Cyclic Adenosine 3',5'-Monophosphate in Human Lymphocytes. Alterations after Phytohemagglutinin Stimulation

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A B S T R A C T We have studied cyclic adenosine 3',5'-monophosphate (cyclic AMP) concentrations in human peripheral blood lymphocytes after stimulation with phytohemagglutinin (PHA), isoproterenol, prostaglandins, and aminophylline. Purified lymphocytes were obtained by nylon fiber chromatography, and low speed centrifugation to remove platelets. Cyclic AMP levels were determined by a highly sensitive radioimmunoassay. At concentrations of 0.1–1.0 mmoles/liter isoproterenol and aminophylline produced moderate increases in cyclic AMP concentrations, whereas prostaglandins produced marked elevations. High concentrations of PHA produced 25–300% increases in cyclic AMP levels, alterations being demonstrated within 1–2 min. The early changes in cyclic AMP concentration appear to precede previously reported metabolic changes in PHA-stimulated cells. After 6 hr cyclic AMP levels in PHA-stimulated cells had usually fallen to the levels of control cells. After 24 hr the level in PHA-stimulated cells was characteristically below that of the control cells.

Adenyl cyclase, the enzyme which converts ATP to cyclic AMP, was measured in lymphocyte homogenates. Adenyl cyclase activity was rapidly stimulated by fluoride, isoproterenol, prostaglandins, and PHA. Since adenyl cyclase is characteristically localized in external cell membranes, our results are consistent with an initial action of PHA at this level.

INTRODUCTION

Cyclic adenosine 3',5'-monophosphate (cyclic AMP) has been widely implicated as a secondary messenger for

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the action of hormones on mammalian tissues (1). Adenyl cyclase, the enzyme which converts adenosine triphosphate to cyclic AMP, has not been isolated in a highly purified state, but it appears to be a component of the external cell membrane where it is rapidly influenced by hormones in the extracellular fluid. Considering the broad applicability of the secondary messenger concept in hormone effects on cells there has been surprisingly little effort to study the role of cyclic AMP in responses of lymphoid cells to antigen and phytohemagglutinin. We have previously reported that isoproterenol, aminophylline, and prostaglandins, agents which elevate cyclic AMP levels in human lymphocytes, markedly influence PHA-stimulated transformation of these cells (2). The present paper describes changes in adenyl cyclase activity and cyclic AMP levels in human lymphocytes with time after exposure to PHA. In the companion paper we present the results of detailed studies on the effects of extracellular and intracellular nucleotides on lymphocyte metabolism.

METHODS

Preparation of lymphocytes. In most experiments, 500 ml of blood from a single healthy human volunteer was used with heparin (1000 U/100 ml of blood) (Liquaemin Sodium, Organon Inc., West Orange, N. J.). Dextran (Grade H, Pharmachem, Bethlehem, Pa.) was added to a final concentration of 0.6% and cells separated by gravity sedimentation at 37°C for 1 hr. The leukocyte-rich supernatant was separated and centrifuged at 250 *g* for 10 min at room temperature. The cell pellet was then washed three times with 0.01 M phosphate–0.15 M NaCl, pH 7.4 (phosphate-saline). This preparation usually contained 40–70% lymphocytes.

Purified lymphocytes were prepared by pouring the leukocyte-rich plasma (unwashed cells) over nylon columns. Before use the nylon fibers (Leuko-Pak, Fenewal Labs., Morton Grove, Ill.) were thoroughly washed with distilled water and air dried. 4 g of dry nylon were packed into each

glass column (200 × 13 mm). Five columns were used per 500 ml of blood. Aliquots of the leukocyte-rich plasma were poured on columns and left to equilibrate for 10 min at 37°C and then eluted with fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Cells in the eluate were pooled, centrifuged at 250 g at room temperature for 10 min, washed three times with phosphate-saline, and centrifuged two times at 100 g at room temperature for 7 min to remove platelets. Purified lymphocyte preparations usually contained 96–99.5% lymphocytes and less than one platelet per three lymphocytes. Platelets purified by the method of Lewis and Majerus (3) were demonstrated to have less than 0.2 pmoles of cyclic AMP per 10^6 platelets. Human polymorphonuclear leukocytes were purified by the method of Böyum (4).

Culture medium. The culture medium contained Eagle's minimum essential medium (Grand Island Biological) 0.02 M Tris·HCl buffer, 20% fetal calf serum, 0.04 M glutamine, nonessential amino acids (Grand Island Biological), and 100 U of penicillin G per ml. The pH of the medium (at 37°C) was adjusted to 7.3 with 0.2 N HCl. Long-term experiments (6 hr or more) were conducted under sterile conditions with streptomycin, 100 μg/ml, also present.

Reagents. Phytohemagglutinin-P (PHA-P), from Difco Labs, Detroit, Mich., was reconstituted in 5.0 cc of sterile water. The reconstituted PHA-P contained the following electrolytes: Na, 129 mEq/liter; K, 2.5 mEq/liter; Cl, 128 mEq/liter; Mg, 0.6 mEq/liter; and Ca, 2.0 mEq/liter. In some experiments the PHA-P was dialyzed versus Gey's solution. Dilutions were made in sterile Gey's balanced salt solution. Leukoagglutinating and erythroagglutinating PHA prepared by the method of Weber, Nordman, and Gräsbeck (5) were generously provided by Dr. S. Kornfeld, Washington University School of Medicine. Aminophylline, norepinephrine, and isoproterenol (Sigma Chemical Co., St. Louis, Mo.) were dissolved in Gey's balanced salt solution and filtered through 0.45 μ filters (Millipore Corp., Bedford, Mass.) before using. D,L-Propanolol was obtained from Sigma, dibenamine from K & K Labs, Inc., Jamaica, N. Y. Phentolamine mesylate was generously provided by Dr. A. J. Plummer of Ciba Pharmaceutical Co. Radioactive ATP-^{14}C (531 mCi/mmole) and cyclic AMP-^{3}H (1.4 Ci/mmole) were obtained from New England Nuclear, Boston, Mass., and Schwarz BioResearch, Orangeburg, N. Y.

Prostaglandins E_1, E_2, A_1, A_2, and $F_{1\alpha}$ were kindly supplied by Dr. J. Pike of The Upjohn Co., Kalamazoo, Mich. 3×10^{-3} M stock solutions were prepared by dissolving the free acids in 95% ethanol, 0.002 M sodium carbonate 1:9, and adjusting the pH to 6.7. A solution containing similar proportions of carbonate and ethanol and adjusted to pH 6.7 was used as a control.

Adenyl cyclase determinations. The column-purified lymphocytes were washed twice with hypotonic phosphate-saline (one part phosphate-saline and four parts H_2O). The cell pellet was then frozen in liquid nitrogen, thawed at room temperature, washed again with hypotonic phosphate-saline, and refrozen in liquid nitrogen. After rethawing the cells were homogenized at 6°C with Teflon pestle with clearance between 0.005 and 0.007 inches in a loose fitting glass homogenizer for 30 sec and distributed in test tubes to contain 6×10^6 cells/tube or 0.14 mg tissue protein (bovine serum albumin standard) as determined by the method of Lowry, Rosebrough, Farr, and Randall (6).

The assay was performed by a modification of recently described methods (7) based on the conversion of uniformly

^{14}C-labeled ATP to cyclic AMP. Each 80 μl reaction volume contained 1 μCi ATP-^{14}C (531 mCi/mmole), 0.375 mM ATP, 10 mM theophylline (an inhibitor of cyclic AMP phosphodiesterase), 5 mM $MgCl_2$, 0.1% bovine serum albumin, 50 mM phosphoenol pyruvate, and 0.05 mg of pyruvate kinase. The lymphocyte preparation was added to the reaction mixture at 0°C and tubes were then incubated for 10 min at 30°C. The reaction was terminated by immersing tubes in a boiling water bath for 3 min. Cell blanks in which the enzyme was inactivated by boiling were included in each experiment. A solution containing 40 mM ATP, 10 mM cyclic AMP, and approximately 0.05 μCi cyclic AMP-^{3}H (for calculating recovery of the cyclic nucleotide during the subsequent steps of isolation) was then added. Cyclic AMP was separated from ATP and other nucleotides by Dowex-50 (H$^+$) cation-exchange resin column chromatography and precipitation with $ZnSO_4$ and $Ba(OH)_2$. Double isotope counting of ^{14}C-labeled and ^{3}H-labeled cyclic AMP was carried out in a Packard liquid scintillation counter. All assays were performed in triplicate. Enzyme activity is expressed as pmoles of cyclic AMP formed per milligram protein per 10 min. Enzyme activity was linear over this time period and proportional to the amount of tissue present.

Cyclic AMP determinations. Nylon column–purified lymphocytes were suspended in supplemented Eagle's minimum usually at a concentration of 16×10^6 cells per ml (for other conditions see Tables V–VII). Before the initiation of the experiment lymphocytes were preincubated in complete medium at 37°C for 30–60 min. In two experiments cell were preincubated at 37°C for 16 hr under sterile conditions. PHA produced similar effects in these experiments. 0.65 ml of this cell suspension and 0.05 ml of solutions containing PHA-P, aminophylline and isoproterenol, prostaglandin, or Gey's solution were then added to 12-ml glass Pyrex homogenizer tubes. The tubes were capped and incubated at 37°C for varying periods of time with manual shaking every 15 min during the early phases of incubation. After incubation cells were centrifuged at 250 g for 2 min at room temperature, the supernatant removed with a capillary pipette and the cell pellet immediately frozen in liquid nitrogen. Experimental conditions were performed in quadruplicate. In very short incubation experiments (less than 2 min) cells were centrifuged before the addition of PHA and PHA was added directly to the cell pellet.

Cyclic AMP was determined by a radioimmunoassay procedure recently developed in these laboratories (8). The frozen cell pellets were homogenized in 6% ice-cold trichloroacetic acid (TCA) in a cold room (6°C) and extracted with 4.0 cc of ether three times. The aqueous phase was heated at 56°C to remove the ether and evaporated to dryness under a stream of nitrogen gas. Cyclic AMP in the residue was dissolved in 300 μl of 0.05 M Na acetate buffer, pH 6.2. ^{125}I-labeled cyclic AMP and rabbit anti–cyclic AMP antibody were added and the reaction mixture allowed to incubate for 3 hr at 6°C. An excess of goat anti-rabbit γ-globulin was then added and after 16 hr of further incubation at 6°C, precipitates were isolated by centrifugation and washed with acetate buffer. Radioactivity in the precipitates was determined by a gamma spectrometer. The ^{125}I-labeled cyclic AMP was prepared by iodination of 2′,0 (succinyl tyrosinemethyl ester)–cyclic adenosine 3′,5′-monophosphate according to the chloramine-T procedure of Hunter and Greenwood (9). The rabbit anti–cyclic AMP antibody was prepared by immunization of rabbits with a bovine serum albumin conjugate of 2′,0-succinyl-cyclic AMP (8).

PHA was extracted with chloroform as follows. 5 ml of undiluted PHA-P in Gey's solution was cooled and acidified to pH 2.0 with 2 N HCl. The acid solution was extracted three times with an equal volume of chloroform over a period of 90 min with continuous shaking. The PHA was quantitatively transferred to a dialysis bag and dialyzed versus Gey's solution.

RESULTS

Adenyl cyclase. The activity of adenyl cyclase in broken cell preparations of lymphocytes is shown in Table I. Sodium fluoride (10 mmoles/liter) markedly increased the activity of the enzyme, a result consistent with what has been observed with broken cell preparations from other tissues (1). The effect of isoproterenol (0.35 mmole/liter), PHA (1:14 to 1:350), and prostaglandins (0.2 mmole/liter) varied from little or no change to 2- to 4-fold increases in enzyme activity. In most experiments PHA, isoproterenol, and prostaglandins clearly stimulated enzyme activity within the 10 min period of the assay (Table I). Failure of these agents to produce increases in adenyl cyclase activity in several early experiments presumably was due to excessive membrane fragmentation. Oye and Sutherland (10) have observed that the responsiveness of nucleated turkey erythrocytes to epinephrine was retained or lost depending on the method of preparing the erythrocyte membranes. In our own experiments with human lymphocytes hormone and PHA responsiveness was observed provided precautions were taken to minimize membrane fragmentation during cell disruption.

Validation of the immunoassay for cyclic AMP (see also reference 8). The specificity of the antibody used in the radioimmunoassay for cyclic AMP is shown in Fig. 1. The ability of the mono-, di-, and triphosphonucleosides of adenine, guanine, and uridine series to inhibit the binding of labeled cyclic AMP by anti–cyclic AMP antibody was minimal; all had less than 0.005% of the inhibitory potency of cyclic AMP, (e.g. more than
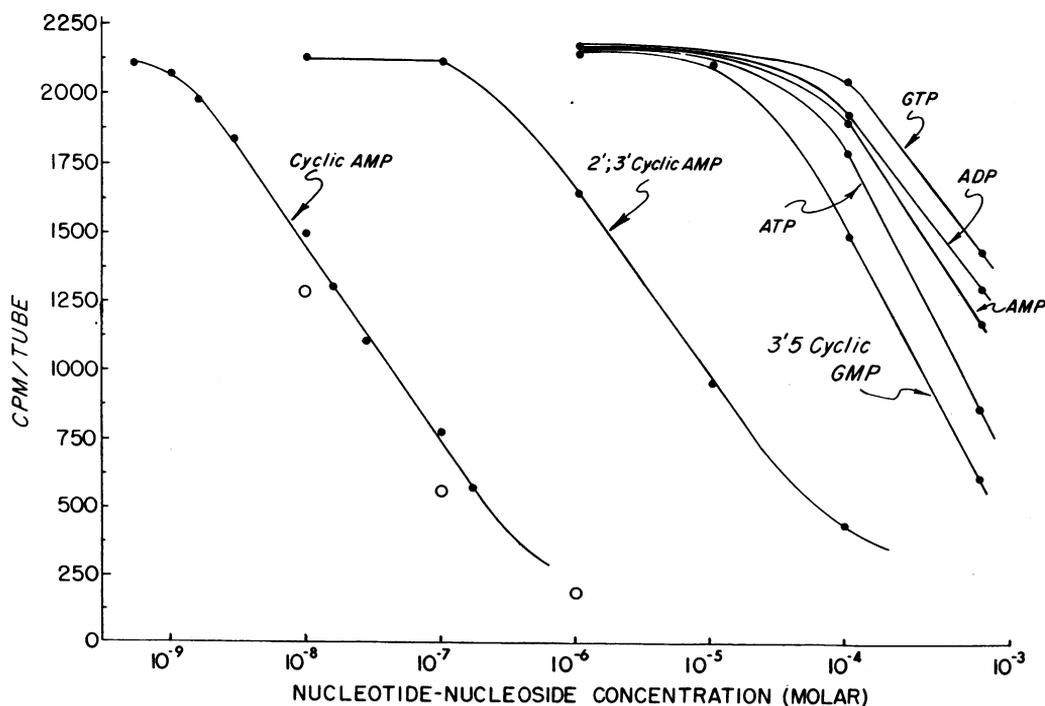

FIGURE 1 The inhibition of ^{125}I-labeled 2'0 (succinyl-tyrosine methyl ester) cyclic AMP binding to anti-cyclic AMP antibody by various nucleotides. The open circles are 2-deoxyribose 3',5'-cyclic AMP (taken from reference 7).

20,000-fold higher concentrations were needed to give inhibition equal to that of cyclic AMP). Even the $3',5'$-cyclic nucleotides of these purine and pyrimidine bases showed negligible cross-reactivity. Although $2',3'$ cyclic AMP did inhibit binding somewhat, its reactivity was less than 1% of that obtained with cyclic AMP.

Data on the recovery of cyclic AMP from lymphocytes are shown in Table II. In this experimental 10 pmoles of cyclic AMP were added to the lymphocyte preparation just before freezing in liquid nitrogen. 72% (8.2 pmoles) of the cyclic AMP theoretically present was recovered. Adding phosphodiesterase after the ether extraction essentially eliminated cyclic AMP activity in the immunoassay. Doubling of the number of lymphocytes produced a 2-fold increase in cyclic AMP recovery.

The effects of aminophylline, isoproterenol, and prostaglandins on lymphocyte cyclic AMP levels. The effects of isoproterenol and prostaglandin (PGE₁) on cyclic AMP levels in purified lymphocytes correlated well with their effects on adenyl cyclase activity in broken cells. In eight experiments isoproterenol at 0.35 mmole/liter uniformly produced moderate increases (usually 1.5- to 3-fold) in cyclic AMP levels; at 0.2 mmole/liter PGE₁ produced a 4- to 8-fold increase in the cyclic AMP concentration. Representative data are shown in Table III. The stimulation of lymphocyte adenyl cyclase activity by isoproterenol and PGE₁ is in accord with the effects of these agents in most other mammalian tissues (11, 12). Norepinephrine (0.02–20 mmoles/liter in Gey's solution) also stimulated lymphocyte cyclic AMP levels. Low concentrations of norepinephrine (2–100 mmoles/liter) lowered cyclic AMP levels by about 25% in two of three experiments.

Aminophylline at 0.35 mmole/liter also induced increases in lymphocyte cyclic AMP levels (Table III), presumably due to inhibition of the phosphodiesterase which hydrolyzes cyclic AMP. When aminophylline and isoproterenol were both present, an additive and in some instances a synergistic effect was observed.

In four experiments various prostaglandins (PGE₁, PGE₂, PGA₁, PGA₂, and PGF₁ₐ) were evaluated at a 0.2 mM concentration and all determinations demon-

TABLE II

Recovery of Cyclic AMP in Immunoassay Experiments

	Amount of cyclic AMP recovered*
Lymphocytes (10^7)	1.3
Lymphocytes (10^7) + 10 pmoles cyclic AMP	8.2
Lymphocytes (10^7) + phosphodiesterase	<0.5
Lymphocytes (2×10^7)	2.4

* Each value represents the average of four determinations.

TABLE III

Effects of Aminophylline, Isoproterenol, and Prostaglandin (PGE₁) on Cyclic AMP Levels in Lymphocytes

Experiment 1		Experiment 2		Experiment 3	
Control	Amino-phylline	Control	Isopro-terenol	Control	PGE₁
2.6	5.6	1.6	2.0	2.4	30
2.3	5.7	1.4	3.0	2.5	34
2.8	6.1	2.0	3.5	2.6	29
1.8	5.0	1.6	4.8	1.4	29
(2.4 ±0.4)	(5.6 ±0.4)	(1.6 ±0.3)	(3.3 ±1.0)	(2.2 ±0.5)	(30.5 ±1.8)

Aminophylline and isoproterenol (3×10^{-4} mole/liter) and PGE₁ (2×10^{-4} mole/liter) were incubated with 10^7 lymphocytes in a volume of 0.7 ml for 1 hr at 37°C. Results are expressed in picomoles of cyclic AMP ±SEM per 10^7 lymphocytes (uncorrected for recovery).

strated marked elevation of cyclic AMP levels. Results of a representative experiment are shown in Table IV. PGF₁ₐ was the least potent of the prostaglandins at this concentration which correlates with its lesser effects on lymphocyte transformation (see accompanying paper).

The effects of phytohemagglutinin on lymphocyte cyclic AMP levels. The influence of PHA on lymphocyte cyclic AMP levels varied with the duration of the incubation. With lymphocytes that were at least 98% pure, cells that had been incubated with PHA for 1 hr or less characteristically exhibited moderate increases in cyclic AMP levels as compared with control cells (Table V). Most experiments were conducted at a PHA dilution of 1:14 (approximately 400 μg PHA protein per ml). Data on changes in cyclic AMP levels (at 1 hr) at different concentrations of PHA are shown in Table V, M. N. and M. H. At a dilution of 1:70 PHA-P consistently stimulated cyclic AMP levels, although usually to a lesser degree than PHA-P, 1:14 (Table V) PHA-P, 1:350 produced smaller increases in cyclic AMP concentrations or in two instances no change at all.

TABLE IV

Effect of Prostaglandins on Lymphocyte Cyclic AMP

Prostaglandin	Cyclic AMP
PGE₁	30
PGE₂	28
PGA₁	24
PGA₂	21
PGF₁ₐ	14
EtOH-NaCl control	2.5
Gey's control	2.2

Prostaglandins at 2×10^{-4} mole/liter and lymphocytes (10^7 per ml) were incubated at 37°C for 1 hr. Each value represents the average of four determinations. Results are expressed in picomoles per 10^7 cells (uncorrected for recovery).

TABLE V

Effect of Phytohemagglutinin on Cyclic AMP Levels in Lymphocytes after 1 hr

Experi-ment	Cell density $\times 10^6$	PHA concen-tration	Platelet/WBC	% of lymphs	Cyclic AMP con-trol	PHA-P
M. N.*	5.0	1:14	—	99.0	3.5	5.6 $P < 0.01$
M. N.*	5.0	1:70	—	99.0	3.5	7.8 $P < 0.01$
D. A.	1.0	1:30	0.2	99.0	3.2	5.2
M. C.	14.0	1:14	0.75	>99.5	4.2	5.4
B. K.	14.0	1:14	—	>99.5	4.2	4.1
D. G.	14.0	1:14	<0.05	99.5	3.8	5.3 $P < 0.01$
J. Be.	12.6	1:14	1.0	>99.5	1.1	1.4
L. R.	14.0	1:14	—	>99.5	5.0	7.3
P. K.	14.0	1:14	—	99.0	3.5	10.0 $P < 0.01$
M. H.	14.0	1:14	—	98.0	1.8	10.2 $P < 0.01$
	14.0	1:70	—	98.0	1.8	5.4 $P < 0.01$
	14.0	1:350	—	98.0	1.8	4.0 $P < 0.01$

PHA-P and 1×10^7 lymphocytes were incubated at 37°C for 1 hr. Results are expressed in picomoles cyclic AMP per 1×10^7 lymphocytes. Each value represents the average of four determinations (uncorrected for recovery).
* Experiment in complete medium diluted with 0.2 volumes of distilled water.

PHA dialyzed extensively versus Gey's solution and purified leuko- and erythroagglutinating PHA's also produced early increases in lymphocyte cyclic AMP levels (usually measured at 1 hr).

Nearly all experiments have been carried out at a cell density of 1.4×10^7 lymphocytes per ml. A quantitative evaluation of the effect of cell density on the response to PHA is not available. Preliminary results indicate that stimulatory effects of PHA on the cyclic AMP levels of lymphocytes at a density of 1×10^6 cells/ml can be obtained.
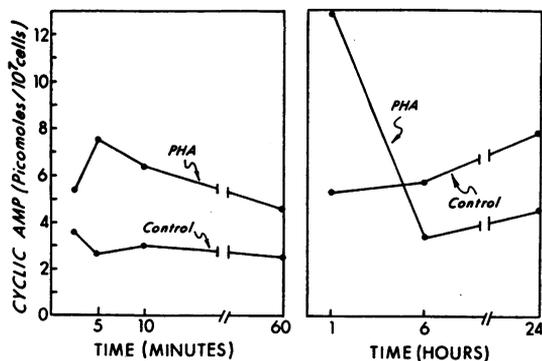

FIGURE 2 Cyclic AMP vs. time in PHA-stimulated cells. 10^7 lymphocytes in a final volume of 0.7 ml were incubated in Eagle's medium with either 0.05 ml of PHA-P or 0.05 ml of Gey's balanced salt solution for various times at 37°C. The cells were then centrifuged for 2 min at 250 g, the supernatants removed, and the cell pellets immediately frozen. Cyclic AMP determinations were then made as described in the text. Two separate experiments are shown.

In the adenyl cyclase experiments it was established that PHA is capable of producing increased adenyl cyclase activity within 5–10 min. The rapidity of the increase in enzyme activity was further studied by following early changes in cyclic AMP levels in stimulated and control cells. In cell suspension experiments significant increases in cyclic AMP levels occurred within 2 min after the addition of PHA (Fig. 2, left). This is the minimal period of time required to add PHA and the control buffer solution and rapidly centrifuge the cells. In experiments on lymphocyte pellets, at 0 time and 30 sec after the addition of PHA definite increases in cyclic AMP levels were not demonstrable. By 1 min, however, clear increases in cyclic AMP levels had occurred.

In experiments in which lymphocyte cyclic AMP levels were followed for many hours a characteristic sequence of changes took place (Fig. 2, right). After the early rise in cyclic AMP levels in PHA-stimulated cells, the cyclic AMP concentration was observed to fall so that by 6 hr the concentration of the cyclic nucleotide in stimulated cells was the same or below that of the control cells. The cyclic AMP level in PHA-stimulated cells was definitely below that of control at 16–20 hr.

To exclude the possibility that the late fall in cyclic AMP levels in PHA-stimulated cells might be due to nutritional deficiency secondary to high cell density, a timed experiment at a cell density of 1×10^6 cells/ml was carried out. The same late fall in cyclic AMP level in PHA-stimulated cells was observed.

PHA in combination with other cyclic AMP stimulators. Combinations of PHA with α- and β-stimulators

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(norepinephrine and isoproterenol) produced greater increases in cyclic AMP concentration than any of the three agents alone (at maximal stimulatory concentrations of each) (Table VI). Similar results were obtained with aminophylline in combination with PHA (not shown).

The effect of α- and β-adrenergic blocking agents. The effect of α(dibenamine, phentolamine) and β(D,L-propanolol) blocking agents on stimulation of lymphocyte cyclic AMP levels by PHA, isoproterenol, norepinephrine, and prostaglandin was investigated (Table VII). PHA stimulation was inhibited incompletely by propanolol (>10 μmoles/liter) and completely by dibenamine (>20 μmoles/liter). The inhibition by dibenamine suggested the possibility of an α-stimulatory effect of PHA on the lymphocyte. However phentolamine, a reversible blocking agent (in contrast to dibenamine which combines irreversibly with cells), did not inhibit PHA at all, even at concentrations above 100 μmoles/liter. This suggested that the dibenamine inhibition was probably "nonspecific," presumably involving the alkylation of a chemically reactive region of the cell distinct from the α-receptor site. Dibenamine inhibition was also observed with norepinephrine- and isoproterenol-stimulated lymphocytes but again a nonspecific inhibition seemed indicated. Propanolol was highly effective as an inhibitor whereas phentolamine was noninhibitory or

TABLE VI

The Effect of PHA Alone and in Combination with Norepinephrine and Isoproterenol on Lymphocyte Cyclic AMP Concentrations

Condition	Cyclic AMP
	pmoles/10⁷ lymphocytes
Buffer control	1.3
PHA 1:3	16.0
PHA 1:7	15.5
PHA 1:14	10.0
PHA 1:3 + Isoproterenol, 40 mmoles/liter	28.0
PHA 1:7 + Isoproterenol, 20 mmoles/liter	31.0
PHA 1:14 + Isoproterenol, 10 mmoles/liter	30.0
Isoproterenol, 40 mmoles/liter	13.0
Isoproterenol, 20 mmoles/liter	12.0
Isoproterenol, 10 mmoles/liter	9.0
PHA 1:3 + Norepinephrine, 20 mmoles/liter	27.0
PHA 1:14 + Norepinephrine, 10 mmoles/liter	50.0
Norepinephrine, 20 mmoles/liter	8.2
Norepinephrine, 10 mmoles/liter	24.0
Norepinephrine, 1 mmole/liter	10.0
Norepinephrine, 0.2 mmole/liter	8.0

1×10^7 lymphocytes were incubated at 37°C for 5 min in Gey's solution. (Cyclic AMP values are uncorrected for recovery.)

TABLE VII

Effect of Dibenamine, Phentolamine, and D, L-Propanolol on Cyclic AMP Stimulation by PHA, Norepinephrine, Isoproterenol, and PGE₁

Condition	Cyclic AMP
	pmoles/10⁷ lymphocytes
Buffer control	1.1
PHA 1:14	6.4
PHA 1:14 + phentolamine, 20 μmoles/liter	7.0
PHA 1:14 + propanolol 20 μmoles/liter	4.0
PHA 1:14 + dibenamine 20 μmoles/liter	1.2
Isoproterenol, 10 mmoles/liter	12.0
Isoproterenol, 10 mmoles/liter + phentolamine, 20 μmoles/liter	11.0
Isoproterenol, 10 mmoles/liter + propanolol, 50 μmoles/liter	2.0
Isoproterenol, 10 mmoles/liter + propanolol, 20 μmoles/liter	5.0
Isoproterenol, 10 mmoles/liter + dibenamine, 20 μmoles/liter	5.0
Norepinephrine, 10 mmoles/liter	15.0
Norepinephrine, 10 mmoles/liter + phentolamine, 20 μmoles/liter	18.0
Norepinephrine, 10 mmoles/liter + propanolol, 20 μmoles/liter	1.3
Norepinephrine, 10 mmolec/liter + dibenamine, 20 μmoles/liter	10.0
PGE₁ 1 × 10⁻⁷ mole/liter	18.0
PGE₁ 1 × 10⁻⁷ mole/liter + phentolamine, 20 μmoles/liter	20.0
PGE₁ 1 × 10⁻⁷ mole/liter + propanolol, 50 μmoles/liter	10.0
PGE₁ 1 × 10⁻⁷ mole/liter + propanolol, 20 μmoles/liter	13.0
PGE₁ 1 × 10⁻⁷ mole/liter + dibenamine, 20 μmoles/liter	9.0
Phentolamine, 20 μmoles/liter	1.4
Propanolol, 20 μmoles/liter	1.0
Dibenamine, 20 μmoles/liter	1.0

Each tube contained 5×10^6 lymphocytes in 0.6 ml of Gey's solution. The lymphocytes were incubated with blocking agents (or control buffer solution) for 10 min at 37°C before the addition of the adenyl cyclase stimulators. The cells were harvested after an additional 5 min at 37°C. (Cyclic AMP values are uncorrected for recovery.)

even weakly stimulatory. Thus it appeared that both isoproteronol and norepinephrine were functioning primarily as β-stimulatory agents.

PGE₁ was partially inhibited by dibenamine (>10 μmoles/liter) and propanolol (>10 μmoles/liter) but not by phentolamine at similar and higher concentrations. The inhibition pattern thus corresponded reasonably closely to what was observed with PHA itself. Since the prostaglandins are widely distributed in the plant and animal kingdom it seemed possible that the PHA-P preparations used in these experiments contained prostaglandin, accounting for their adenyl cyclase–stimulating activity. Observations bearing on this possibility were the following. (*a*) The PHA-P remained active following extensive dialysis and column chromatography (in preparation of erythro- and leukoagglutinating fractions), conditions which would ordinarily remove prostaglandins. (*b*) PHA-P at a final dilution of 1:14 failed to elevate cyclic AMP levels in purified human polymorphonuclear leukocytes and rabbit kidney cortex

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TABLE VIII
Effect of PHA on Cyclic AMP Concentrations in Various Cells

	Cyclic AMP, picomoles			
	Con-trol	PHA 1:14	PHA 1:350	PGE₁ threshold
1. Lymphocytes 1 X 10 cells	2.0	5.6	3.9	$\sim$30 mμmoles/liter
2. Platelets 6 X 10⁷ cells	3.5	4.8	3.6	$\sim$10 mμmoles/liter
3. Polymorphonuclear leukocytes 1 X 10⁷ cells	3.5	3.6	3.4	$\sim$100 mμmoles/liter
4. Rabbit renal cortex 10 g (wet wt)	5.0	4.8		$\sim$1500 mμmoles/liter

Lymphocytes, platelets, and polymorphonuclear leukocytes were incubated for 10 min at 37°C in Gey's solution. Rabbit renal cortex incubated for 30 min in Krebs-Ringer bicarbonate in the presence of 1×10^{-2} M theophylline, 2.50 mg/ml glucose and 2.5 mg/ml BSA. (Cyclic AMP values are uncorrected for recovery.)

slices, although there was a 25% increase in cyclic AMP in purified human platelets (Table VIII). On the basis of the minimal concentrations of PGE₁ capable of stimulating the various tissues (Table VIII) and the fact that the PHA-P preparation used in these experiments stimulated lymphocyte cyclic AMP at a dilution of 1 : 350, more marked effects on leukocyte and platelet cyclic AMP levels by concentrated solutions of PHA might have been expected if PGE were involved in lymphocyte cyclic AMP stimulation by PHA. (c) We have been unable to remove lymphocyte cyclic AMP–stimulating activity from PHA by repeated extraction with chloroform at pH 2. (d) Using a newly developed radioimmunoassay for prostaglandin capable of detecting less than 1 mμg of PGE₁ and PGA₁,[1] we were unable to detect any prostaglandin-like activity in unextracted PHA.

DISCUSSION

These studies document the presence of adenyl cyclase in human lymphocytes. The failure of Wolfe and Shulman (13) to detect adenyl cyclase in these cells may have been due to their use of extensively sonicated cell preparations. In our own studies with human lymphocytes we have failed to detect significant cyclase activity in cells sonicated even for relatively brief periods. Levels of lymphocyte adenyl cyclase activity differed considerably with the lymphocyte preparation. The basis for this variation (lymphocyte donor versus lymphocyte processing) requires further exploration. Other investigators have noted marked variations in early metabolic responses of human lymphocytes to PHA (14) and have suggested that much of this may be due to the use of different cell donors. The enzyme exhibited conventional

[1] Jaffe, B., J. W. Smith, and C. W. Parker. 1970. A radioimmunoassay for prostaglandin. In preparation.

responsiveness to fluoride, PGE₁, and isoproterenol. A systematic evaluation of the distribution of the enzyme in lymphocyte subcellular fractions has not yet been made. However, the apparent diminution of hormonal responsiveness with increasing cell fragmentation is consistent with a localization in a particular cell fraction, presumably the external cell membrane, judging from the results of studies with nearly all other mammalian and avian cells (10).

Lymphocytes exposed to high concentrations of *Phaseolus vulgaris* PHA underwent an early increase in adenyl cyclase activity, apparently due to a direct action of the mitogen on the enzyme. To our knowledge this is the first *direct* evidence that a nonhormonal protein can mediate alterations in cyclase activity. Lichtenstein and Margolis (15) have obtained *indirect* evidence that antigen inhibits adenyl cyclase activity in sensitized human leukocytes. They observed that antigen-mediated release of histamine from these cells was inhibited by aminophylline, isoproterenol, and dibutyryl cyclic AMP. While this evidence is consistent with a localized action of antigen on leukocyte adenyl cyclase, measurements of adenyl cyclase activity and cyclic AMP levels in antigen-stimulated and control cells are needed.

The demonstration that cyclic AMP levels rise very rapidly in lymphocytes after PHA stimulation is consistent with an early primary effect at the external cell membrane. The fact that adenyl cyclase in mammalian cells is characteristically at or near the external cell membrane is in accord with this assumption. The basis for the ability of PHA to elevate cyclic AMP levels in lymphocytes is uncertain. PHA-P and purified erythro- and lymphoagglutinating fractions of PHA are known to agglutinate lymphocytes and no doubt produce alterations in the configuration of the external cell membrane. Allosteric increases in adenyl cyclase activity might occur in this situation. Stimulation under these conditions

might be considered to be due to a surfactant action of PHA analogous to the stimulatory effect of detergents and chelating agents in other adenyl cyclase systems. Alternatively, clumping of lymphocytes might interfere with the transport of essential nutrients into the cell, with secondary changes in intracellular metabolism and altered adenyl cyclase activity. However, the latter possibility seems unlikely because of the rapidity with which changes in enzyme activity occur. The role of agglutination as such in the stimulation of the enzyme is currently under evaluation. Goldberg, Rosenau, and Burke (16) recently described a highly purified PHA preparation with a low protein content which is very active in stimulating DNA and RNA synthesis but which has little or no lymphoagglutinating activity. Studies with this material are in progress.

The combination of isoproterenol or norepinephrine with PHA produced greater increases in cyclic AMP concentration than the maximal increase of any of the three agents alone. Presumably, stimulation by the adrenergic agents and PHA involves independent receptors on a single adenyl cyclase system although the possibility of separate cyclase systems as suggested by Levy and Epstein for myocardial adenyl cyclase (17) is not excluded.[2] Judging from the results of studies with adrenergic blocking agents, PHA stimulation probably involves a β-stimulatory effect. This is in accord with results of studies in other tissues where β-stimulation characteristically increases adenyl cyclase activity. In some tissues α-blocking agents also stimulate adenyl cyclase suggesting a reciprocal relationship between α- and β-receptors in regard to their effects on adenyl cyclase (18, 19). Weak stimulatory effects of phentolamine on lymphocyte cyclic AMP levels have been observed in several experiments but the effects are small and of borderline $(P \sim 0.10)$ statistical significance.

The observation that there is marked fluctuation in cyclic AMP levels with time after exposure to PHA is of considerable interest. Following an early rise there was a prolonged fall in cyclic AMP levels lasting for many hours. The early response occurred within 1 min, sufficiently fast to suggest that cyclic AMP could play a major role in the early metabolic effects of PHA. Other early changes in lymphocyte metabolism in PHA-stimulated cells include increased incorporation of ^{32}P orthophosphate into phosphatidylinositol (20), an increased rate of phosphorylation and acetylation of nuclear protein (21), and an increased rate of incorporation of radioactive precursors into RNA and protein

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[2] It is possible that effects on different lymphocyte populations (thymus dependent and bursa dependent) are involved here.

(22). These changes appear to occur less rapidly than the early elevation in cyclic AMP concentration. Since cyclic AMP can mediate alterations in phosphatidyl inositol metabolism (23) and histone phosphorylation (24) in other tissues it would be attractive to assume that these metabolic alterations are a direct consequence of the increase in cyclic AMP concentration. However, parallel metabolic studies with the same cell preparations are not yet available and the extent (if any) to which cyclic AMP acts as an intracellular messenger for PHA during the early phases of the cellular response is still uncertain. More thorough evaluation of this question will require studies on early effects of prostaglandins, aminophylline, and isoproterenol on lymphocyte metabolism as well as an investigation of the metabolic effects of cyclic AMP and dibutyryl cyclic AMP on intact lymphocytes and subcellular lymphocyte fractions. However, work in progress already indicates that some of the early metabolic effects of PHA are *not* readily ascribable to cyclic AMP. Thus PHA stimulates an early (30–90 min) increase in the uptake of [32]PO$_4$ into lymphocyte phospholipid, but dibutyryl cyclic AMP, aminophylline, and isoproterenol do not. It would therefore seem probable that other mediators are responsible for the rapid increase in phospholipid synthesis; moreover, from the results in the companion paper, although cyclic AMP does initiate lymphocyte transformation, it is considerably less effective than PHA in this regard.

The basis for the late decrease in cyclic AMP levels in PHA-stimulated lymphocytes is not known. Leakage of cyclic AMP through the cell membrane has been demonstrated in *E. coli* (25). In a single experiment cyclic AMP could be demonstrated in the medium 6 and 24 hr after PHA stimulation. Further investigation of this possibility is needed since phosphodiesterase in the fetal calf serum might destroy cyclic AMP as soon as it leaves the cell. A substantial fall in available ATP, the substrate in the adenyl cyclase reaction, seems unlikely but has not been specifically excluded. Quite conceivably there is a feedback control mechanism which ultimately produces a reduction in adenyl cyclase activity or an increase in cyclic mononucleotide phosphodiesterase activity. Still another contributing factor might be the enzymatic degradation of PHA molecules attached to the lymphocyte membrane so that adenyl cyclase is no longer stimulated. In dose response curves of lymphoid cells to PHA, high doses of the mitogen often result in decreased DNA synthesis (26). High PHA concentrations would favor the availability of a continuing supply of undegraded PHA. Under these circumstances adenyl cyclase stimulation might continue at a high level preventing the late fall in cyclic AMP levels. Judging from the results

in the companion paper *persistently* elevated cyclic AMP levels would be expected to interfere with DNA synthesis and cellular replication. Similar arguments could be used to explain the poor immunogenicity of poly D amino acids (27) and the occurrence of immunological tolerance in vivo in response to very high doses of immunogen. In both circumstances persistence or replenishment of intact antigen on the lymphocyte membrane might interfere with the late fall in cyclic AMP levels resulting in arrest of the cellular response. Experiments designed to test adenyl cyclase responsiveness to additional amounts of PHA or to isoproterenol at various times after PHA stimulation should help to settle some of these questions.

The requirement for high concentrations of PHA to obtain maximal early increases in lymphocyte cyclic AMP levels deserves comment. In lymphocyte transformation experiments these levels of PHA would produce less marked stimulation of DNA synthesis than intermediate PHA concentrations. On the other hand Kay and Cooper (22) have noted that high PHA concentrations are needed for *maximal early* (within 1–4 hr) stimulation of RNA synthesis and could not demonstrate a PHA excess effect. Our own observations on the effect of PHA on early synthesis (after 1 hr) are in accord with theirs and suggest a general correlation between RNA synthesis and increases in cyclic AMP concentration.

Human lymphocytes undergoing a maximal PHA response are a synchronized population of cells and as such provide one of the most useful experimental models for the study of the mammalian cell cycle. Evaluation of the G_1 phase of the mammalian cell cycle has presented particular difficulties because of its variable duration and the relative absence of suitable markers to provide a basis for further subdivision. It will be interesting to see if the fall in cyclic AMP concentration observable after several hours in PHA-stimulated lymphocytes is also present during some portion of the G_1 phase of synchronized cells of other types.

In view of the widespread metabolic effects of cyclic AMP in the cell, one might anticipate that persistent alterations of cyclic AMP levels might interfere with the complex machinery of lymphocyte transformation. This subject is considered in detail in the next paper.

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