The Possible Mediation by Cyclic AMP of the Stimulation of Thymocyte Proliferation by Cyclic GMP[1] (35598)

J. F. WHITFIELD, J. P. MACMANUS, D. J. FRANKS,[2] D. J. GILLAN, AND T. YOUDALE
(Introduced by Helen J. Morton)
Division of Biology, National Research Council of Canada, Ottawa K1A OR6, Canada

Low concentrations (10^{-8} to 10^{-6} *M*) of cyclic AMP stimulate the proliferation of rat thymic lymphoblasts *in vitro* and *in vivo*, as well as the proliferation of bone marrow cells *in vivo* (8, 10, 14, 17, 18). Furthermore, this cyclic nucleotide probably mediates the mitogenic actions of a variety of hormones such as epinephrine, growth hormone, parathyroid hormone, and vasopressin on thymocyte proliferation *in vitro* (7, 9–11, 17, 18). The mitogenic levels of cyclic AMP also induce the transformation of small, mitotically inactive blood lymphocytes into proliferating lymphoblasts and the cyclic nucleotide probably mediates the transforming action of phytohemagglutinin on these cells (2, 15). Finally, very low concentrations (10^{-9} to 10^{-6} *M*) of exogenous cyclic AMP also stimulate the mitotic activity of other cell types such as strain HeLa human cells cultivated *in vitro* (13).

In contrast to the large body of information on the various physiological actions of cyclic AMP, almost nothing is known about the biological role of cyclic guanosine 3′, 5′-monophosphate (cyclic GMP). Since cyclic GMP can mimic some of the actions of cyclic AMP (4, 12), we suspected that it might also affect thymocyte proliferation. In the present communication, we show that cyclic GMP has two distinct mitogenic actions both of which might be mediated by cyclic AMP.

Materials and Methods. Thymocytes from the thymus glands of 180–200 g, albino, male (specific-pathogen-free) rats were suspended in a complex serum-free tissue culture medium, MAC-1 (8, 10, 11, 17, 18). The 5-ml cell suspensions (containing about 2 × 10^8 cells/ml) were incubated at 37° while revolving in (25 × 150 mm) roller tubes at 40 rpm. Since only 10 to 20% of the cells in a thymocyte population are actively proliferating, or at least immediately capable of proliferating (17, 18), colchicine (0.06 m*M*) was added to the thymocyte suspensions and the level of cell proliferation was estimated from the subsequent progressive accumulation in the numerically constant population of cells having the characteristic morphology of colchicine metaphase. To determine the proportion of metaphase cells, we used a much improved version of the procedure described by Whitfield *et al.* (16). After various times of incubation, 1 drop of cell suspension was placed on a slide and mixed with 3 drops of a solution consisting of 9 vol of phosphate-buffered 10% formalin and 1 vol of glacial acetic acid. The mixture was allowed to dry slowly in air at room temperature. The fixed cells were then stained with Harris' hematoxylin, dehydrated, and mounted in a synthetic mounting medium. The full details of these procedures and detailed discussions of the relevance of observations made under these conditions to the control of cell proliferation in the thymus gland *in vivo* have been presented in several recent publications (7–11, 14, 17, 18).

Cyclic GMP-induced changes in the cellular cyclic AMP content were determined using a method which has already been fully described (11). Briefly, however, the cellular cyclic AMP-yielding adenosine triphosphate (ATP) pool was first labeled by incubating thymocytes for 2 hr in medium containing ^{3}H-adenine (5, 6, 11). The labeled cells were removed from the ^{3}H-adenine-containing medium (11), exposed to various concentra-

[1] Issued as NRCC No. 11986.

[2] NRCC Postdoctorate Fellow 1970–71.

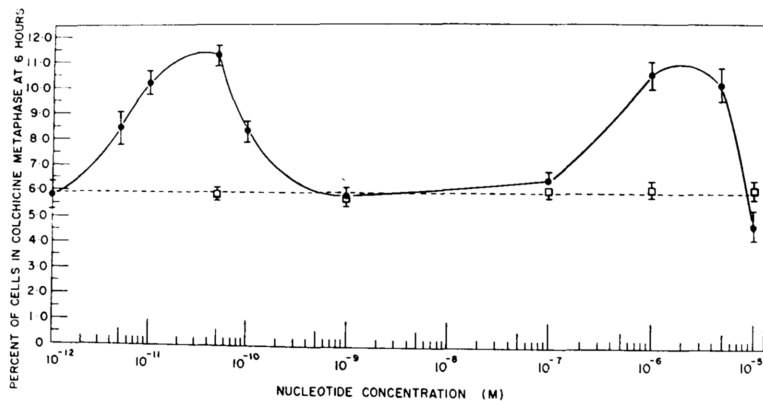

FIG. 1. The effects of various concentrations of cyclic guanosine 3′,5′-monophosphate (cyclic GMP) on thymocyte proliferation *in vitro*. Thymocytes were first incubated for 6 hr in colchicine-containing medium. The total proportion of cells which had reached metaphase during this period of incubation was then determined. (●) cell populations incubated in MAC-1, medium containing colchicine and various concentrations of cyclic GMP; (□) cell populations incubated in MAC-1 medium containing colchicine and various concentrations of guanosine 5′-monophosphate. The points are the means ± SEM of 4 to 23 (for 5×10^{-11} and 10^{-6} *M* cGMP) experiments. The level of mitotic activity in untreated cell populations maintained in colchicine-containing MAC-1 medium is represented by the broken line and its value is 5.9 ± 0.1% ($n = 40$).

tions of cyclic GMP (from Sigma Chemical Co., St. Louis) and their content of ^{3}H-cyclic AMP was determined at various times thereafter. Absolute amounts of cyclic AMP were not estimated since the particular ATP pool specifically responsible for cyclic AMP synthesis cannot be isolated. However, the radioactivity in the total cellular ATP was determined and found to be constant (about 130,000 cpm/10^8 cells) (Fig. 3). Therefore, the increases observed in the ^{3}H-cyclic AMP content (Fig. 3) could not be artifacts due to changes in the ^{3}H-ATP content. It should be noted that cyclic GMP did not cause a leakage of ^{3}H-cyclic AMP from the prelabeled cell.

The identity of the ^{3}H-cyclic AMP formed from ^{3}H-ATP was established by its cochromatography with authentic cyclic AMP on thin-layer chromatograms using the specific cyclic nucleotide separation method of Dighe *et al.* (3). This identification was verified by the demonstration that the tritiated compound was almost completely (95%) hydrolyzed by a preparation of cyclic AMP-phosphodiesterase obtained from brain tissue according to the procedure of Brooker *et al.* (1).

To prepare rat thymocyte cyclic AMP-phosphodiesterase, cells, suspended in a solution containing 130 m*M* NaCl and 5.0 m*M* Tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.0), were centrifuged at 700*g* for 10 min at 20°. The cells were resuspended in a solution of 60 m*M* Tris buffer (pH 7.5) and homogenized at 4° with a Sorvall omnimixer. The particulate fraction of the homogenate was sedimented by centrifugation at 20,000*g* for 10 min. The pellet containing the phosphodiesterase activity was resuspended in Tris solution and stored at 4°. The activity of the cyclic AMP-phosphodiesterase was assayed according to Brooker *et al.* (1).

Results. When thymocyte populations were suspended in MAC-1 medium containing colchicine and exposed to various concentrations of cyclic GMP for 6 hr, it was found that the progression of cells into mitosis was increased by either very low (5×10^{-12} to 10^{-10} *M*), or much higher (10^{-6} to 5×10^{-6} *M*) concentrations of the cyclic nucleotide (Fig. 1). However, intermediate (10^{-9} to 10^{-7} *M*) and very high (10^{-5} *M*) concentrations of cyclic GMP did not affect the flow of cells into mitosis (Fig. 1). Furthermore, the mitogenic actions were specific effects of cyclic guanosine

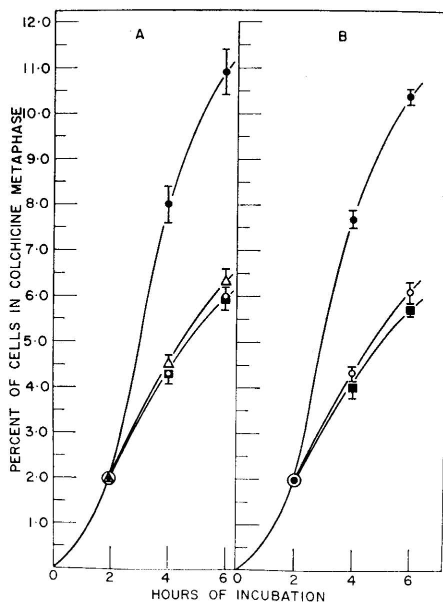

FIG. 2. The effects of cyclic guanosine 3′, 5′-monophosphate and other guanine nucleotides on the flow of cells into mitosis in thymocyte populations maintained in colchicine-containing MAC-1 medium: (A) (●) the medium contained 5×10^{-11} *M* cyclic guanosine 3′,5′-monophosphate; (○) the medium contained 5×10^{-11} *M* cyclic guanosine 2′,3′-monophosphate; (■) the medium contained 5×10^{-11} *M* guanosine 5′-monophosphate (5′-GMP); (△), normal medium. (B) (●) 10^{-6} *M* cyclic 3′,5′-GMP; (○), 10^{-6} *M* cyclic 2′,3′-GMP; (■), 10^{-6} *M* 5′-GMP. The points are means ± SEM of 10 experiments.

3′,5′-monophosphate since neither guanosine 5′-monophosphate (5′-GMP, the principal breakdown product of cyclic GMP) nor cyclic guanosine 2′,3′-monophosphate (cyclic 2′,3′-GMP) affected thymocyte proliferation (Figs. 1 and 2).

Experiments were also carried out to determine the effects of cyclic GMP on the progression of cells into mitosis during the 6-hr incubation period. After suspension of thymocyte populations in colchicine-containing MAC-1 medium or in medium containing colchicine and either 5′-GMP, or cyclic 2′,3′-GMP, the proliferating lymphoblasts continued to enter mitosis and the proportion of colchicine-metaphase cells increased with time although it did so at a progressively declining rate (Fig. 2A, B). When the medium contained maximally effective concentrations of cyclic GMP, an additional group of cells was induced to proceed toward mitosis and these stimulated cells began to arrive at metaphase sometime between 2 and 4 hr after the beginning of the exposure to the cyclic nucleotide (Fig. 2). This delayed response of thymocyte mitotic activity to cyclic GMP *in vitro* is identical to the mitotic response *in vivo* or *in vitro* to maximally effective levels of cyclic AMP or to the several hormones which use cyclic AMP as the mediator of their mitogenic actions on these cells (8–11, 14, 17, 18).

Both the low and high cyclic GMP concentrations which maximally stimulated thymocyte proliferation also caused rapid and significant increases in the cellular cyclic AMP content. For example, during the first 10 to 20 min of exposure to 5×10^{-11} or 5×10^{-6} *M* cyclic GMP, the cellular cyclic AMP content rose to a maximum value which was 1.7 to 1.9 times higher ($p < 0.001$) than the constant level in untreated cells (Fig. 3A, C). However, a nonmitogenic, intermediate cyclic GMP concentration (10^{-8} *M*) did not affect the cellular cyclic AMP content (Fig. 3B). Thus, when cyclic GMP stimulates thymocyte proliferation, it raises the cellular cyclic AMP level within a few minutes and this is followed, 2 to 4 hr later, by an increase in the proportion of cells arriving at metaphase.

The cyclic GMP-induced increases in the cellular cyclic AMP content could be due to a stimulation of cyclic AMP formation by adenyl cyclase, or to an inhibition of its degradation by cyclic AMP-phosphodiesterase. We have so far been unable to detect either a stimulation of adenyl cyclase activity or an inhibition of phosphodiesterase activity by the lower mitogenic concentrations of cyclic GMP. However, the higher mitogenic cyclic GMP concentrations strongly reduced the activity of a preparation of thymocyte cyclic AMP-phosphodiesterase (Fig. 4).

Discussion. The present observations show that, depending on its concentration, cyclic GMP can stimulate thymocyte proliferation by one of two processes. Since the two mitogenic ranges of cyclic GMP concentrations

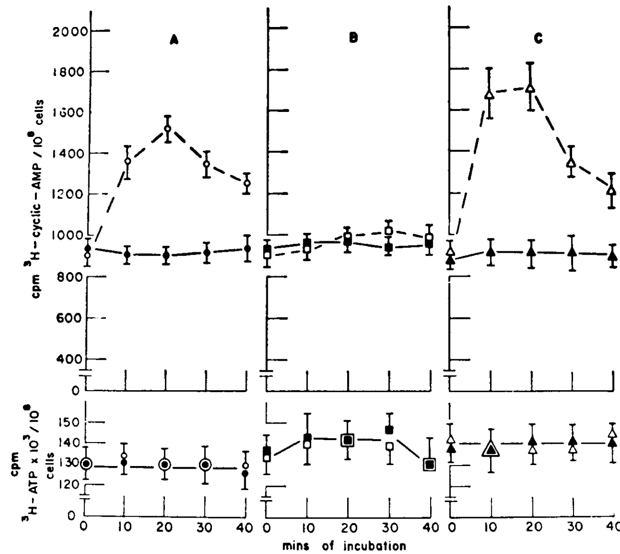

FIG. 3. The effects of various concentrations of cyclic GMP on the cellular content of ^{3}H-cyclic AMP and ^{3}H-ATP in thymocytes prelabeled with ^{3}H-adenine: (A) (○) the effect of 5×10^{-11} *M* cyclic GMP; (●) untreated cells; (B) (□) the effect of 10^{-8} *M* cyclic GMP; (■) untreated cells; (C) (△) the effect of 5×10^{-6} *M* cyclic GMP; (▲) untreated cells. The results are the means ± SEM of between 6 and 16 separate determinations. Note that cpm = counts per minute.

also caused preliminary, rapid rises in the cellular cyclic AMP level while nonmitogenic, intermediate concentrations did not, it is possible that both mitogenic processes are ultimately mediated by cyclic AMP.

The mechanism by which the extremely low concentrations of cyclic GMP induce the cellular cyclic AMP level to rise is unknown. These low concentrations do not seem to affect the activities of *isolated* thymocyte adenyl cyclase, or cyclic AMP-phosphodiesterase. The remarkable potency of these low concentrations is best appreciated when it is noted that the mitogenic 10^{-11} and 5×10^{-11} *M* cyclic GMP represent only 30 to 150 molecules of the cyclic nucleotide per cell in a thymocyte suspension containing 2×10^8 cells/ml. In view of the very small number of cyclic GMP molecules required to set the cyclic AMP-mediated, mitogenic process in motion, it is unlikely that significant quantities of cyclic GMP enter the cell and the nucleotide may therefore affect some enzyme system located on the cell surface. The mechanism by which the higher cyclic GMP concentrations raise the cellular cyclic AMP level can be much more easily explained since they can considerably reduce cyclic AMP degradation by strongly inhibiting the action of cyclic AMP-phosphodiesterase.

Although the present communication is concerned only with the mitogenic actions of cyclic GMP, it should be noted in passing that the mitogenic ineffectiveness of the intermediate (10^{-9} to 10^{-7} *M*) concentrations of cyclic GMP indicates that the nucleotide can initiate a third type of reaction. This reaction might either suppress cyclic AMP formation, or increase its degradation. However, more information must be obtained to ascertain which of these two possibilities is involved.

Summary. Very low (5×10^{-12} to 10^{-10} *M*), as well as higher (10^{-6} to 5×10^{-6} *M*) concentrations of cyclic guanosine 3′, 5′-monophosphate (cyclic GMP) stimulate the proliferation of rat thymic lymphocytes *in vitro*, but intermediate concentrations (10^{-9} to 10^{-7} *M*) of the cyclic nucleotide do not affect cell proliferation. Other guanosine monophosphates such as guanosine 5′-monophosphate (5′-GMP) and cyclic guanosine 2′,3′-monophosphate (cyclic 2′,3′-GMP) do

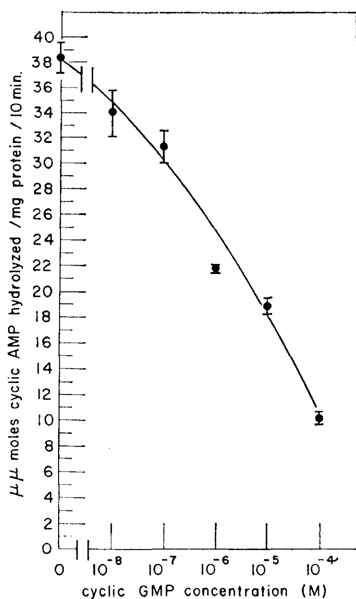

FIG. 4. The inhibition of thymocyte cyclic AMP-phosphodiesterase by cyclic guanosine 3′-5′-monophosphate (cyclic GMP). The enzyme was prepared as described in Materials and Methods and the concentration of the cyclic AMP substrate used in the assay of the enzyme's activity was 9.7×10^{-8} M. The points are means ± SEM of four determinations.

not affect thymocyte proliferation.

The maximally mitogenic concentrations (5×10^{-11} and 5×10^{-6} *M*) of cyclic GMP cause rapid (within 10 to 20 min) rises in the cellular cyclic AMP content, but nonmitogenic, intermediate cyclic GMP concentrations do not affect the cellular cyclic AMP content. The higher cyclic GMP levels probably cause the cellular cyclic AMP level to rise by reducing the cyclic AMP-degrading activity of cyclic AMP-phosphodiesterase. The nature of the cyclic AMP-elevating action of the very low cyclic GMP levels is unknown. It is concluded that cyclic GMP can stimulate thymocyte proliferation by two distinct mechanisms both of which are mediated by cyclic AMP.

We acknowledge the technical assistance of Mr. B. Braceland.

1. Brooker, G., Thomas, L. J., Jr., and Appleman, M. M., Biochemistry 7, 4177 (1968).
2. Cross, M. E., and Ord, M. G., Biochem. J., 120, 21P (1970).
3. Dighe, P. K., Pahuja, D. N., and Shah, D. H., J. Chromatogr. 40, 449 (1969).
4. Glinsmann, W. M., Hern, E. P., Linarelli, L. G., and Farese, R. V., Endocrinology 85, 711 (1969).
5. Humes, J. L., Rounbehler, M., and Kuehl, F. A., Anal. Biochem. 32, 210 (1969).
6. Kuo, J. F., and De Renzo, E. C., J. Biol. Chem. 244, 2252 (1969).
7. MacManus, J. P., Perris, A. D., Whitfield, J. F., and Rixon, R. H., Proc. Leukocyte Culture Conf., 5th, 1970, p. 125. Academic Press, New York.
8. MacManus, J. P., and Whitfield, J. F., Exp. Cell Res. 58, 188 (1969).
9. MacManus, J. P., and Whitfield, J. F., Proc. Soc. Exp. Biol. Med. 132, 409 (1969).
10. MacManus, J. P., and Whitfield, J. F., Endocrinology 86, 934 (1970).
11. MacManus, J. P., Whitfield, J. F., and Youdale, T., J. Cell. Physiol. 77, 103 (1971).
12. Murad, F., Manganiello, V., and Vaughan, M., J. Biol. Chem. 245, 3352 (1970).
13. Rebhun, L. I., and Monroy, E., J. Cell Biol. 47, 168a (1970).
14. Rixon, R. H., Whitfield, J. F., and MacManus, J. P., Exp. Cell Res. 63, 110 (1970).
15. Smith, J. W., Steiner, A. L., and Parker, C. W., Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29, 701 (1970).
16. Whitfield, J. F., Brohée, H., and Youdale, T., Exp. Cell Res. 36, 341 (1964).
17. Whitfield, J. F., MacManus, J. P., and Gillan, D. J., J. Cell. Physiol. 76, 65 (1970).
18. Whitfield, J. F., MacManus, J. P., and Rixon, R. H., J. Cell. Physiol. 75, 213 (1970).

Received Dec. 6, 1970. P.S.E.B.M., 1971, Vol. 137.