

## Guanosine 3':5'-Cyclic Monophosphate: A Possible Intracellular Mediator of Mitogenic Influences in Lymphocytes

(phytohemagglutinin/concanavalin A/cyclic AMP/proliferation)

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**ABSTRACT** Adenosine 3':5'-cyclic monophosphate (cyclic AMP) has been shown to have an antimitotic role in various cell types, and it has been hypothesized that a decrease of cyclic AMP concentration in the cell initiates or permits cell division. This hypothesis has been evaluated with respect to clonal proliferation of lymphocytes. Two potent mitogenic agents, phytohemagglutinin and concanavalin A, which induce thymic-dependent lymphocytes to undergo clonal proliferation, were examined for their ability to initiate proliferation and to alter the concentrations of cyclic AMP and guanosine 3':5'-cyclic monophosphate (cyclic GMP) in purified human peripheral blood-lymphocytes. Optimal mitogenic concentrations of phytohemagglutinin and concanavalin A produced 10- to 50-fold increases in the concentration of lymphocyte cyclic GMP within the first 20 min of exposure to the mitogens. No changes were seen in the concentration of cyclic AMP after stimulation with either mitogen in purified form. Increases of less than 2-fold in the concentration of lymphocyte cyclic AMP observed with a less purified preparation of phytohemagglutinin could be attributed to the agglutinating rather than the mitogenic properties of the mitogen. A revised hypothesis is presented in which a temporally discrete rise in lymphocyte cyclic GMP concentration is viewed as an active signal to induce proliferation, while the elevation of cyclic AMP concentration in these cells is viewed as a regulatory influence that limits or inhibits mitogenic action.

The lymphocyte, a cell whose normal functions include clonal proliferation after stimulation by antigen, provides a model for studying the biochemical events related to initiation of cellular division. Mitogens, such as phytohemagglutinin (PHA) and concanavalin A (Con A), have been used to induce clonal proliferation in lymphocytes, particularly those derived from the thymus. Several experiments (1-6) have been performed in which the relationship between cyclic AMP concentration and mitogen-induced lymphocyte division has been investigated. The results indicate (a) that cyclic AMP itself does not significantly stimulate division of lymphocytes in the resting phase ("G<sub>0</sub>" or "G<sub>1</sub>" phase) of the cell cycle (1), (b) that increases in cyclic AMP concentration resulting from stimulation by hormones and polynucleotides do not induce lymphocytes to divide (2, 3), and (c) that agents that promote increases in lymphocyte cyclic AMP concentration inhibit mitogen-induced clonal proliferation (4-6).

Abbreviations: PHA, phytohemagglutinin; Con A, concanavalin A.

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In contrast to the foregoing is the observation that high concentrations of PHA can induce small increases in the amounts of cyclic AMP in lymphocytes (4). These observations have been interpreted as support for the concept that PHA exerts its mitogenic effects through an increase in cellular cyclic AMP concentration. Support for the observation that PHA can induce increases in lymphocyte cyclic AMP concentrations have been conflicting (3, 7, 8). Since the relationship of cyclic AMP concentration to the mitogenic action of PHA is still unresolved, and recent evidence has been offered implicating guanosine cyclic 3':5'-monophosphate (cyclic GMP) in the mediation of events antagonistic to those promoted by cyclic AMP in certain biological systems (9, 10§), changes in concentrations of cyclic AMP and cyclic GMP during the early stages of lymphocyte stimulation by mitogens were investigated.

In this report, evidence is presented implicating cyclic GMP as an intracellular effector in mitogenic action. These observations add further support to the hypothesis that cyclic GMP and cyclic AMP promote antagonistic cellular events and introduce the concept that the elevation of the concentration of cellular cyclic GMP may be involved in the initiation of events leading to cell division.

### MATERIAL AND METHODS

Human peripheral-blood lymphocytes were obtained in a pure state (>95%) by differential centrifugation (2) and Ficoll-Hypaque gradient separation (11).

For cyclic nucleotide measurements,  $2.5 \times 10^7$  lymphocytes were incubated in 1-ml aliquots of Hank's Balanced Salt Solution with the experimental compounds for the times indicated, and the reactions were terminated by the addition of an equal volume of 20% trichloroacetic acid.

For the estimation of DNA synthesis,  $10^6$  lymphocytes were incubated in culture medium for 24 and 72 hr, and the amount of tritiated thymidine incorporated into acid-insoluble material was determined (2).

§ In ref. 10, it is demonstrated that cholinergically mediated events in heart, lung, uterus, brain, and lymphocytes that are antagonistic to those promoted by an elevation of cyclic AMP concentration are associated with elevation in the concentration of tissue cyclic GMP. In addition, the cholinergic-like effects of oxytocin and serotonin on uterine contractility and the hypocalcemic action of calcitonin are similarly associated with elevated concentrations of cyclic GMP.

Cyclic AMP was determined by the method of Gilman (12), and cyclic GMP by the procedure of Goldberg and O'Toole (13).

Preparations of experimental compounds obtained from commercial sources were freshly made. PHA-M (Difco Laboratories, Detroit, Mich.) is a semipurified preparation of phytohemagglutinin derived from the red kidney bean (*Phaseolus vulgaris*) that contains both mitogenic and hemagglutinating characteristics. PHA-MR69 (Wallace Diagnostic Reagents; Burroughs Wellcome Co., Research Triangle Park, N.C.) is a purified fraction of PHA that is highly mitogenic and has 1:100 of the agglutinating properties of PHA-M. Another phytomitogen derived from the jack bean (*Canavalia ensiformis*), concanavalin-A, was obtained from Calbiochem (La Jolla, Calif.). Each of the mitogens was tested over a wide range of concentrations for effectiveness in inducing lymphocyte DNA synthesis by the method described; optimal mitogenic doses were used.

Optimal mitogenic concentrations were evaluated for their agglutinating potential by the method of Yachnin *et al.* (14). The incubations were in tissue culture chambers mounted on glass microslides (Lab-Tek Products, Westmont, Ill.), and were evaluated by an inverted phase contrast microscope. Agglutination was estimated on a scale of 0 to + + + + (14).

## RESULTS

Phytohemagglutinin has been shown to be separable, upon purification, into two fractions, one possessing primarily mitogenic activity and another possessing primarily agglutinating properties (15). A less highly purified preparation of PHA (PHA-M) containing both agglutinating and mitogenic properties was used in the first series of experiments. The effects of PHA-M on concentrations of cyclic nucleotides in lymphocytes at various times during the first 30 min of

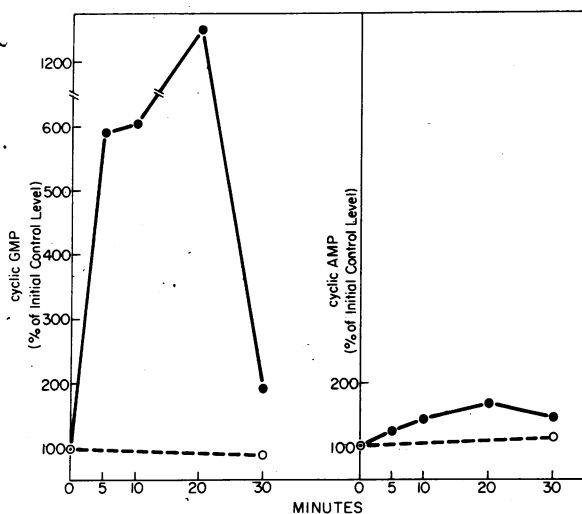


FIG. 1. The effect of a partially purified preparation of phytohemagglutinin (PHA-M) on lymphocyte cyclic GMP (left) and cyclic AMP (right) concentrations. Human peripheral-blood lymphocytes ( $2.5 \times 10^7$  cells) were incubated in 1 ml of Hank's Balanced Salt Solution with (●—●), or without (○—○) PHA-M (250  $\mu$ g/ml) for the times indicated before the addition of an equal volume of 20% trichloroacetic acid. Cyclic GMP was measured by the method of Goldberg and O'Toole (13), and cyclic AMP by the method of Gilman (12).

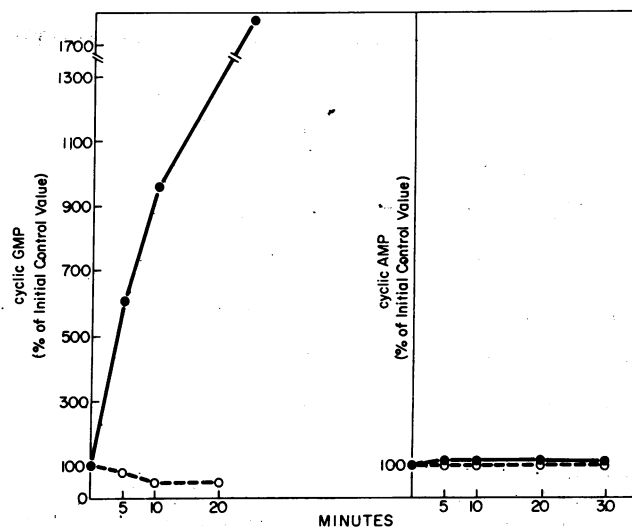


FIG. 2. The effect of a highly purified preparation of phytohemagglutinin PHA-MR69 (3  $\mu$ g/ml; ●—●) on lymphocyte cyclic GMP (left) and cyclic AMP (right) concentrations. The incubations and assays were performed as described in Fig. 1. ○—○, control.

exposure to the mitogen are shown in Fig. 1. The concentrations of cyclic GMP and cyclic AMP in cells not treated with the mitogen ranged between 0.09 to 0.15 and 3.04 to 4.42 pmol per  $10^6$  cells, respectively, during the 30 min incubation. Treatment with PHA-M resulted in increases in the concentrations of both cyclic GMP and cyclic AMP. The concentration of cyclic GMP increased within 5 min to a value six times higher than that of the initial control, and by 20 min, to a peak concentration that was about 12-fold higher than the control. In contrast, the maximum increase in cyclic AMP concentration, which also occurred at 20 min, was less than 2-fold (i.e., 70%).

The effects of a more highly purified mitogenic form of phytohemagglutinin (PHA-MR69) on concentrations of lymphocyte cyclic nucleotides are shown in Fig. 2. Cyclic GMP concentrations increased about 6-, 10-, and 17-fold at 5, 10, and 20 min, respectively, after addition of the mitogen. The concentrations of lymphocyte cyclic AMP, however, were unaffected by PHA-MR69 (Fig. 2).

The data in Figs. 1 and 2, when viewed collectively, indicate that the mitogenic properties of PHA are associated with greater than 10-fold increases in concentrations of cellular cyclic GMP. It appears that properties of PHA other than those related to mitogenic action are associated with the relatively small increases in cyclic AMP concentration.

The effects of another mitogen, concanavalin A (Con A), on concentrations of cyclic GMP and cyclic AMP in lymphocytes at 0, 10, and 20 min of incubation are shown in Table 1. Con A produced a pattern of cyclic nucleotide response similar to that seen with PHA-MR69. Increases in the concentrations of cyclic GMP at 10 and 20 min were about 15- and 50-fold, respectively, relative to the respective control values. No changes in lymphocyte cyclic AMP concentrations were observed after stimulation by Con A. Therefore, two different phytomitogens can be shown to have similar effects to promote striking increases in lymphocyte cyclic GMP concentrations.

TABLE 1. The effects of Con A on the concentrations of cyclic GMP and cyclic AMP in human peripheral-blood lymphocytes

Minutes of incubation	Cyclic GMP			Cyclic AMP		
	0	10	20	0	10	20
Control		0.18	0.12		4.90	5.71
	0.22			7.02		
Con A*		2.82	5.77		5.03	5.88

\* Con A concentration was 25  $\mu\text{g/ml}$ .

The comparative effects of the three mitogens on DNA synthesis in lymphocytes (Table 2) were measured at 24 hr, the time calculated to include only the first division cycle, and at 72 hr, the time of maximum DNA synthesis. A comparison of the transformation indices at 24 and 72 hr serves to underscore the exponential nature of the expansion involved in clonal proliferation in these lymphocyte cultures. At optimum mitogenic concentrations, the three mitogens produced equivalent increases in DNA synthesis at both time periods. These data indicate that increases in cyclic GMP concentration ranging from 12- to 50-fold are associated with maximum DNA synthesis. The data in Table 2 also confirm that PHA-M is markedly agglutinating while PHA-MR69 at the concentrations used has minimal but detectable agglutinating activity. Con A has minimal agglutinating activity comparable to that of PHA-MR69.

#### DISCUSSION

The data presented indicate that a marked increase in cyclic GMP concentration is associated with the initiation of mitogen-induced proliferation of peripheral blood lymphocytes.

Minor alterations in the concentration of lymphocyte cyclic AMP were observed with stimulation by PHA-M but not with stimulation by equally mitogenic but nonagglutinating PHA-MR69 or Con A. The relatively small increases in cyclic AMP concentration, therefore, can be attributed to the agglutinating properties of PHA-M. It is possible, then, to conclude that an elevation of cyclic AMP concentration is not a significant event in the initiation of proliferation of lymphocytes and to concur with the conclusion that cyclic AMP may have an antimitotic action when increases of an appropriate magnitude occur concomitantly with mitogen stimulation (4-6). However, once clonal proliferation has been initiated in lymphocyte cultures, the influence of cyclic AMP on proliferation may change depending on the period of the cell cycle. Evidence (6) indicates that a pharmacologically-induced increase in the concentration of lymphocyte cyclic AMP is considerably less effective in inhibiting subsequent proliferation initiated 1 hr or later after exposure to a mitogen. Furthermore, when lymphocytes that have entered the synthetic phase ("S" phase) of the cell cycle are stimulated by hormonal agents, which increase the concentration of cellular cyclic AMP, enhanced metabolism and DNA synthesis (5, 16) and hastened mitoses (17) may result.

What then can be said of the marked elevation in cyclic GMP content in relation to the induction of cell division? The increase in the concentration of cellular cyclic GMP during the first 20 min of PHA stimulation is temporally associated with, and has similar characteristics to, the binding of PHA to lymphocytes, a process shown to be complete within

TABLE 2. The effects of optimal concentrations of PHA-M, PHA-MR69, and Con A on lymphocyte DNA synthesis and agglutination

Mitogen	Optimal dose ( $\mu\text{g/ml}$ )	Transformation index*		Agglutination index†
		24 hr	72 hr	
PHA-M	250	2.1	274.4	++++
PHA-MR69	3	2.1	280.1	±
Con A	25	2.1	280.0	±

\* Transformation index is calculated as cpm of tritiated thymidine incorporated into trichloroacetic acid-insoluble material per mitogen-stimulated control culture.

† Agglutination index indicates degree of aggregation based on a 0 to +++++ scale.

30 min (18). A comparison of the rate of binding of PHA with the time-course of cyclic GMP elevation induced by PHA-M (Fig. 1) suggests that cyclic GMP is generated only during the rapid binding of PHA to the cell membrane. A unique feature of PHA interaction with lymphocytes is that a short exposure of cells to this mitogen (followed by multiple washes) is sufficient to initiate clonal proliferation (2). PHA once introduced, produces multiple generations of proliferating daughter lymphocytes that apparently have little further requirement for stimulation by PHA and seem to lack a receptor for it (19). The profile of the changes in concentration of cyclic GMP appears to conform to the general characteristics of this "trigger type" of signal of mitogen action.

It has recently been shown that internalization of PHA by the cell is not a requisite for the induction of transformation (20), which suggests that the final action of PHA, like many hormones, is with the plasma membrane. This observation makes the idea compelling that a "second messenger" is required for the intracellular expression of the mitogenic signal. Our studies would indicate that cyclic GMP may qualify as the intracellular messenger for expression of this particular signal.

Of equal importance in establishing cyclic GMP as an intracellular effector in the expression of the mitogenic signal is the identification of a site of cyclic GMP action that would serve to initiate the sequence of events leading to cell division. Some insight into this possible site of action may be gained from an analysis of the alterations known to occur in lymphocyte nuclei within minutes after stimulation of whole lymphocytes by PHA. These changes include, in the sequence observed: increased histone acetylation, increased phosphorylation of nuclear proteins, and increased nuclear RNA synthesis (21). These alterations are believed to be associated with transcription of DNA and can be envisioned to relate to subsequent cell division. Preliminary observations (J. Hadden and G. Meetz, unpublished observations) indicate that cyclic GMP in the concentration range of 0.1-1 nM enhances RNA synthesis in isolated lymphocyte nuclei. The nature of the mechanism involved is unknown; however, the hypothesis can be put forth at this time that, for the mitogenic signal, cyclic GMP fulfills a role as membrane to nucleus messenger.

¶The "second messenger" concept was put forth by Dr. E. W. Sutherland to describe the role played by cyclic AMP as an intracellular mediator of the actions of hormones that interact with the cell membrane.

Various other events have been observed within the first 30 min of PHA action, including enhanced membrane transport of leucine, uridine, choline, and glucose (22), enhanced sodium and potassium exchange-pump activity (23), and increased incorporation of phosphate into membrane phosphatidylinositol as well as into other lipids (24). The relationship of these concomitant changes in membrane transport and metabolism to mitogenic action, changes in concentration of cyclic GMP, or nuclear events remains to be established.

An association of changes in endogenous concentration of cyclic GMP to proliferation of other cell types has not been described; however, evidence has accumulated that supports a role for cyclic AMP as an inhibitor of proliferation in other cell types. This evidence derives primarily from experiments in which the division of cells grown in culture has been shown to be inhibited by exogenous cyclic AMP or by exposure to agents that induce increases in intracellular cyclic AMP concentration. This view of the antiproliferative action of cyclic AMP has been clarified by the recent observations of Otten *et al.* (25) and Sheppard (26) that indicate that high concentrations of intracellular cyclic AMP are associated with the limitation of "normal" proliferation and acquisition of contact inhibition, whereas low cellular concentrations are related to "malignant" transformation and uncontrolled proliferation without contact inhibition. These studies imply that biochemical events involving high intracellular concentration of cyclic AMP promote cellular differentiation and that lowering of the concentration of cyclic AMP allows antagonistic events associated with cell proliferation to proceed. We have not found an association between lowering of cyclic AMP concentration and mitogenic action. From our observations that agents inducing lymphocyte proliferation produce increases in concentration of cellular cyclic GMP (an event that can be tentatively related to biochemical events involved in nuclear activation) viewed in the light of the reciprocal events recently attributed to cyclic AMP and cyclic GMP (9, 10), we suggest a revised working hypothesis concerning the regulation of cell division: an increase in the concentration of cellular cyclic GMP may represent at least one of the active signals that may induce cell division, while the elevation of the concentration of cyclic AMP or sustained high steady-state concentration may limit or inhibit the initiation of cell division.

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