The Effect of Cyclic AMP on Heme Synthesis by Rat Bone Marrow Cells *in Vitro*[1] (36743)

STANLEY E. GRABER, MARTA CARRILLO, AND SANFORD B. KRANTZ
(Introduced by R. M. Des Prez)

Department of Medicine, Division of Hematology, Veterans Administration Hospital and Vanderbilt University School of Medicine, Nashville, Tennessee 37203

Since adenosine 3′,5′-cyclic-monophosphate (cAMP) is known to mediate the action of many peptide hormones (1–2), it is possible that erythropoietin (Ep) might regulate erythropoiesis through a change in the cellular concentration of this nucleotide. Several investigators (3–6) have shown that injection of cAMP into rodents produces a marked increase in erythropoiesis. This is most likely due to the release of endogenous Ep, since the effect is blocked by an antibody to the hormone (3, 6). Whether or not cAMP exerts a direct effect on marrow cells to increase erythropoiesis is still not clear. Gorsheim and Gardern (7) found that N^6,$O^{2'}$-dibutyryl adenosine 3′,5′-cyclic-monophosphate (db-cAMP) increased heme synthesis in human marrow explants and Dukes (8) was able to show a potentiation of Ep-stimulated heme synthesis when db-cAMP was added to marrow cultures. In contrast, Bottomley *et al.* (5) found a stimulatory effect on ALA synthetase, when cAMP was added to cultures of rabbit marrow, but failed to show any effect on heme synthesis. We have used fasted rat marrow cell cultures, which are very sensitive to Ep, to study the effect of cAMP and other nucleotides on heme synthesis and have also tested the effect of additional substances that modify intracellular production or destruction of cAMP.

Materials and Methods. Male Sprague-Dawley rats (180–250 g), fasted 3 days were used as a source of bone marrow for all experiments. Marrow cells were cultured in small petri dishes (Falcon Plastics) as outlined by Krantz, Gallien-Lartique and Goldwasser (9). The medium consisted of 0.8 ml NCTC-109, 0.1 ml rat serum and 0.1 ml calf serum. In all experiments each point was the mean of triplicate or quadruplicate cultures. Nucleated cell concentrations were uniform for each experiment, but ranged from 5 to 12 $\times$ 10^6 cells/ml for different experiments. The cultures were incubated at 37° in an atmosphere of 5% CO_2 and 95% air for 36–40 hr. At this time 0.4 to 0.5 μCi of transferrin bound ^{59}Fe was added to each plate and the incubation continued for another 8 hr. The cells were then transferred to glass tubes and centrifuged at 1000*g*. The supernatants were removed and the cells were resuspended in phosphate buffered saline. After recentrifugation and removal of the supernatant fluid the cells were lysed in 1.2 ml H_2O and 0.6 ml Drabkin's solution. Heme was dissociated from globin by the addition of 0.2 ml of 1 *N* HCl and was then extracted into 2 ml of cyclohexanone (10). An aliquot of the cyclohexanone phase was then transferred to another glass tube and the radioactivity measured with a Nuclear Chicago auto gamma counter to determine the amount of ^{59}Fe incorporated into heme. In each experiment the test materials were added at the beginning of the incubation to cultures which contained either no added Ep[2] or a 45% maximal dose of Ep. In addition, a series of control plates containing no additives, or a 45% maximal dose of Ep only, were run. The materials tested were adenosine 3′,5′-cyclic-monophosphate (cAMP), N^6,$O^{2'}$-dibutyryl

[1] Supported by Veterans Administration Hematology Training Grant TR No. 93 and NIH Grant No. AM-15555-01.

[2] Step III erythropoietin obtained from Connaught Laboratories, Toronto, Canada.

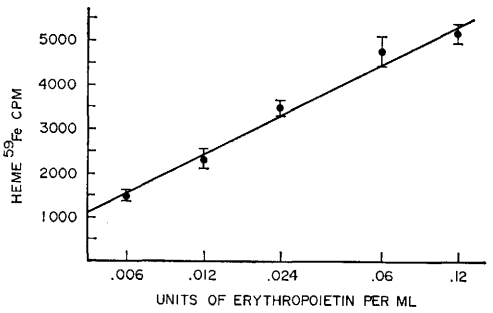

FIG. 1. Erythropoietin dose response in rat bone marrow cultures, as measured by incorporation of ^{59}Fe into heme. Each point represents the mean of quadruplicate cultures ± 1 SD.

adenosine 3′,5′-cyclic-monophosphate (db-cAMP), guanosine 3′,5′-cyclic-monophosphate (cGMP), adenosine 5′-triphosphate (ATP), adenosine 5′-monophosphate (AMP), 1-methyl-3-isobutyl xanthine (MIX), epinephrine, insulin, and imidazole. They were dissolved in NCTC-109 and were sterilized by passage through a 0.45 μ filter (Millipore) prior to addition to the cultures. Appropriate dilutions were made with NCTC-109.

Results. Figure 1 demonstrates that the dose–response relationship between Ep and the rate of heme synthesis by rat marrow cells was linear through a concentration of 0.12 U/ml. In all subsequent experiments 0.012 U/ml of Ep were employed. This amount of hormone produced a 2–3-fold (45% maximal) increase in the rate of heme synthesis, but was small enough to allow measurement of any potentiation by a test substance.

A typical experiment using db-cAMP is shown in Fig. 2. As shown, db-cAMP did not stimulate base line heme synthesis or potentiate the effect of a 45% maximal dose of Ep at any of the concentrations tested. Higher concentrations (10^{-3} and 10^{-4} *M*), however, inhibited both base line and Ep stimulated heme synthesis. The results of experiments performed with the other test substances are shown for base line heme synthesis in Table I, and for erythropoietin-stimulated heme synthesis in Table II. In the former case all values were normalized to the control cultures (no-additives) which were set equal to 100, while in the latter case all values were normalized to the Ep cultures (0.012 units Ep only) which were set equal to 100. No increase in the rate of heme synthesis was seen with any of the nucleotides, and all produced a marked inhibition of both base line and Ep-stimulated ^{59}Fe uptake into heme at concentrations of 10^{-3} *M*. MIX, epinephrine, and imidazole gave essentially the same results as the nucleotides, *i.e.*, they all failed to stimulate heme synthesis at any concentration tested and inhibited at higher concentrations. In contrast, insulin stimulated base line heme synthesis approximately 3-fold and also increased the rate of heme synthesis of the erythropoietin treated cells.

Discussion. When erythropoietin is added to bone marrow cells *in vitro*, RNA synthesis is increased within 15 min (11). This stimulation is the earliest known effect of the hormone and appears to be its primary mode of action (11, 12). Both the rapidity of the effect and the molecular weight (13) of the hormone (45,800) suggest that erythropoietin may be interacting with the membrane of the responsive cell to produce a second messenger (cAMP), which in turn triggers the increased RNA synthesis. To test this hypothesis the present studies to determine the effect of exogenous cyclic nucleotides on heme synthesis by marrow cells *in vitro* were undertaken.

Addition of cAMP, db-cAMP, and cGMP to rat marrow cultures did not stimulate heme synthesis and at higher concentrations inhibited both base line and Ep stimulated ^{59}Fe incorporation into heme. A similar pattern was observed with the noncyclic nucleotides, AMP and ATP, and with epinephrine and MIX, two substances known to raise intracellular cyclic AMP levels (14, 15). These results, which are in agreement with Bottomley and co-workers' (5) experiments on heme synthesis, fail to support the concept that the effect of Ep is mediated solely through an increase in cellular cAMP levels. Similar observations with cyclic nucleotides have been made in two other cell culture systems. Exogenous cAMP, db-cAMP, AMP, ATP, and aminophylline prevented mouse marrow cells from forming granulocyte colonies *in vitro* (16), and inhibited the effect of

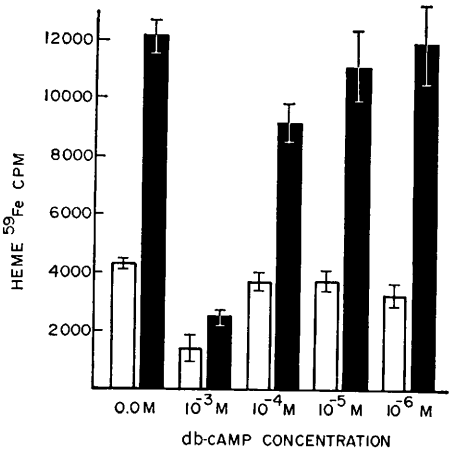

FIG. 2. Effect of dibutyryl cyclic AMP on base line and erythropoietin stimulated heme synthesis. Open bars contain no erythropoietin, solid bars contain a 45% maximal dose of Ep. Each bar represents the mean of triplicate cultures ± 1 SD.

phytohemagglutinin (PHA) on DNA, RNA, and protein synthesis in human blood lymphocytes (17). These systems are analogous to the erythroid system studied here in that they require cell division and maturation in order to observe the final response. Because such a complex sequence of reactions is necessary to obtain Ep-stimulated heme synthesis, the possibility that the hormone might affect the adenyl cyclase system remains viable. More precisely, the action of Ep involves RNA synthesis, followed by DNA synthesis, cell division and maturation in the responsive cells (18). Increased heme synthesis is a late event and is generally not measured for many hours after initiation of the cultures. Therefore, the net effect of cAMP on this parameter may be the resultant of multiple actions of the nucleotide on the erythropoietin responsive cell (ERC), *i.e.*, precise modulation of cAMP levels both up and down may be necessary for the responsive cells to properly divide and synthesize hemoglobin. Thus, in the lymphocyte system, elevations of intracellular cAMP following treatment by phytohemagglutinin have been clearly demonstrated despite the fact that the addition of extracellular cAMP inhibited the PHA induced transformation of these cells (17, 19). Furthermore, Dukes (8) recently reported experiments are also compatible with the concept of multiple actions of cAMP on a dividing cell system. He showed that db-cAMP potentiated the Ep stimulation of heme synthesis in rat marrow cultures. How-

TABLE I. Effect of Various Nucleotides, Epinephrine, MIX, Imidazole and Insulin on Base Line[a] Heme Synthesis[b,c] in Rat Marrow Cultures.

	Concn (moles/liter)					
Test substance	0.0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
db-cAMP	100 ± 18		97 ± 24	89 ± 4	82 ± 9	20 ± 11
cAMP	100 ± 10	90 ± 12		95 ± 37		53 ± 29
cGMP	100 ± 13	104 ± 20		115 ± 11		45 ± 7
AMP	100 ± 11		110 ± 8	118 ± 12	87 ± 8	30 ± 16
ATP	100 ± 12		121 ± 32	122 ± 40	94 ± 39	52 ± 27
Epinephrine	100 ± 16	97 ± 10	108 ± 15	101 ± 10	23 ± 10	
MIX	100 ± 18			57 ± 18[d]		
Imidazole	100 ± 10		77 ± 22[e]	84 ± 20[e]	56 ± 10[e]	52 ± 2[e]

	Concn (mU/ml)				
	0.0	0.8	8.0	80	800
Insulin	100 ± 15	134 ± 33	240 ± 63	301 ± 82	287 ± 62

[a] No erythropoietin was added to any of the cultures presented in the table.

[b] ^{59}Fe incorporation into heme is expressed relative to the control cultures which contained no additives and were set equal to 100.

[c] Values = the mean of 3–6 replicate cultures ± 1 SD.

[d] Actual concn = 5×10^{-5} *M*.

[e] Actual concn = 4×10^{-6}, 4×10^{-5}, 4×10^{-4}, 4×10^{-3} *M*, respectively.

TABLE II. Effect of Various Nucleotides, Epinephrine, MIX, Imidazole and Insulin on Erythropoietin-Stimulated[a] Heme Synthesis[b,c] in Rat Marrow Cultures.[d]

	Concn (moles/liter)					
Test substance	0.0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
db-cAMP	100 ± 35		96 ± 22	98 ± 24	77 ± 19	10 ± 9
cAMP	100 ± 10	86 ± 7		91 ± 5	±	42 ± 2
cGMP	100 ± 13	88 ± 12		95 ± 12	±	35 ± 3
AMP	100 ± 26		109 ± 16	117 ± 10	112 ± 17	42 ± 13
ATP	100 ± 17		106 ± 12	103 ± 13	94 ± 4	42 ± 15
Epinephrine	100 ± 15	93 ± 8	87 ± 21	95 ± 14	33 ± 11	
MIX	100 ± 5		92 ± 12[e]	94 ± 5	57 ± 3[e]	36 ± 4
Imidazole	100 ± 16		105 ± 17[f]	89 ± 14[f]	77 ± 9[f]	5 ± 2[f]

	Concn (mU/ml)				
	0.0	0.8	8.0	80	800
Insulin	100 ± 21	112 ± 41	139 ± 36	169 ± 25	167 ± 31

[a] 0.012 units of erythropoietin were added to each of the cultures presented in the table.

[b] ^{59}Fe incorporation into heme is expressed relative to the cultures containing erythropoietin only, which were set equal to 100.

[c] Values = the mean of 3–6 replicate cultures ± 1 SD.

[d] Note that the value 100 in this table represents heme synthesis 2–4 times greater than the value 100 in Table I.

[e] Actual concn = 5×10^{-6}, 0.5×10^{-4} *M*, respectively.

[f] Actual concn = 4×10^{-6}, 4×10^{-5}, 4×10^{-4}, 4×10^{-3} *M*, respectively.

ever, the effect occurred in a very narrow concentration range (2.32×10^{-6} to 4.18×10^{-5} *M*) and amounts of 6.96×10^{-5} *M* or greater were inhibitory. This finding of two divergent effects by very similar concentrations of db-cAMP suggests that net heme synthesis might be the resultant of two or more actions of the nucleotide. Therefore, despite the failure to stimulate heme synthesis, our results do not disprove an effect of Ep on the adenyl cyclase system.

The physiological significance of the inhibitory effect of cAMP on heme synthesis is unclear, as it only occurs at high concentrations of the nucleotide and is seen when other noncyclic compounds such as AMP and ATP are employed. This may represent a nonspecific effect possibly related to a direct toxic action of large doses of the nucleotides on bone marrow cells. Another possible interpretation is that Ep acts by lowering the amount of cAMP in the cell. Additional support for this hypothesis comes from the insulin experiments, since insulin, which is known to lower intracellular cyclic AMP levels (20), stimulated base line heme synthesis approximately 3-fold. Alternatively, the well-known effect of insulin on glucose metabolism might be related to the heme synthetic effect, especially since imidazole, another compound known to lower cAMP concentration (2) did not stimulate heme synthesis. With regard to this, Peets and Gordon (21) have reported an early effect of Ep on the uptake of glucose by rat bone marrow cultures. In conclusion, the question of whether cAMP mediates the action of Ep, either in part or *in toto*, remains unproven and probably awaits actual measurement of cAMP levels in tissue rich in erythropoietin responsive cells.

Summary. Cyclic AMP, db-cAMP, epinephrine, and MIX failed to stimulate heme synthesis in rat marrow cultures at all concentrations tested and in relatively large amounts (10^{-3} *M*) greatly inhibited both base line and Ep-stimulated ^{59}Fe incorporation into heme. The inhibition may be nonspecific since identical concentrations of ATP and AMP produce a similar effect. Insulin,

which is known to lower cyclic AMP levels, was the only material tested other than Ep that stimulated heme synthesis. The possibility that cAMP mediates the action of Ep is discussed.

1. Robison, A. G., J. Reprod. Fert. Suppl. **10**, 55 (1970).

2. Robison, A. G., Butcher, R. W., and Sutherland, E. W., Annu. Rev. Biochem. **37**, 149 (1968).

3. Gidari, A. S., Zanjani, E. D., and Gordon, A. S., Life Sci. **10**, 895 (1971).

4. Winkert, J., and Birchette, C., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 843 (1970).

5. Bottomley, S. S., Whitcomb, W. H., Smithee, G. A., and Moore, M. Z., J. Lab. Clin. Med. **77**, 793 (1971).

6. Schooley, J. C., and Mahlman, L. J., Proc. Soc. Exp. Biol. Med. **137**, 1289 (1971).

7. Gorsheim, D., and Gardner, F. H., Blood **36**, 847 (1970).

8. Dukes, P. P., Blood **38**, 822 (1971).

9. Krantz, S. B., Gallien-Lartique, O., and Goldwasser, E., J. Biol. Chem. **238**, 4085 (1963).

10. Hrinda, M. E., and Goldwasser, E., Biochim. Biophys. Acta **195**, 165 (1969).

11. Krantz, S. B., and Goldwasser, E., Biochim. Biophys. Acta **103**, 325 (1965).

12. Djaldetti, M., Preisler, H., Marks, P. A., and Rifkind, R. A., J. Biol. Chem. **247**, 731 (1972).

13. Goldwasser, E., and Kung, C. K. H., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **30**, 1128 (1971).

14. Sutherland, E. W., Robison, A. G., and Butcher, R. W., Circulation **37**, 279 (1968).

15. Beavo, J. A., Rogers, N. L., Crofford, O. B., Hardman, J. G., Sutherland, E. W., and Newman, E. V., Mol. Pharmacol. **6**, 597 (1970).

16. Morley, A., Quesenberry, P., Garrity, M., and Stohlman, F., Jr., Proc. Soc. Exp. Biol. Med. **138**, 57 (1971).

17. Smith, J. W., Steiner, A. L., and Parker, C. W., J. Clin. Invest. **50**, 442 (1971).

18. Krantz, S. B., and Jacobson, L. O., "Erythropoietin and the Regulation of Erythropoiesis," pp. 123–135. Univ. of Chicago Press, Chicago (1970).

19. Smith, J. W., Steiner, A. L., Newberry, W. M., Jr., and Parker, C. W., J. Clin. Invest. **50**, 432 (1971).

20. Butcher, R. W., Sneyd, J. G. T., Park, C. R., and Sutherland, E. W., J. Biol. Chem. **241**, 1651 (1966).

21. Peets, E. A., and Gordon, A. S., Life Sci. **7**, 561 (1968).

Received May 4, 1972. P.S.E.B.M., 1972, Vol. 141.