Enhanced Effects of Prostaglandin E_1 and Dibutyryl Cyclic AMP upon Human Lymphocytes in the Presence of Cortisol

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ABSTRACT The combined effects of cortisol and agents acting through a cyclic AMP-mediated mechanism have been studied in cultures of highly purified human peripheral lymphocytes. Incubation with prostaglandin E_1 (PGE_1), dibutyryl cyclic AMP, or cortisol results in a concentration-dependent inhibition of [^{3}H]-thymidine incorporation by both unstimulated and phytohemagglutinin (PHA)-stimulated lymphocytes, and PHA-induced morphologic transformation is prevented. When cortisol and PGE_1 (or dibutyryl cyclic AMP) are added together to lymphocyte cultures, enhanced inhibitory effects are observed.

Incubation of unstimulated or PHA-stimulated lymphocytes with PGE_1 results in an elevation of intracellular cyclic AMP levels within 20 min. The concentration of cyclic AMP gradually returns to base-line levels over a 1–6 h period of time. Cortisol alone does not significantly alter cyclic AMP concentrations. However, incubation with PGE_1 in the presence of cortisol results in a greater stimulation of intracellular cyclic AMP levels than that observed with PGE_1 alone. These findings suggest that cortisol may act synergistically with PGE_1 to elevate lymphocyte cyclic AMP levels and to regulate [^{3}H]thymidine incorporation and transformation.

INTRODUCTION

Cultures of lymphocytes obtained from human peripheral blood maintain a low, stable rate of [^{3}H]thymidine incorporation into DNA for longer than a week (1). When incubated with phytohemagglutinin (PHA),[1] the majority of these cells undergo a morphologic transformation accompanied by a markedly increased rate of [^{3}H]thymidine incorporation (2). Studies of healthy individuals and of patients with a variety of illnesses have shown that the capacity for this transformation response to PHA correlates well with the donor's ability to generate a cellular (delayed) immune response (3).

PHA-induced lymphocyte transformation can be diminished or prevented by incubating cultures with glucocorticosteroid hormones (4–6). Recently it was observed that incubation with dibutyryl cyclic AMP or hormones which elevate intracellular cyclic AMP concentrations can also result in a suppressed response to PHA (7, 8). Because corticosteroid hormones and hormones which act through a cyclic AMP-mediated mechanism have been shown to interact in a number of biochemical systems (9), we undertook to determine whether such an interaction could be demonstrated in human peripheral lymphocyte cultures. The effect of these hormones and of exogenous cyclic AMP upon both unstimulated and PHA-stimulated lymphocytes was assayed by measuring [^{3}H]thymidine incorporation. In addition, measurements of intracellular cyclic AMP concentrations suggested a mechanism for the observed hormone interaction.

METHODS

Isolation of lymphocytes. Peripheral blood lymphocytes were obtained from a unit of normal donor blood by previously reported methods (10). The preparative steps

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[1] *Abbreviations used in this paper:* cyclic AMP, cyclic adenosine 3′,5′-monophosphate; dibutyryl cyclic AMP, N_6,-O'_2-dibutyryl cyclic adenosine 3′,5′-monophosphate; PGE_1, prostaglandin E_1; PHA, phytohemagglutinin.

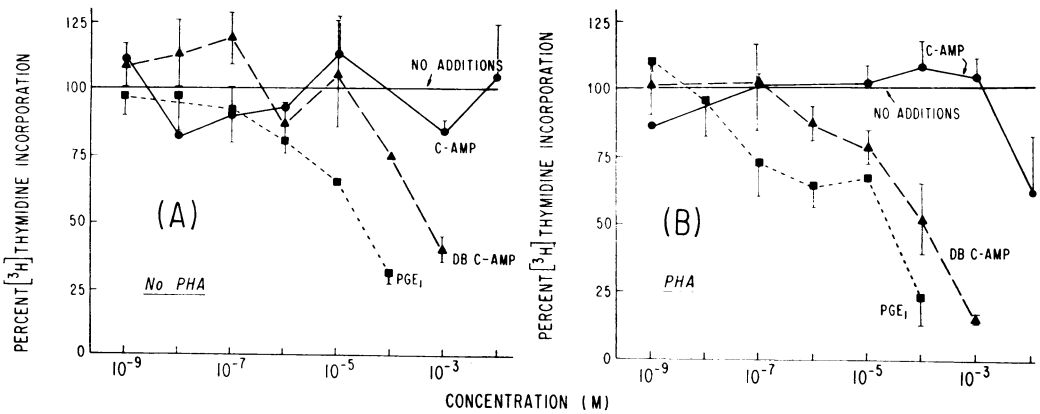

FIGURE 1 Concentration-dependent effects of dibutyryl cyclic AMP, cyclic AMP, and PGE_1 upon lymphocyte [^{3}H]thymidine incorporation. (A) In multiple experiments, replicate lymphocyte cultures were exposed initially to varying concentrations of dibutyryl cyclic AMP, cyclic AMP, or PGE_1. The level of [^{3}H]thymidine incorporation was determined after 72 h of incubation. The plotted data compare treated cultures with controls that had no additions. Brackets show SEM for points obtained from more than two observations. (B) Conditions were identical with (A) except that PHA was added to each culture.

involve: (*a*) defibrination, which removes platelets and many polymorphonuclear leukocytes; (*b*) sedimentation in 1% dextran (grade H, mol wt 180,000, Pharmachem Corp., Bethlehem, Pa.) followed by centrifugational banding upon an aqueous solution of Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and Hypaque sodium (diatrizoate sodium, Winthrop Labs., New York) to remove erythrocytes; (*c*) passage through a nylon fiber (E. I. DuPont de Nemours & Co., Wilmington, Del., type 200) column to remove granulocytes and monocytes. The final cell population contains more than 99% small (diameter less than 10 μm) lymphocytes, with approximately four erythrocytes per 100 lymphocytes.

Transformation studies. The lymphocytes were distributed in duplicate 1-ml cultures containing $1\text{–}4 \times 10^6$ cells in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) fortified with 10% autologous serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Phytohemagglutinin-E, purified from Difco PHA-P (Difco Laboratories, Detroit, Mich.) (11), was used at a concentration of 5 μg/ml, which produced maximal transformation at 72 h under these conditions (10- to 40-fold stimulation of [^{3}H]thymidine incorporation). In later experiments, PHA-P at a concentration of 20 μg/ml was used, with similar results. Hormones and cyclic AMP derivatives were added in total volumes of 10 μl or less. The rate of [^{3}H]thymidine incorporation in cultures was determined by measuring the acid precipitable counts incorporated after 2 h of incubation with 0.3 μM [^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., 7 mCi/μM), during which incorporation increases linearly with time. After incubation with the isotope, the cultures were washed twice by centrifugation with saline, precipitated with 5% trichloroacetic acid (TCA) at 4°C, disrupted by sonication, washed three times with 5% TCA on Whatman GF/C fiberglass filters, and counted in a liquid scintillation spectrometer. Duplicates agreed to within 8% for unstimulated cultures and 16% for PHA-stimulated cultures. Autoradiographic studies were performed on cultures exposed to [^{3}H]thymidine for the last 15 h of a 72 h incubation with PHA. After exposing slides to Kodak NTB emulsion (Eastman Kodak Co., Rochester, N. Y.) for 2 wk, grains were counted over $5\text{–}10 \times 10^3$ cells (12). The DNA content per culture was measured by the method of Burton (13).

Cyclic AMP assay. For these studies, $5\text{–}10 \times 10^6$ lymphocytes were suspended in 1–2 ml of complete incubation medium (containing 10% autologous fresh serum) and additions of PHA, PGE_1, and/or cortisol were then made. After further incubation for appropriate time intervals, the cultures were collected by centrifugation at 200 *g* for 3 min, and 1 ml of 5% TCA at 4°C was added immediately. The samples were frozen and thawed, sonicated, and centrifuged at 2,000 *g* for 15 min. The acid-soluble supernate was removed, extracted five times with ether to remove TCA, heated at 85°C for 30 min to remove the ether, and then stored at −20°C. Parallel extractions performed after addition of [^{3}H]cyclic AMP showed that approximately 100% of free cyclic AMP was recovered. When replicate short-term incubations were performed in the presence and absence of serum, it was found that intracellular cyclic AMP levels were reduced by more than 50% after 30 min incubation without serum. Cyclic AMP levels were slightly reduced when autologous fresh serum was replaced by fetal calf serum.

Cyclic AMP concentrations were measured by a competitive binding assay (14), employing a specific binding protein partially purified from the cytoplasm of diploid permanent lymphocyte line 8866, kindly provided by Dr. Richard Lerner. To obtain the binding protein, cells in the logarithmic phase of growth were harvested, washed by centrifugation, and resuspended in TMG (0.01 M Tris, pH 7.4, 6 mM 6-mercaptoethanol, 10% glycerol). After disruption by freezing, thawing, and sonication, the cytosol was obtained by successive centrifugations at low speed and high speed (100,000 *g* for 90 min). The supernate was brought to 45% saturation by addition of solid ammonium sulfate, and the resulting precipitate was redissolved, dialyzed against TMG at 4°C, and stored at −20°C. This cytosol extract contained a protein with a specific high affinity binding site for cyclic AMP. The equilibrium dissociation constant for the reaction (cyclic AMP) + (binding site) $\rightleftharpoons$

(cyclic AMP-binding site complex) was approximately 1 nM. Binding of cyclic AMP was not inhibited by AMP, ADP, ATP, or cortisol at 100-μM concentrations, and more than 100-fold excess cyclic guanosine 3',5'-monophosphate was required for competition with the binding of cyclic AMP.

This assay of cyclic AMP levels depends on competition between the free cyclic AMP in the cell extract and added [^{3}H]cyclic AMP for sites on the binding protein. The following components were added in the listed order to give a final volume of 200 μl: 20 μl cell extract or cyclic AMP standard of known concentration; 1.65 pmol (10 μl) of [^{3}H]cyclic AMP (24.1 Ci/mM); 120 μl 0.1 M $NaHPO_4$, pH 7.0, containing 16 mM theophylline, an inhibitor of cyclic AMP phosphodiesterase; 50 μl of the binding protein extract containing 25 μg protein. After incubation at room temperature for more than 2 h, the nucleotide-binding protein complexes were chilled and collected by filtration onto a nitrocellulose filter (Millipore Corp., Bedford, Mass., 24 mm diameter, 0.45 μm) and washed with 4°C buffer containing 0.02 M $NaHPO_4$, pH 7.0, and 0.1 M $MgCl_2$. The filters were dissolved in 10 ml Bray's solution and counted in a liquid scintillation spectrometer. A standard curve was run with each assay. Each extract from a lymphocyte culture was assayed for cyclic AMP in duplicate, with agreement in the determined cyclic AMP levels usually to within 5%. On occasion, when greater variation was observed, the assay was repeated. When extracts from paired lymphocyte cultures were assayed, the intracellular cyclic AMP concentrations agreed to within 14%.

The validity of the assay was verified by the following observations: (*a*) The measured concentration of cyclic AMP was shown to be independent of the number of cells in the lymphocyte cultures. (*b*) When known amounts of cyclic AMP were added to a portion from a previously assayed lymphocyte extract, the recovery of total cyclic AMP in a repeat assay was 95±9% (mean ±SEM, $n=7$). (*c*) Incubation of lymphocytes in the presence of 2 mM theophylline for 20 min resulted in a 4.4-fold elevation of cyclic AMP levels; with 5 mM theophylline, there was a 7-fold rise. (*d*) Incubation of lymphocytes in the presence of PGE_1 resulted in elevation of cyclic AMP levels, as detailed in Results. (*e*) The material extracted from lymphocytes and assayed as cyclic AMP in the binding assay was destroyed by preincubation with cyclic AMP phosphodiesterase for 2 h (15).

Materials and reagents. Dibutyryl cyclic AMP (P-L Biochemicals, Inc., Milwaukee, Wis.) was extracted with ether at pH 2 to remove butyric acid (16). The concentration of dibutyryl cyclic AMP was then determined spectrophotometrically. Cyclic AMP (P-L Biochemicals, Inc.) was neutralized with NaOH. [^{3}H]Cyclic AMP was obtained from New England Nuclear (Boston, Mass.) and the purity was verified by paper chromatography (17). Cortisol (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol. PGE_1 (lots 10323-JHK-71E and 10323-JHK-153A) was generously donated by Dr. John E. Pike (The Upjohn Co., Kalamazoo,, Mich.) and was dissolved in ethanol. Cyclic GMP was obtained from Boehringer Mannheim Corp. (New York). AMP, ADP, ATP, and cyclic AMP phosphodiesterase were from Sigma Chemical Co.

RESULTS

Effect of cyclic AMP, dibutyryl cyclic AMP, and PGE_1 upon lymphocytes. Studies were performed to determine the effects of agents acting through a cyclic AMP-mediated mechanism upon [^{3}H]thymidine incorporation by cultured lymphocytes. The cultures were exposed to varying concentrations of cyclic AMP, dibutyryl cyclic AMP, or PGE_1. Incubation with dibutyryl cyclic AMP or PGE_1 for 72 h resulted in a concentration-dependent reduction in [^{3}H]thymidine incorporation by normal lymphocytes, unstimulated by PHA (Fig. 1A). At the highest inhibitory concentrations tested, incorporation was reduced to levels below 35% of untreated cultures. Similar studies of the effects of dibutyryl cyclic AMP and PGE_1 upon cultures incubated with PHA demonstrate an even greater reduction in [^{3}H]thymidine incorporation, confirming previously reported observations on PHA-stimulated lymphocytes (Fig. 1B). The similarity of the dose-response curves when both unstimulated and stimulated cultures are exposed to these agents is apparent.

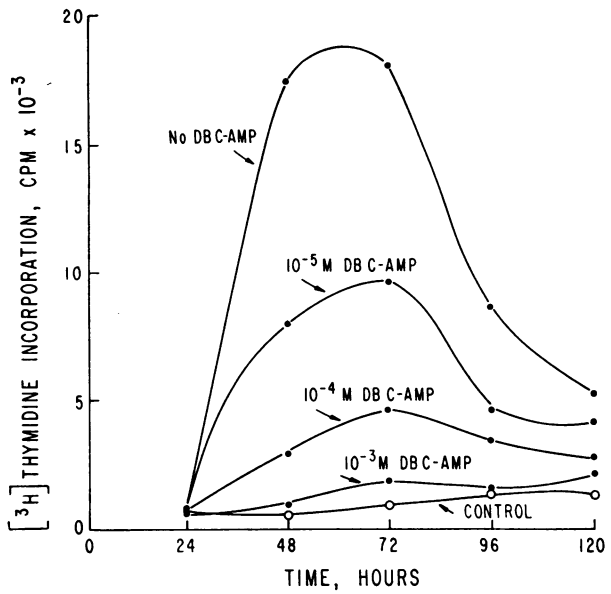

FIGURE 2 Kinetics of lymphocyte [^{3}H]thymidine incorporation. Cultures were incubated with PHA (●) or without PHA (○). Additions of dibutyryl cyclic AMP were made to the appropriate culture initially. After the designated time intervals, the levels of [^{3}H]thymidine incorporation were determined.

The cultured lymphocytes were examined morphologically to determine the incidence of blastic transformation under these experimental conditions. After incubation of the unstimulated lymphocytes for 72 h, blastic transformation into enlarged cells, with cytoplasm: nucleus ratios of >1 and with greatly enlarged nuclei containing enlarged or multiple nucleoli, was limited to less than 2% of the cells. In contrast, PHA-stimulated cultures contained between 50 and 70% blastic transformed cells after 72 h of incubation. Incubation of PHA-treated cultures with dibutyryl cyclic AMP or

TABLE I

*Effect of Cortisol plus Dibutyryl Cyclic AMP on [^{3}H]Thymidine Incorporation by Lymphocytes**

Dibutyryl cyclic AMP	Cortisol					
	Unstimulated lymphocytes			PHA-stimulated lymphocytes		
	0	10^{-6} M	10^{-4} M	0	10^{-6} M	10^{-4} M
	cpm			*cpm*		
0	1,400	660	420	18,540	3,040	500
10^{-5} M	1,470	570	380	12,040	2,360	320
10^{-3} M	880	220	130	1,830	410	50

* All additions were made to replicate lymphocyte cultures initially, and the level of [^{3}H]thymidine incorporation was determined after 72 h of incubation. The data obtained from paired cultures agreed to within 8% for unstimulated cultures and within 16% for stimulated cultures. Observations from one of six similar experiments are presented.

PGE_1 resulted in a reduction in the percent of transformed cells which corresponded with the reduction in [^{3}H]thymidine incorporation.

Cultures were also incubated in the presence of exogenous cyclic AMP for 72 h. At concentrations of 10 mM, some inhibition of [^{3}H]thymidine uptake by PHA-stimulated cultures was observed in four of six experiments, whereas no effect was observed upon unstimulated lymphocytes. At lower concentrations, cyclic AMP did not inhibit [^{3}H]thymidine incorporation or PHA-induced transformation (Figs. 1A, 1B).

The observed inhibition of lymphocyte [^{3}H]thymidine incorporation and of PHA-induced morphologic transformation, cannot be attributed to cell death. After 72 h of culture in the presence or absence of dibutyryl cyclic AMP, PGE_1, or cortisol (the latter referred to below), cell counts of PHA-stimulated cultures remained stable within 12%, cell viability (judged by trypan blue dye exclusion) was always greater than 96%, and the DNA content per culture remained stable within 16%. Furthermore, removal of dibutyryl cyclic AMP from the culture medium after more than 48 h of incubation permitted full recovery of the PHA-induced increase in [^{3}H]thymidine incorporation.

Studies were also performed to determine the effects of dibutyryl cyclic AMP on the kinetic pattern of [^{3}H]-thymidine incorporation by PHA-stimulated lymphocytes. In response to PHA, there was a peak in the rate of incorporation at 48–72 h, as shown in Fig. 2. When dibutyryl cyclic AMP was initially added to the cultures, a concentration-dependent, quantitative depression of [^{3}H]thymidine incorporation was observed. The time course of [^{3}H]thymidine incorporation continued to show a physiologic kinetic pattern at all concentrations of dibutyryl cyclic AMP, with persistence of the peak rate of incorporation after 48–72 h of incubation (Fig. 2).

Additive effects of cortisol and agents acting through cyclic AMP. When replicate cultures of lymphocytes were incubated with varying concentrations of cortisol, a concentration-dependent inhibition of [^{3}H]thymidine uptake by unstimulated lymphocytes was observed (Table I). To test for an interaction of cortisol with agents acting through cyclic AMP, varying concentrations of dibutyryl cyclic AMP were also added to the cultures. It was found that addition of cortisol together with dibutyryl cyclic AMP resulted in enhanced suppression of [^{3}H]thymidine incorporation. At each inhibitory concentration tested, addition of the other agent augmented the inhibition, and at maximal concentrations of both agents together, [^{3}H]thymidine incorporation was reduced to levels less than 10% of control cultures. As was the case with either agent alone, cell death was not observed, and the cell count and DNA content per culture remained stable.

Similar inhibitory effects upon [^{3}H]thymidine incorporation were observed when PHA-stimulated lymphocyte cultures were exposed to cortisol, confirming previously reported observations (Table I). As with the unstimulated cultures, a summation of concentration-dependent inhibitory effects upon [^{3}H]thymidine incorporation was observed when cortisol and dibutyryl cyclic AMP were added together to replicate cultures (Table I). At maximal concentrations of the two agents, the level of incorporation was reduced by 99%. Comparable enhancement of inhibitory effects upon morphologic transformation was confirmed by microscopic examination. With the highest concentrations of both inhibitory agents, the suppression of transformation resulted in lymphocyte morphology indistinguishable from control cultures, unstimulated by PHA.

The interaction between cortisol and exogenous agents acting through cyclic AMP was also observed when dibutyryl cyclic AMP was replaced by PGE_1. In both unstimulated and PHA-stimulated cultures, exposure to cortisol and PGE_1 together resulted in greater reduction in [^{3}H]thymidine incorporation than was ob-

TABLE II

*Effect of Cortisol plus PGE_1 on [^{3}H]Thymidine Incorporation by Lymphocytes**

PGE_1	Cortisol			
	Unstimulated lymphocytes		PHA-stimulated lymphocytes	
	0	2×10^{-6} M	0	2×10^{-6} M
	cpm		*cpm*	
0	1,120	466	13,286	2,166
2×10^{-6} M	993	261	5,804	412

* Conditions similar to Table I.

TABLE III

*Concentration of Cyclic AMP in Human Peripheral Lymphocytes**

Experiment	Cyclic AMP concentration: Hormone addition: None	Cyclic AMP concentration: Hormone addition: Cort.	Cyclic AMP concentration: Hormone addition: PGE_1	Cyclic AMP concentration: Hormone addition: PGE_1 + Cort.	Ratio cyclic AMP concentrations [Hormone addition / No addition]: Cort. / None	Ratio cyclic AMP concentrations [Hormone addition / No addition]: PGE_1 / None	Ratio cyclic AMP concentrations [Hormone addition / No addition]: PGE_1 + Cort. / None
	pmol/10^6 lymphocytes						
1	0.5	1.5	1.9	4.1	3.0	3.8	8.2
2	1.7	1.9	4.9	6.1	1.1	2.9	3.6
3	1.8	<0.2	3.9	7.4		2.2	4.1
4	4.2	3.0	6.7	9.1	0.7	1.6	2.2
5	2.4	1.5	3.4	4.8	0.6	1.4	2.0
6	1.5	1.1	2.0	7.3	0.7	1.3	4.9
7	2.7	2.2	9.6	23.1	0.8	3.6	8.6
8	1.4	2.3	4.9	8.9	1.6	3.5	6.4
9	1.1	2.4	6.1	12.0	2.2	5.6	10.9
10	2.8	1.7	15.0	22.0	0.6	5.4	7.9
11	2.4	2.5	18.4	30.1	1.0	7.7	12.5
12	2.8	3.8	10.0	12.4	1.4	3.6	4.4
13	3.1	4.2	11.3	9.4	1.4	3.7	3.0
14	1.0	1.5	1.9	8.7	1.5	1.9	8.7
15	3.0	3.3	4.1	6.1	1.1	1.4	2.0
16	2.6	1.5	10.2	29.0	0.6	3.9	11.2
17	2.5	1.9	8.4	26.7	0.7	3.4	10.7
Mean	2.2	2.1	7.2	13.4	1.2	3.3	6.5
SEM	±0.2	±0.2	±1.1	±2.2	±0.2	±0.4	±0.8
Significance		$P > 0.7$	$P < 0.001$	$P < 0.001$	$P > 0.2$	$P < 0.001$	$P < 0.001$
						$\neq (P < 0.001)$	

* Replicate cultures contained 5 to 10 × 10^6 lymphocytes in complete medium. Cortisol 0.1 mM and PGE_1 0.1 mM were added initially to the appropriate cultures. The cultures were then incubated at 37°C for 20 min and harvested for cyclic AMP assays as described in Methods. Cyclic AMP concentrations in hormone-stimulated lymphocytes were tested for statistically significant differences from cyclic AMP levels in control lymphocytes by Student's *t* test, small sample method. The significance of the additional rise in cyclic AMP levels, when PGE_1-stimulated cultures were concurrently exposed to cortisol, was tested by comparing the increases observed under the two incubation conditions.

served with either agent alone (Table II). Autoradiographic studies of [^{3}H]thymidine incorporation were performed with PHA-stimulated cultures after exposure to 0.1 mM PGE_1 or cortisol, and to the two hormones together. Under the experimental conditions, 50% of the lymphocytes were morphologically transformed by PHA and 24% of the cells contained > 200 grains. A small percent of cells had between 10 and 200 grains. In the presence of either PGE_1 or cortisol, 2–4% of the cells contained > 200 grains, and there was a corresponding decrease in the percent of cells with 10–200 grains. Incubation with the two hormones together resulted in 0.3% of cells with > 200 grains. The reduction in the percent of cells with a high density grain count was comparable with the reduction in [^{3}H]thymidine incorporation assayed as total acid precipitable counts per culture.

The observed interaction of cortisol and agents acting through a cyclic AMP-mediated mechanism, both in suppressing lymphocyte [^{3}H]thymidine incorporation and in preventing PHA-induced morphologic transformation, may represent true synergism, or it may be due to additive independent inhibitory effects. To attempt to resolve these two possibilities, assays of intracellular cyclic AMP levels were performed on lymphocytes after incubation with PGE_1 alone, cortisol alone, and the two agents together.

Effects of PGE_1 and cortisol upon lymphocyte cyclic AMP levels. Cell suspensions obtained from the peripheral blood of normal human donors, containing > 99% lymphocytes, were assayed for intracellular cyclic AMP concentrations. A concentration of 2.2±0.2 (mean ±SEM, $n = 17$) pmol cyclic AMP/10^6 lymphocytes was observed (Table III).

TABLE IV

*Concentration of Cyclic AMP in PHA-Stimulated Human Peripheral Lymphocytes**

Experiment	Cyclic AMP concentration: Hormone addition: None	Cort.	PGE_1	PGE_1 + Cort.	Ratio cyclic AMP concentrations [Hormone addition / No addition]: Cort. / None	PGE_1 / None	PGE_1 + Cort. / None
	pmol/10^6 lymphocytes						
1	2.2	2.9	8.8	11.6	1.3	4.0	5.3
2	4.6	3.1	9.5	13.6	0.7	2.1	3.0
3	2.3	2.5	5.8	9.4	1.1	2.5	4.1
4	3.2	3.2	6.5	11.5	1.0	2.0	3.6
5	9.3	6.0	21.4	28.3	0.7	2.3	3.0
18	6.3	0.3	15.0	16.2	0.1	2.8	2.6
19	4.7	5.1	11.7	19.4	1.1	2.5	4.1
Mean	4.7	3.3	11.2	15.7	0.8	2.6	3.7
SEM	±0.9	±0.7	±2.1	±2.4	±0.2	±0.2	±0.4
Significance		$P > 0.1$	$P < 0.01$	$P < 0.001$	$P > 0.3$	$P < 0.001$	$P < 0.001$
							$\neq (P = 0.02)$

* Conditions similar to legend, Table III, except for addition of PHA to each culture.

Experiments were performed to determine the effects of PGE_1 and cortisol, alone and in combination, upon lymphocyte cyclic AMP levels (Table III). Addition of 0.1 mM PGE_1 resulted in a rapid elevation of intracellular cyclic AMP levels which peaked by 20–30 min, whereas at concentrations of 0.01–0.001 mM PGE_1, the response was variable. Cyclic AMP levels rose 3.3±0.4-fold (mean ±SEM, $n = 17$), 20 min after incubation with 0.1 mM PGE_1. Cortisol alone, in concentrations up to 0.1 mM, had no significant effect upon cyclic AMP levels. However, when the cultures were exposed to PGE_1 and cortisol concurrently, there was a further rise in cyclic AMP levels, resulting in a 6.5±0.8-fold increase compared with control cultures. The additional rise in intracellular cyclic AMP levels, observed when PGE_1-stimulated cultures were also exposed to cortisol, is highly significant ($P < 0.001$ by Student's t test [18]). Thus cortisol and PGE_1, at concentrations which have additive inhibitory effects upon unstimulated lymphocyte [^{3}H]thymidine incorporation, act synergistically to cause an early rise in cyclic AMP levels.

TABLE V

*Effect of Hormones on Lymphocyte Cyclic AMP Concentrations**

Hormone addition	Ratio cyclic AMP concentrations, [Hormone addition / No addition]	
(A) PGE_1 0.01 mM	—	0.9
Cortisol 0.001 mM + PGE_1 0.01 mM	—	1.3
Cortisol 0.1 mM + PGE_1 0.01 mM	—	2.8
PGE_1 0.1 mM	2.8	2.6
Cortisol 0.001 mM + PGE_1 0.1 mM	2.9	4.0
Cortisol 0.1 mM + PGE_1 0.1 mM	5.0	5.9
Cortisol 0.001 mM	1.8	—
Cortisol 0.1 mM	1.3	—
(B) Dexamethasone 0.1 mM	1.2	1.0
PGE_1 0.1 mM	2.1	1.5
Dexamethasone 0.1 mM + PGE_1 0.1 mM	3.7	2.2
(C) Cholesterol 0.1 mM	—	1.5
PGE_1 0.1 mM	2.3	7.5
Cholesterol 0.1 mM + PGE_1 0.1 mM	1.5	4.6
Cortisol 0.1 mM + PGE_1 0.1 mM	4.1	20.0
(D) Ethanol control, 10 µl/ml	1.1±0.1	(n = 9)

* Conditions similar to legend, Table III. The results of a number of separate experiments are presented.

On some occasions, lymphocytes from apparently healthy donors, on no medications, contained amounts of cyclic AMP far in excess of the levels recorded in Table III, e.g., more than 10 pmol/10^6 lymphocytes. No explanation for these sporadic observations could be discovered. In these cases, further elevations of cyclic AMP levels were observed after incubation with PGE_1 and with the combination of PGE_1 plus cortisol.

A similar elevation of cyclic AMP levels in response to hormones was observed in lymphocyte cultures stimulated by the addition of PHA immediately before (or after) addition of hormones (Table IV). The addition of 0.1 mM PGE_1 resulted in a 2.6±0.2-fold (mean ±SEM, $n = 7$) rise in cyclic AMP levels after 20 min. Cortisol further augmented the effect of PGE_1 resulting in a 3.7±0.4-fold increase in cyclic AMP levels, which

is a statistically significant change ($P = 0.02$). In a larger series of experiments, cyclic AMP levels were measured 20 min after addition of PHA to lymphocyte cultures paired with unstimulated cultures from the same donor. In the presence of PHA, a 1.3±0.16-fold (mean ±SEM, $n = 23$) rise in cyclic AMP levels was observed, which is not a statistically significant change ($0.1 > P > 0.05$).

The kinetics of changes in lymphocyte intracellular cyclic AMP levels, in response to PGE_1 and cortisol, are shown in Fig. 3. In this case, the response of PHA-stimulated cultures is plotted; a similar time course was observed with unstimulated cultures. The peak elevation in cyclic AMP levels was observed at the earliest time point, 20 min after addition of PGE_1. During the next 1–6 h, the concentrations gradually declined to nearly base-line levels. The kinetics of intracellular cyclic AMP concentrations followed a similar pattern when the levels were further augmented by the interaction of cortisol with PGE_1 (Fig. 3).

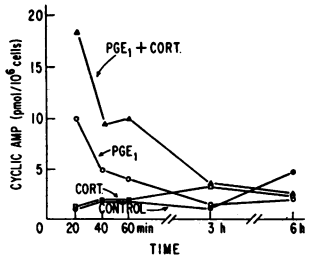

FIGURE 3 Changes in lymphocyte cyclic AMP levels in response to hormones. Replicate cultures of lymphocytes were incubated in complete medium in the presence of PHA. Additions of hormones were made to the appropriate cultures initially. After the designated periods of incubation, the cultures were harvested for assays of cyclic AMP levels, as described in Methods.

The enhanced response to PGE_1, induced by addition of cortisol to lymphocyte cultures, was further characterized by experiments summarized in Table V. The data in Table VA are from two experiments that demonstrate concentration dependence of the augmented cyclic AMP stimulation induced by simultaneous addition of PGE_1 and cortisol to lymphocyte cultures. Dexamethasone can substitute for cortisol in stimulating a further response to PGE_1 (Table VB). However, the steroid molecule, cholesterol, in similar concentrations, does not augment the effect of PGE_1 upon lymphocyte cyclic AMP levels (Table VC). Addition of ethanol (solvent for hormones) to cultures caused inconsequential changes in cyclic AMP levels (Table VD).

DISCUSSION

Our studies with both unstimulated and PHA-stimulated lymphocyte cultures demonstrate that [^{3}H]thymidine incorporation into lymphocyte DNA is regulatable by a glucocorticoid hormone and by agents acting through a cyclic AMP-mediated mechanism. Morphologic transformation by PHA was also prevented by these agents. The observations on PHA-stimulated cultures confirm previous reports (4–8). At the initiation of in vitro cultures, peripheral small lymphocytes are felt to be in a resting or G_0 phase of the cell cycle (19). The function of DNA synthesized by unstimulated peripheral lymphocytes in short-term culture has not been explored. It could be due to stimulation of a very small proportion of the cultured cells by exogenous stimuli in the medium, or it may represent repair synthesis. The nature and function of DNA synthesized after PHA stimulation is also not fully understood. Some newly synthesized DNA may subsequently be removed from transformed cells (20, 21). However, at least a portion of it must be utilized for genome replication, since an increase in the mitotic rate of PHA-transformed lymphocytes has been observed (22).

We observed cooperative inhibitory effects of cortisol and agents acting through cyclic AMP. Incubation of lymphocytes with cortisol alone did not significantly alter intracellular cyclic AMP concentrations. However, when cortisol was added to cultures concomitant with PGE_1, we observed a rapid further rise in cyclic AMP concentrations to levels higher than with PGE_1 alone. Thus cortisol may augment the suppressive effects of PGE_1 upon lymphocytes through a mechanism involving further elevation of intracellular cyclic AMP concentrations. Whether the observed increase in cyclic AMP levels is due to increased adenyl cyclase activity, inhibition of phosphodiesterase, or a more indirect mechaism remains to be tested and is the subject of further investigation in this laboratory. Since the potentiation of PGE_1-induced rises in cyclic AMP levels occurred within minutes, the mechanism is unrelated to the events associated with glucocorticosteroid-stimulated synthesis of specific proteins, which involves increased transcription of messenger RNA as a result of steroid-receptor complex binding to DNA (23). It should be stressed that the further stimulation of PGE_1-induced increases in cyclic AMP levels does not account for the inhibitory effect of cortisol when added alone to peripheral lymphocyte cultures. The varied effects of cortisol upon biochemical events in different lymphocyte populations have been reviewed recently (24).

Interactions between glucocorticosteroid hormones and agents acting through cyclic AMP have been described in a variety of experimental systems. The mechanism of this interaction is not understood, and a "permissive" role of corticosteroids has been postulated (see reference 9). This was attributed to a decrease in cyclic AMP phosphodiesterase activity in a recent study

of cultured hepatoma cells (25). A requirement for glucocorticoids in the stimulation of increased adenyl cyclase activity by isoproterenol has been demonstrated in a recent study of cultured peripheral leukocytes from asthmatic patients (26). Our data on lymphocytes from normal donors indicate that interaction between cortisol and agents stimulating increased cyclic AMP levels may be a more general phenomenon, which could possibly be exploited in clinical situations where lymphocyte suppression is desirable.

Exogenous cyclic AMP and dibutyryl cyclic AMP, in low concentrations, have been implicated as primary stimulators of peripheral lymphocyte transformation (7, 8, 27). In each of these reports, small increases in [^{3}H]thymidine incorporation were observed. We find no evidence that cyclic AMP, in concentrations ranging from 1 nM to 1 mM, stimulates a significant increase in [^{3}H]thymidine incorporation, a conclusion in agreement with the findings of Novogrodsky and Katchalski (28).

When cultures exposed to optimal concentrations of PHA were compared with unstimulated cultures, a small rise in cyclic AMP levels was often observed, but statistical analysis showed this to be nonsignificant. Smith, Steiner, Newberry, and Parker (29), demonstrated a transient stimulation of human lymphocyte cyclic AMP levels by a range of PHA concentrations which were in many cases far higher than those used to induce lymphocyte transformation and which can inhibit optimal transformation. Within 1–6 h, the cyclic AMP levels in PHA-stimulated lymphocytes fell to below the levels in control cultures (29). Others have not observed an early rise in intracellular cyclic AMP after stimulation with various preparations of PHA (28, 30). The observation that incubation of both unstimulated and PHA-stimulated lymphocytes with PGE_1 resulted in early elevation of cyclic AMP levels and marked inhibition of [^{3}H]thymidine incorporation supports the conclusion reached by Smith et al. (29), that elevated intracellular cyclic AMP levels are associated with inhibition of peripheral lymphocyte nucleic acid synthesis and transformation. Hormone-induced stimulation of lymphocyte cyclic AMP levels also has been found to inhibit the cytotoxic activity of lymphocytes from preimmunized animals against allogeneic cells in vitro (31, 32).

Support for a general role of cyclic AMP in controlling cell division and cell differentiation is derived from recent studies with permanent cell culture lines. The doubling time of 3T3 cells correlates with intracellular cyclic AMP levels (33). Incubation of a permanent Chinese hamster ovarian fibroblast line with dibutyryl cyclic AMP has been shown to induce contact inhibition of growth and reacquisition of an elongated fibroblast-like morphology, with initiation of collagen synthesis (34). In this case, as in our studies, the response to an agent raising intracellular cyclic AMP levels was augmented by concurrent exposure to a steroid hormone, testosterone.

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