Effect of Cyclic 3′,5′-Adenosine Monophosphate and Theophylline on Lymphocyte Transformation (34690)

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Shortly after polypeptide hormones act on target tissues, the tissues undergo a series of morphologic changes that can be related to the rearrangement of vacuolar systems. Thus thyroid-stimulating hormone induces endocytosis of thyroglobulin, parathyroid hormone induces the bulk exocytosis of lysosomal hydrolases and hydrogen ions from osteoclasts, glucagon stimulates the formation of autophagic vacuoles in liver, and melanophore-stimulating hormone induces the rearrangement of melanosomes in skin (1, 2). Since cyclic 3′, 5′-adenosine monophosphate (cA MP) has been implicated as the "second messenger" for these effects of hormones upon target tissues, it appeared possible that adenine nucleotides might regulate other functions of the vacuolar system in various cell types. Evidence suggesting that increments in the level of intracellular cAMP may inhibit granule flow and merger has been obtained by Lichtenstein and Margolis (3), who found that both theophylline and dibutyryl cyclic AMP (dcAMP) inhibited the release of histamine from leukocytes. Indeed, further experiments with peripheral blood leukocytes showed that these compounds inhibited both the antigenic release of histamine and the phagocytic release of β-glucuronidase (4).

Since redistribution of acid hydrolases follows enhanced endocytosis in cultures of human lymphocytes exposed to phytohemagglutinin (PHA) (5, 6), these findings suggested that the effects of PHA on lymphocytes might also be mediated by cyclic AMP. We have therefore studied the effect of cyclic AMP, its dibutyryl derivative and theophylline (an inhibitor of cyclic AMP degradation) (1, 2) upon the stimulation of lymphocytes.

Materials and Methods. Separation and culturing of cells. Human peripheral blood lymphocytes were obtained by methods previously described. Briefly, heparinized blood was allowed to sediment spontaneously at 37°, the supernatant plasma was removed, treated with adenosine diphosphate to aggregate platelets and the filtrate was passed through a prewarmed nylon fiber column at 37°. The lymphocyte suspension was then washed 3 times, suspended in complete media [minimal essential media Spinner (MEMS), 20% heat-inactivated fetal calf serum (FCS), 1%, 200 m*M* glutamine, 100 units of penicillin, and 100 μg of streptomycin/ml] and usually allowed to stand overnight at room temperature at a cell concentration of 5–10 × 10^6/ml.

Culturing procedures. Cell suspensions were adjusted to concentrations of 7–9 × 10^5 cells/ml and 4 ml were dispensed into 15-ml screw-top glass culture tubes and incubated in a water bath at 37° for varying times.

Cell counts and viability. White blood cell counts were determined by standard methods using a 1:20 dilution of cell suspension with 0.6% acetic acid containing methylene blue; cell counts from individual cultures were performed using a 1:1 dilution. Cell viability was determined after 5-min incubation of a 1:1 dilution of cell suspension with erythrocin B in phosphate-buffered saline (final concentration of 0.067 gms./100 ml).

Determination of rates of macromolecular synthesis. DNA-incorporation of ^{14}C-thymidine (sp act ≅ 53.8 mCi/mmoles) into TCA-precipitable material was used to measure rates of DNA synthesis. 0.3–0.45 μCi (as indicated) were added to 4-ml cultures for 24-hr periods, the cultures were then

cooled to 4° and centrifuged at 1200 rpm in an International PR 2 centrifuge. The cell pellet was washed twice in ice-cold normal saline, precipitated with ice-cold 15% trichloroacetic acid (TCA) for 1 hr, centrifuged at 2000 rpm and washed twice with 5-ml aliquots of 15% ice-cold TCA. The precipitate was dissolved in 0.5 ml of hyamine by heating at 56° for 0.5–1 hr, transferred with three 5-ml aliquots of PPO-POPOP in toluene to counting vials. Cultures to which ^{14}C-thymidine was added just prior to harvest contained less than 30–50 cpm acid-insoluble counts.

RNA. Incorporation of ^{14}C-uridine (sp act $\cong$ 54.5 mCi/mmole) into acid precipitable counts was used as a measure of RNA synthesis. One μCi/4-ml culture was added for the last 2 or 3 hr, as indicated, prior to harvesting. The same harvest procedure as for DNA was utilized.

Protein. Two μCi of ^{14}C leucine (sp act $\cong$ 160 mCi/mmole)/4-ml culture were added for 3–4 hr (as indicated) prior to harvest. The cells were then centrifuged at 1200 rpm at 4°, washed twice with cold normal saline and 0.5 ml of 1 *N* NaOH was added at 56° for 5 min. Two ml of cold 15% TCA was added and the precipitate was allowed to stand overnight at 4°, centrifuged at 2000 rpm for 15 min, and the precipitate was washed 3 times with 5-ml aliquots of 10% TCA. The precipitate was dissolved and counted as described above.

Morphology. The percentage of cells which were morphologically altered (% transformation) was determined by the method of Hirschhorn *et al.* (6).

Results. When added 15 min prior to PHA, cyclic AMP, dcAMP, and theophylline (Schwarz Boichemicals) each inhibited PHA-induced incorporation of ^{14}C-thymidine into DNA (Fig. 1). Inhibition was evident when rates of DNA synthesis were determined both 24–48 hr and 48–64 hr after addition of PHA and the tested compound. Dibutyryl cAMP was the most effective compound, inhibiting significantly between 10^{-4} to 10^{-5} *M*; theophylline was next, inhibiting between 10^{-3} to 10^{-4} *M* and cyclic

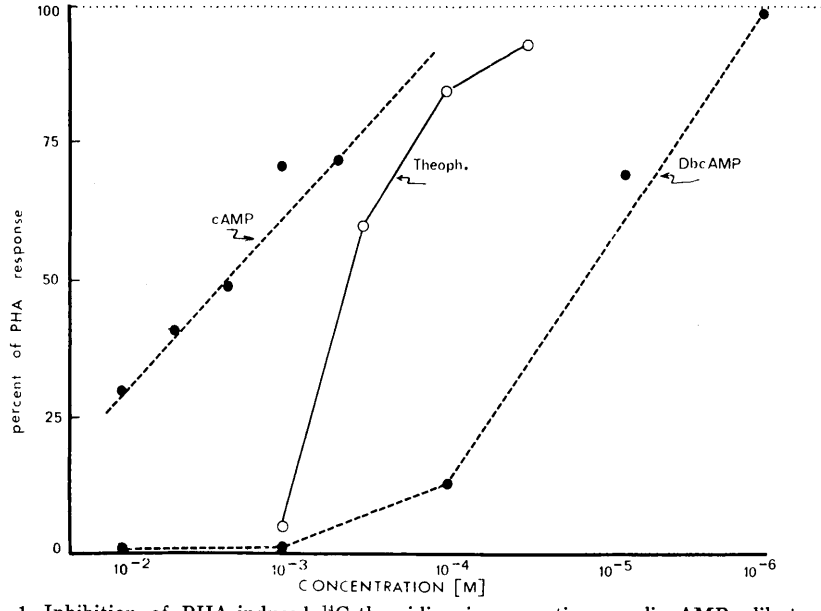

FIG. 1. Inhibition of PHA-induced ^{14}C-thymidine incorporation: cyclic AMP, dibutyryl cyclic AMP and theophylline were added to peripheral blood lymphocytes 15 min prior to the addition of PHA. ^{14}C-thymidine (see text) was added 24 hr later and incorporation was determined 48 hr after onset of culture. Values are reported as percentage of cpm incorporated by the PHA-stimulated cultures alone and represent averages of four replicate cultures.

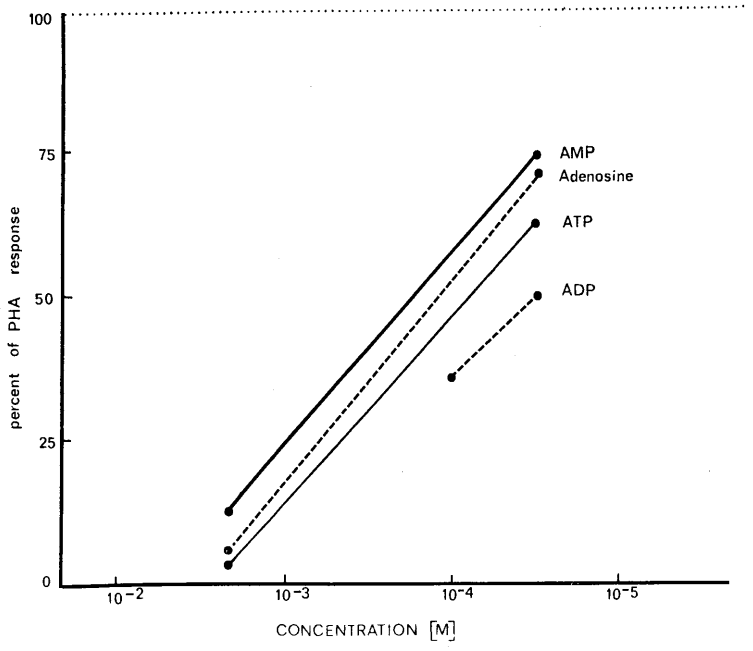

FIG. 2. Inhibition of PHA-induced ^{14}C-thymidine incorporation: adenosine, AMP, ATP, and ADP were added to cultured peripheral blood lymphocytes. Conditions as described in legend for Fig. 1.

AMP was the least effective, requiring concentrations of 3.33×10^{-3} *M* for 50% inhibition. This order of inhibition may reflect the well-documented properties of dcAMP both to exceed cAMP in access to the interior of the cells and to be degraded less rapidly by phosphodiesterase (1).

In order to evaluate the specificity of inhibition, adenosine, 5′-AMP, ATP, and ADP were also tested. Each of these compounds, which have not been reported to affect the levels of cyclic AMP, were nonetheless effective in inhibiting PHA-induced increments in DNA synthesis (Fig. 2) at concentrations between 5×10^{-4} to 5×10^{-5} *M*.

Additionally, combination of theophylline with cyclic AMP or adenosine did not further enhance the inhibition produced by either of these compounds alone. Cyclic AMP (6.67×10^{-4} *M*) inhibited 35.5% of ^{14}C-thymidine incorporation alone and 31.2% in the presence of 10^{-4} *M* theophylline (a dose which alone caused 18.1% inhibition). To determine if these compounds acted chiefly by interfering with DNA synthesis rather than by inhibiting lymphocyte stimulation *per se*, protein synthesis, which proceeds before the onset of significant DNA synthesis in this system (7), was measured 24–48 hr after PHA stimulation. Each of the compounds which inhibited PHA-induced increments of DNA synthesis also inhibited protein synthesis (Table I).

However, when cyclic AMP was added to lymphocytes *not* stimulated by PHA a biphasic response was observed. Thus, although inhibition of incorporation of ^{14}C-thymidine

TABLE I. Effect of Adenine Derivatives on Protein Synthesis by Stimulated Lymphocytes.[a]

	(*M*)	(cpm)	PHA (%)
PHA		7626	100
PHA + cyclic AMP,	3.3×10^{-3}	6181	81
PHA + ADP	3.3×10^{-3}	5337	70
PHA + ATP	3.3×10^{-3}	2652	35
PHA + adenosine	3.3×10^{-3}	2170	28
Control		1793	24

[a] Peripheral blood lymphocytes were cultured for 28 hr with PHA and the indicated compounds. ^{14}C-leucine (see text) was added during the last 4 hr of culture and TCA precipitable cpm were determined.

into DNA was observed at 3.3×10^{-3} *M* cAMP, there was a small but reproducible *rise* in the rate of incorporation at lower concentrations of cAMP. No such increases were found when adenosine, AMP or ATP were added to unstimulated cells (Table II).

In order to determine if this increase reflected an increase in total stimulated lymphocytes in contrast to an isolated alteration in thymidine metabolism, uridine incorporation (presumably into RNA) was determined 96 hr after the onset of culture; again there was an almost twofold increase in rates of incorporation, as seen in Table III. Smaller increments (126% at 6.7×10^{-4} *M* cAMP) were also observed at 24–48 hr after the onset of culture. In contrast, as seen in Table III, addition of 5′-AMP had no such stimulatory effect upon uridine incorporation. However, neither theophylline nor dibutyryl cAMP, at concentrations lower than those resulting in inhibition, had a stimulatory effect. Thus, theophylline in concentrations ranging from 10^{-4} to 10^{-8} *M* and dibutyryl cAMP in concentrations ranging from 5×10^{-5} to 10^{-7} *M* did not result in stimulation.

TABLE II. ^{14}C-Thymidine Incorporation by Unstimulated Lymphocytes: Effect of Cyclic AMP, Adenosine, AMP, and ATP.[a]

	% of control		
Conc (*M*) (hr):	72–96	96–120	144–168
Cyclic AMP			
6.7×10^{-4}	(a) 163	(c) 139	(e) 195
	(b) 164	(d) 161	
6.7×10^{-5}	—	(c) 151	(e) 182
3.3×10^{-5}	—	(c) 151	(e) 147
6.7×10^{-6}	—	(c) 111	(e) 113
6.7×10^{-7}	—	(c) 106	(e) 88
AMP, 6.7×10^{-4}	(b) 102	(d) 111	—
ATP, 6.7×10^{-4}	(b) 96	(d) 104	—
Adenosine			
6.7×10^{-4}	(b) 91	(d) 77	—

[a] Peripheral blood lymphocytes were cultured alone or with addition of the indicated compound at 0 time. Rates of DNA synthesis were measured by labeling with ^{14}C-thymidine during the time intervals indicated. Results are the average of four replicate cultures and are expressed as percentage increase over control rates. Control cpm for experiment (a) = 539; (b) = 1868; (c) = 2926; (d) = 4400; (e) = 2666.

TABLE III. ^{14}C-Uridine Incorporation by Unstimulated Lymphocytes: Effect of Cyclic AMP and 5′-AMP.[a]

	% of control	
Conc (*M*)	Cyclic AMP	5′-AMP
6.7×10^{-4}	156	96
3.3×10^{-4}	186	124
6.7×10^{-5}	162	98
3.3×10^{-5}	151	90
6.7×10^{-6}	119	77

[a] Peripheral blood lymphocytes were cultured alone or with the addition of the indicated components. Rates of RNA synthesis were measured by labeling with ^{14}C-uridine from 96 to 100 hr after the culture was started. Results are the average of triplicate determinations and are expressed as percentage of increase over control rates.

Discussion. There are at least three possible explanations for the observed effects of cAMP, dibutyryl cAMP, and theophylline on lymphocyte stimulation: (a) each of the tested compounds may have effects upon overall metabolic pathways (*e.g.*, depression of glucose uptake) which is manifested by inhibition of the metabolic events following PHA stimulation, (b) the rearrangement of intracellular vacuoles and membranes induced by PHA may be mediated by a system sensitive to the intracellular level of cAMP and such rearrangement is necessary for the further events following stimulation, or (c) cAMP, its dibutyryl derivative, or theophylline may preemptively induce changes in the cell at a locus which is also acted upon by PHA. Since, at low concentrations, cAMP, (but not adenosine or its derivaties) *stimulates* lymphocytes in a manner analogous to PHA itself, the latter possibility would appear plausible. However, it is quite obvious that changes in pool sizes of available nucleotides, effects upon respiration, or other factors could also account for these findings.

At first glance, these data might provide support for the hypothesis that, shortly after PHA stimulation, lymphocytes undergo a rearrangement of their vacuolar system, and

that this process is regulated by the level, within cells, of 3′,5′-adenosine monophosphate. By analogy with systems such as antigenic histamine release by mast cells (3, 4), and insulin release from pancreatic islet cells (8), it might be suggested that cyclic AMP (or the other compounds tested) regulate the state of aggregation of microtubules which control the flow of intracellular vacuoles or their contents. In both of these cell types, mast cells and islet cells, procedures which dissolve microtubules (*e.g.*, colchicine treatment) have effects that can be duplicated by cAMP, its dibutyryl derivative, or methyl xanthines (8). Indeed microtubular proteins contain specific binding sites for nucleotides (9). But any such simplistic account cannot readily explain why adenosine or its derivatives should also have prevented lymphocyte stimulation by PHA. The effects of these compounds cannot easily be reduced to some common property and their mode of action remains unclear at present. A definitive explanation of our observations can only follow the direct assay of adenyl cyclase activity and of alterations in the intracellular levels of cAMP in control and in PHA-stimulated cells.

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