Protein kinase C used in the reconstitution studies was purified from CT6 cell membranes. The specific activity measured in the presence of Ca^{++}, phosphatidylserine and diolein, using H1 histone as a substrate, was 786 nmol ^{32}P/min/mg (18).

RESULTS AND DISCUSSION

The antagonistic effects of PGE_2 on IL 2-stimulated lymphocyte proliferation and differentiation (12) inversely correlated with their effects on cAMP production in intact human T lymphocytes (fig. 1). Cyclic AMP levels were elevated from 1.7 to 3.5,2.7 and 3.6 pmol/10^6 cells/5 min by 1 μM PGE_2, 1 μM isoproterenol and 100 μM forskolin, respectively. However, in the presence of 0.1 U/ml IL 2, both basal and stimulated cAMP levels were reduced to 0.4-0.6/pmol/10^6 cells/5 min. The phorbol ester, TPA, similarly decreased cAMP levels. That IL 2 inhibited

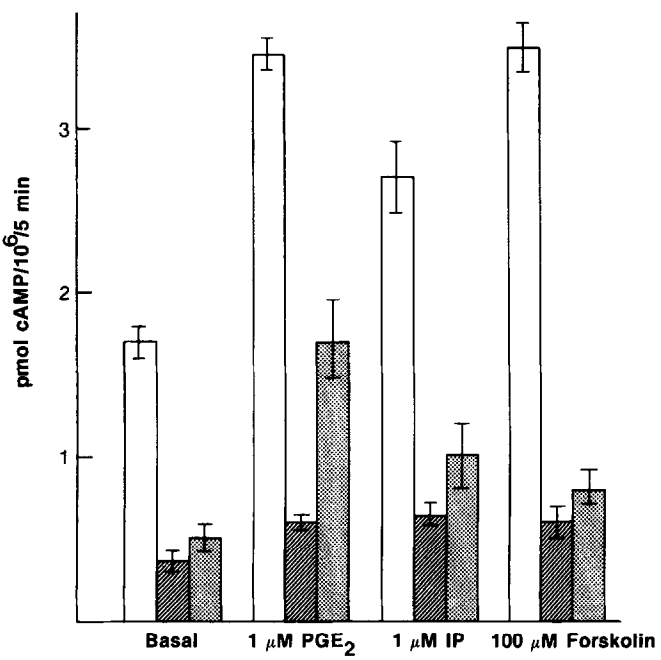

FIG. 1. Inhibition of cAMP Production by IL 2 and TPA. Human T lymphocytes were washed twice with RPMI and resuspended (10x10^6 cells/ml) in RPMI, 20 mM HEPES pH 7.5, 20μlM Ro 20-1724. After 30 min at room temperature, 100 μl cell suspension was added to 10 μl of the indicated compound without (□) or with (▨) 0.1U/ml IL 2, or 1 nM TPA (▩) and cAMP was allowed to accumulate for 5 min. Reaction was terminated with boiling water, protein was removed by centrifugation, and cAMP was determined by radioimmunoassay. Data represent the mean ± S.D. of triplicate determinations of 3 separate experiments.

TABLE I

Effect of IL 2 and TPA on Human Lymphocyte Membrane Adenylate Cyclase Activity

	Basal	1 μM PGE_2	I μM IP	100 μM forskolin
		(pmol cAMP/min/mg)		
Control	30 ± 4	61 ± 6	68 ± 4	62 ± 8
10 U/ml IL 2	28 ± 2	66 ± 8	90 ± 5	81 ± 6
10 nM TPA	34 ± 4	54 ± 6	86 ± 4	68 ± 6

Human T lymphocyte membranes were prepared and adenylate cyclase activity measured for 10 min, 37 in the presence of PGE_2, isoproterenol or forskolin in the absence (control) or presence of IL 2 or TPA. Values represent the mean ± s.d. of triplicate determinations of 2 separate experiments.

forskolin-stimulated cAMP production suggests that IL 2 exerts its inhibitory effect directly on the adenylate cyclase system rather than at the hormone receptor level.

Adenylate cyclase activity is regulated by two distinct protein complexes, G_s and G_i, which stimulate and inhibit activity, respectively (19). To determine if IL 2 decreased cAMP levels by direct interaction with G_i, the effects of IL 2 on plasma membrane adenylate cyclase activity were examined. However, neither IL 2 nor TPA significantly inhibited adenylate cyclase activity (30 pmol/min/mg) of membrane preparations, regardless of the concentration, under conditions where inhibitory regulation through G_i can be observed (Table I). However, PGE_2, isoproterenol and forskolin stimulation of adenylate cyclase through G_s was clearly evident under these conditions. Adenylate cyclase activity was increased from 30 to 61, 68 and 62 pmol/min/mg with 1 μM PGE_2, 1 μM isoproterenol and 100 μM forskolin, respectively. Additionally, there was no effect of IL 2 or TPA on the ability of any of these compounds to stimulate adenylate cyclase.

Both IL 2 and TPA cause a translocation of protein kinase C to human lymphocyte membranes. Maximal protein kinase C activity is associated with the membrane within 10 min (11). To determine if the translocation of protein kinase C was necessary to observe the inhibitory effect of these compounds on adenylate cyclase, washed intact human T cells were pretreated with IL 2 or TPA for 5 min, centrifuged, and membranes were prepared as described. However, the adenylate cyclase activity of membranes from

TABLE II

Inhibition of Adenylate Cyclase Activity by IL 2 and TPA Requires Protein Kinase C

Pretreatment	Basal	1 μM PGE	100 μM forskolin
	(pmol cAMP/min/mg)		
None	32 ± 4	86 ± 5	56 ± 4
+Ca/ATP	28 ± 5	79 ± 4	56 ± 4
IL 2	31 ± 2	88 ± 3	52 ± 5
+Ca/ATP	14 ± 0.5	30 ± 1	35 ± 2
TPA	33 ± 3	84 ± 6	58 ± 3
+Ca/ATP	12 ± 0.6	15 ± 0.4	30 ± 1
None	32 ± 4	86 ± 5	56 ± 4
+PKC	31 ± 3	90 ± 6	54 ± 3
+PKC/Ca/ATP	8 ± 0.5	9 ± 0.4	6 ± 0.4

Intact human T lymphocytes were pretreated for 10 min, 37°C without (none) or with 10 U/ml IL 2 or 20 nM TPA. Membranes were prepared and incubated for 5 min, 31°C without or with 100 μM $CaCl_2$, 25 mM ATP (+Ca/ATP). Another set of parallel control (unpretreated membranes) were incubated with 1.5 μg purified protein kinase C (specific activity 786 nmol ^{32}P/min/mg) under these same conditions. A small aliquot (10 μl) was immediately diluted into 90 μl adenylate cyclase reaction mix and activity measured as described in the absence (basal) or presence of 1 μM PGE or 100 μM forskolin. Data represents the mean ± SD of triplicate determinations from 2 separate experiments.

pretreated cells (Table II) was not significantly different from that of untreated cells (32 pmol/min/mg).

To determine if protein kinase C activation was required to observe an inhibitory effect on membrane adenylate cyclase, membranes from cells pretreated with IL 2 or TPA were incubated for 5 min under conditions utilized to activate protein kinase C (Ca^{2+}, ATP). These samples were then immediately diluted 10-fold into the adenylate cyclase mix and adenylate cyclase activity was measured over a 10-min period. Under these conditions, the adenylate cyclase activity of membranes prepared from cells pretreated with IL 2 or TPA was significantly lower than that of membranes prepared from untreated cells (Table II). Basal adenylate cyclase activity was decreased from 32 to 14 and 12 pmol/min/mg of protein while PGE_2-stimulated activity was decreased from 86 to 30 and 15 pmol/min/mg and that

of forskolin from 56 to 35 and 30 pmol/min/mg of protein, following pretreatment with IL 2 and TPA, respectively.

The role of protein kinase C in mediating the effects of IL 2 and TPA on adenylate cyclase was assessed directly by reconstitution studies. Human T lymphocyte membranes were incubated without (none) or with 1.5 μg of purified protein kinase C in the presence of Ca^{++} and ATP (+ PKC/Ca/ATP), and then immediately diluted 10-fold into the adenylate cyclase reaction mix. As seen in Table II, in control membranes treated with purified protein kinase C, Ca^{++}, and ATP, basal adenylate cyclase activity decreased from 32 to 8 pmol/min/mg; PGE_2- and forskolin-stimulated activity from 86 and 56 to 9 and 6 pmol/min/mg, respectively. Significant inhibition was observed only if Ca^{++} and ATP were present during protein kinase C activation. The degree of inhibition of adenylate cyclase activity of membranes incubated with purified protein kinase C was comparable to that of membranes prepared from cells pretreated with IL 2 or TPA and protein kinase C activated in the presence of calcium and ATP.

That both pretreatment of intact cells with IL 2 (translocation) and activation of protein kinase C are required to observe the inhibition of adenylate cyclase by IL 2 suggests that direct interaction between these two distinct kinase systems regulates the ultimate biological response to IL 2. The simplest explanation would be that protein kinase C phosphorylates either adenylate cyclase itself or one of the G proteins. Studies are currently underway to determine if this is the case.

Several studies have demonstrated an inhibition of adenylate cyclase by phorbol esters. In some systems (20-24), this reflects desensitization at the receptor level, while in hepatacytes (25) and murine T lymphocytes, (18) exogenous Ca^{++} and ATP were required, as in the present study. In yet other systems, phorbol esters seem to enhance hormone stimulated adenylate cyclase activity (26-28). In studies with S49 lymphoma cells (27) this enhancement by TPA seems a result of an increased interaction of G_s with adenylate cyclase.

Although the regulation of human lymphocyte growth and LAK cell differentiation are extremely complex processes, the present studies indicate that the regulation of adenylate cyclase by IL 2 represents an important component of its transmembrane signalling process. Better understanding of subsequent biochemical events will provide insight into the complex coordination of cellular growth and will also be applicable to the design of future cancer clinical trials involving IL 2 and LAK cell therapy.

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