Cyclic AMP phosphodiesterase activity during differentiation of rabbit erythroid bone marrow cells

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Changes in the activity of cyclic AMP phosphodiesterase during differentiation of rabbit bone marrow erythroid cells were investigated. The cells were separated by velocity sedimentation at unit gravity into six fractions corresponding to different stages of development: proerythroblasts, basophilic cells, polychromatic cells, early orthochromatic and late orthochromatic cells and reticulocytes. Cyclic AMP phosphodiesterase was found to be very active in the most immature cells, the proerythroblasts, which also have the highest content of cyclic AMP. After differentiation into basophilic erythroblasts, a 4-fold decrease in cyclic AMP phosphodiesterase activity was observed. In these cells the amount of cyclic AMP was about 80% lower than that in proerythroblasts. In polychromatic cells a further drop in phosphodiesterase activity occurred. After the final cell division the enzyme activity was very low and the levels of cyclic AMP in the early and late orthochromatic cells remained constant. Kinetic studies demonstrated a heterogeneity of erythroid cell cyclic AMP phosphodiesterase: high affinity, low- K_m (5.5×10^{-6} M) and low affinity, high- K_m (0.1×10^{-3} M) enzymes were found. The phosphodiesterase activity was dependent on the presence of Mg^{2+} and was activated by Ca^{2+} at low Mg^{2+} concentrations (1 mM). The changes in cyclic AMP phosphodiesterase activity during differentiation and maturation of erythroid cells suggest the possible importance of this enzyme in the physiological control of cyclic AMP concentrations in developing erythroblasts. The loss of cyclic AMP phosphodiesterase activity after cessation of cell division supports the concept of the significance of the final cell division in erythroblast differentiation.

Cyclic AMP has been reported to be involved in many different regulatory functions in both prokaryotic and eukaryotic cells, including growth, differentiation and morphogenesis (Sutherland, 1972; Pastan & Perlman, 1970; Jost & Rickenberg, 1971). The development of erythrocytes from undifferentiated pluripotent stem cells in the bone marrow involves both cell proliferation and differentiation by a series of complex morphological, physiological, biochemical and biophysical changes. Little is known, however, about the molecular basis of the control mechanisms involved. Immature erythroid cells are stimulated to proliferate and initiate globin synthesis by erythropoietin, but the precise mode of action of this hormone has not yet been elucidated (Paul, 1976). The role of the adenylate cyclase/cyclic AMP system in the regulation of erythropoiesis remains uncertain. Quantita-

tive data about the activities of cyclic AMP-generating, cyclic AMP-degrading and cyclic AMP-utilizing enzymes in relation to the differentiation of bone marrow erythroid cells might therefore provide useful information about the molecular basis for the hormonal control of erythroid cell differentiation.

We have found previously that differentiation and maturation of rabbit bone marrow erythroid cells is accompanied by a 15-fold decrease in basal adenylate cyclase activity (Setchenska *et al.*, 1979). It was suggested that the continuous decrease in adenylate cyclase activity during the maturation process may be due to structural and conformational changes of the erythroid cell membrane in the later stages of differentiation, resulting in either loss or inhibition of enzyme activity in erythrocytes.

The content of cyclic AMP and cyclic GMP in

erythrocytes was determined for the first time by Patterson *et al.* (1974), who found striking species differences in cyclic nucleotide levels. When we measured the content of cyclic AMP in different rabbit bone marrow erythroid cell fractions (Setchenska *et al.*, 1979) we found that there was a very marked decrease in content in basophilic and polychromatic cells in comparison with that in the early proerythroblasts. Subsequently, the cyclic AMP content remained fairly constant in the non-dividing bone marrow cells.

Since the cellular concentration of cyclic nucleotides may be controlled not only by their biosynthesis but also by degradation we have now investigated the activity of cyclic AMP phosphodiesterase in rabbit bone marrow erythroid cells of increasing maturity, namely: proerythroblasts, basophilic cells, polychromatic cells, early orthochromatic and late orthochromatic cells. Dramatic changes in the enzyme activity were found during the early period of erythroid cell development when erythroblasts are actively dividing. After condensation of the nucleus, however, the cyclic AMP phosphodiesterase activity remained constant, but was extremely low. In circulating reticulocytes there was only negligible activity.

Experimental

Materials

The animals were white New Zealand rabbits. [^3H]Adenosine 3':5'-phosphate (ammonium salt) in 50% aq. ethanol (30 Ci/mmol) was purchased from The Radiochemical Centre. Unlabelled cyclic AMP and dithiothreitol were obtained from Boehringer. 5'-Nucleotidase from *Crotalus atrox* venom and Dowex 1 (X2; 200–400 mesh) were from Sigma. All other reagents were AnalaR from BDH.

Isolation and fractionation of erythroid cells

Rabbits were made anaemic by five daily injections of 0.3 ml of neutralized 2.5% (w/v) phenylhydrazine/kg body weight (Arnstein *et al.*, 1964). The animals were killed on the fifth day after the last injection by bleeding from the heart. Blood reticulocytes were separated from the plasma by centrifugation at 1000 g for 10 min and the 'buffy' coat of white cells at the top was removed by aspiration. The cells were washed twice with 0.145 M-NaCl in 0.02 M-sodium phosphate buffer, pH 7.4 (phosphate-buffered saline). Bone marrow was scraped from the femur and humerus and the cells were disaggregated in ice-cold phosphate-buffered saline (approx. 10 vol.) by gentle pipetting. The suspension was filtered through a double layer of muslin, the cells were collected by centrifugation

at 500 g for 10 min and washed twice in the same medium.

Bone marrow erythroid cells were fractionated by velocity sedimentation in a Ficoll/bovine serum albumin gradient in a chamber designed by Denton & Arnstein (1973). As mammalian erythroid cells differentiate, they become smaller and the change in size is sufficient to allow efficient fractionation (Clissold *et al.*, 1977). Disaggregated marrow at a density of 3×10^6 cells/ml was allowed to sediment for 4–5 h, 30 fractions were collected and appropriate consecutive fractions were pooled to give the following cell fractions: proerythroblasts, basophilic cells, polychromatic cells, early orthochromatic cells and late orthochromatic cells. Details of the separation achieved have been reported previously (Denton & Arnstein, 1973; Clissold *et al.*, 1977). Cell numbers were determined with a Coulter counter.

Bone marrow cells and circulating reticulocytes were lysed in 10 mM-Tris/HCl buffer, pH 7.4, and cell debris was removed by centrifugation at 12 000 g for 30 min.

Cyclic AMP phosphodiesterase assay

Low- and high- K_m cyclic AMP phosphodiesterase activity was measured by the radioisotope method of Boudreau & Drummond (1975) as described by Azhar & Menon (1979), using snake venom 5'-nucleotidase in the second step in order to promote the conversion of AMP to adenosine. The incubation mixture (0.2 ml) contained 10 mM-Tris/HCl buffer, pH 8.0, 0.6 mM-dithiothreitol and 5 mM-MgCl₂. When the effect of Ca²⁺ on the cyclic AMP phosphodiesterase activity was studied, the concentration of MgCl₂ was kept at 1 mM per assay. In all except the kinetic studies, two different substrate concentrations were used: 10^{-6} M-cyclic AMP for the low- K_m phosphodiesterase and 10^{-4} M-cyclic AMP for the high- K_m phosphodiesterase. Each assay contained approx. 200 000 c.p.m. of cyclic [^3H]AMP. The reaction was started with an appropriate amount of cell lysate. After incubation at 30°C for 15 min the reaction was terminated by immersing the tubes for 2 min in boiling water. Snake venom (50 µg) was added to each tube and the incubation was continued for another 15 min at 30°C. At the end of that period, 1 ml of a slurry of Dowex 1 anion-exchange resin (X2; 200–400 mesh) in 3 mM-acetic acid (1:2 v/v) was added. After centrifugation, 0.5 ml of the clear supernatant was counted in a liquid-scintillation spectrometer. Enzyme activity is expressed as pmol of cyclic AMP hydrolysed/20 min per 10^6 cells. Since changes in specific enzyme activity during erythroid cell differentiation may be due to dilution with increasing amounts of haemoglobin, it is more meaningful to calculate enzyme activity per cell rather than per mg of protein. Moreover, when enzyme activity is

expressed in this way, a comparison with the levels of cyclic AMP in these cells is possible. Results are given as the mean for triplicate determinations $\pm$ S.E.M.

Results

When cyclic AMP phosphodiesterase activity of total bone marrow cell lysates was compared with that of circulating reticulocytes, a striking difference was found. The low- K_m phosphodiesterase activity in bone marrow erythroid cells was 5.5 ± 0.5 pmol of cyclic AMP hydrolysed per 10^6 cells and the high- K_m enzyme activity was 3.1 ± 0.3 pmol of cyclic AMP per 10^6 cells, whereas in reticulocytes both activities were equal within the sensitivity of the method (about 0.06 pmol of cyclic AMP per 10^6 cells).

The developmental changes in cyclic AMP phosphodiesterase activity of the erythroid bone marrow cells are presented in Table 1. In proerythroblasts, both low- K_m and high- K_m activities were very high. These cells were also richest in cyclic AMP which is present at a cellular concentration of approx. $1.4 \mu\text{M}$. After differentiation into basophilic erythroblasts, a dramatic decrease in cyclic AMP phosphodiesterase activity was found: 4-fold for the low- K_m enzyme and 6.5-fold for the high- K_m enzyme. In these cells the high affinity phosphodiesterase was twice as active as the low affinity enzyme. The cyclic AMP level was about 80% lower than in proerythroblasts. In polychromatic cells, a further drop of phosphodiesterase activity occurred, and both low- K_m and high- K_m enzyme activities became equal. The cyclic AMP content of these cells was only 10% of that of proerythroblasts. In both early and late orthochromatic cells cyclic

AMP phosphodiesterase activities were extremely low. No further changes in cyclic AMP levels were found after the final cell division.

Some kinetic studies were carried out with proerythroblast fractions pooled from five different rabbits. Only high affinity, low- K_m phosphodiesterase activity was measured because of the small amount of material available. These results are mean values from duplicate determinations. The reaction rate was linear up to 15 min incubation time and for between 0.5×10^6 and 1.5×10^6 cells per assay (results not shown). The effect of pH was studied between 6.5 and 8.5. Maximal activity was observed at pH 8.0 (Fig. 1). Cyclic AMP hydrolysis showed an absolute requirement for Mg^{2+} with an optimal concentration of approx. 5 mM (Fig. 2). Ca^{2+} activated the reaction when Mg^{2+} was below the optimal concentration. The concentration curve had a sigmoid shape (Fig. 3). Above 3 mM- Mg^{2+} the cyclic AMP phosphodiesterase activity appeared to be independent of Ca^{2+} .

Cyclic AMP phosphodiesterase activity was measured under the standard assay conditions with ten different cyclic AMP concentrations from 2.5 to $500 \mu\text{M}$. The plot of the reciprocal of cyclic nucleotide concentration against the reciprocal of activity for cyclic AMP phosphodiesterase showed the presence of a high affinity and a low affinity enzyme (Fig. 4). The apparent K_m values were approx. 5.5 and $100 \mu\text{M}$, respectively. The K_m of the high affinity diesterase is thus of the same order as the cellular concentration of cyclic AMP in proerythroblasts (Table 1.)

With total bone marrow cells, it was found that the optimal temperature was in the range of 20 to 30°C and that the reaction was completely inhibited by 10 mM-papaverine, -theophylline and -caffeine

Table 1. Cyclic AMP phosphodiesterase activity of developing bone marrow erythroblasts

Erythroid cell fractions were obtained after fractionating the bone marrow cells by velocity sedimentation as described in the Experimental section. The cells were lysed in 10 mM-Tris/HCl buffer, pH 7.4, and the 12000 g supernatant was analysed for cyclic AMP phosphodiesterase activity by the radioisotope method described by Azhar & Menon (1979). Two substrate concentrations of cyclic AMP were used: 10^{-6}M for the low- K_m enzyme and 10^{-4}M for the high- K_m enzyme. In each assay, the cyclic [^3H]AMP contained 200 000 c.p.m.

Cell type	Enzyme activity (pmol of cAMP hydrolysed/ 20 min per 10^6 cells)		Cyclic AMP content* (pmol/ 10^6 cells)
	Low- K_m	High- K_m	
Proerythroblasts	20.8 ± 1.5	17.5 ± 0.7	1.358 ± 0.185
Basophilic erythroblasts	5.0 ± 0.4	2.7 ± 0.4	0.326 ± 0.072
Polychromatic erythroblasts	1.4 ± 0.2	1.4 ± 0.2	0.153 ± 0.028
Early orthochromatic cells	0.7 ± 0.03	0.7 ± 0.03	0.141 ± 0.032
Late orthochromatic cells	0.7 ± 0.06	0.6 ± 0.09	0.131 ± 0.024

* Data from Setchenska *et al.* (1979).

Fig. 1. Effect of pH on cyclic AMP phosphodiesterase activity of proerythroblasts

The assay mixture was as described in the Experimental section except that the pH of the incubation buffer was varied. Each assay tube contained lysate from 0.2×10^6 cells.

Fig. 2. Effect of Mg^{2+} concentration on cyclic AMP phosphodiesterase activity of proerythroblasts

Incubation conditions were as described in the Experimental section except for the variation of Mg^{2+} concentration. Each assay tube contained lysate from 0.2×10^6 cells.

(results not shown). The enzyme was stable in frozen cells, but homogenates preincubated for 1 h at 30°C and 4°C , respectively, lost 52% and 30% of their activity.

Fig. 3. Effect of Ca^{2+} concentration on cyclic AMP phosphodiesterase activity of proerythroblasts

The assay mixture was as described in the Experimental section except that the Mg^{2+} concentration was 1 mM and the Ca^{2+} concentration was varied. Each incubation tube contained lysate from 0.2×10^6 cells.

Fig. 4. Effect of cyclic AMP concentration on phosphodiesterase activity of proerythroblasts

Assay conditions were as described in the Experimental section except for the variation of cyclic AMP concentration (from 2.5 to $500 \mu\text{M}$). Each assay tube contained lysate from 0.2×10^6 cells. Results are shown as a double-reciprocal plot. v is expressed as pmol of cyclic AMP hydrolysed/20 min per 10^6 cells.

Discussion

During development of rabbit bone marrow erythroid cells, changes in the activities of several enzymes have been observed to occur at the time of condensation of the nucleus in orthochromatic erythroblasts (Denton *et al.*, 1975; Hulea *et al.*, 1975; Spencer & Peller, 1976; Hulea & Arnstein, 1977; Setchenska & Arnstein, 1978, 1979). In the present study, we have found that the activity of

cyclic AMP phosphodiesterase also changes markedly during erythroid cell development. The observed decline in enzyme activity, particularly during the early stages of development, is much greater than the 2-fold decrease in cell size during each cell division. These changes are therefore not explicable solely by the reduction of cell volume which accompanies the differentiation of erythroblasts but must also involve a decrease in enzyme activity or concentration. After the final cell division, the residual enzyme activity is almost undetectable. It should be noted, however, that even the highest activities of both low- and high- K_m enzymes in the proerythroblast stage of development are much lower than the value reported previously for total rat bone marrow cells (Smoake *et al.*, 1974). This may be due to species differences.

The effects of Mg^{2+} and Ca^{2+} on the activity of erythroblast cyclic AMP phosphodiesterase were interdependent. In the presence of 5 mM- Mg^{2+} , which gives maximal activity, the addition of 30 μ M- Ca^{2+} had no effect. At low Mg^{2+} concentration (1 mM), however, cyclic AMP phosphodiesterase activity was stimulated by Ca^{2+} and similar results have been obtained by Methven *et al.* (1980) with phosphodiesterase from guinea-pig pancreas. A Ca^{2+} -dependent protein activator may be involved in this activation (Larsen & Vincenzi, 1979) since a Ca^{2+} -dependent, low molecular weight, thermostable regulatory protein for cyclic AMP phosphodiesterases has been reported in many tissues (Kakiuchi *et al.*, 1975; Wolf *et al.*, 1977; Dedman *et al.*, 1977). The activation requires micromolar levels of calcium (Kakiuchi *et al.*, 1975) and our data indicate the possible existence of a similar protein in early erythroid cells. The sigmoid shape of the concentration curve suggests co-operative binding.

The physiological significance of the changes in cyclic AMP phosphodiesterase activity during erythroid cell development may be related to the functioning of the adenylate cyclase/cyclic AMP/protein kinase system. Since the K_m of the high affinity enzyme (approx. 6 μ M) is somewhat above even the comparatively high cellular concentration of cyclic AMP in proerythroblasts (1.4 μ M, Table 1), increased cyclic AMP production following stimulation of adenylate cyclase will be followed by accelerated degradation of the cyclic nucleotide. It is significant also that adenylate cyclase activity is stimulated by erythropoietin only in relatively immature erythroid cells, i.e. proerythroblasts and basophilic erythroblasts (Setchenska *et al.*, 1979) and that plasma-membrane associated cyclic AMP-dependent protein kinase activity is found mainly in these cells (Setchenska *et al.*, 1980). It seems reasonable, therefore, to consider cyclic AMP phosphodiesterase to be concerned with the disposal of the

increased amounts of cyclic AMP produced when immature erythroblasts are stimulated by erythropoietin. During erythroid cell development adenylate cyclase activity, cellular cyclic AMP concentrations and cyclic AMP phosphodiesterase activity all decline in an apparently co-ordinate fashion. Most significantly, however, the residual adenylate cyclase in the more mature non-dividing erythroid cells is no longer stimulated by erythropoietin (Setchenska *et al.*, 1979) and the residual protein kinase in plasma membranes is cyclic AMP-independent (Setchenska *et al.*, 1980). On the other hand, cyclic AMP-dependent protein kinase activity in the cytosol declines during differentiation but is still present in mature erythroblasts although at a low level (Setchenska *et al.*, 1981). In mature erythroblasts, cyclic AMP phosphodiesterase virtually disappears, since there is presumably no longer any need to regulate the cellular concentration of this cyclic nucleotide in response to hormonal stimulation.

If the hormonal signal for erythroid cell differentiation acts through the adenylate cyclase/cyclic AMP/protein kinase system, the disappearance of the response of adenylate cyclase to erythropoietin may be related to the life-time of the receptors (Vassileva-Popova & Yanev, 1975). The decrease in phosphodiesterase activity is probably due to cessation of the synthesis of the relevant messenger RNA following condensation of the nucleus in orthochromatic erythroblasts (Denton & Arnstein, 1973), as well as to protein degradation, which has been shown to be a selective process (Setchenska & Arnstein, 1978) and thus could preferentially affect specific enzymes.

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References

- Arnstein, H. R. V., Cox, R. A. & Hunt, J. A. (1964) *Biochem. J.* **92**, 648–661
- Azhar, S. & Menon, K. M. J. (1979) *Biochem. J.* **180**, 201–211
- Boudreau, R. J. & Drummond, G. I. (1975) *Anal. Biochem.* **63**, 388–399
- Clissold, P. M., Arnstein, H. R. V. & Chesterton, C. J. (1977) *Cell* **11**, 353–361
- Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252**, 8415–8422
- Denton, M. J. & Arnstein, H. R. V. (1973) *Br. J. Haematol.* **24**, 7–17
- Denton, M. J., Spencer, N. & Arnstein, H. R. V. (1975) *Biochem. J.* **146**, 205–211
- Drummond, G. I. & Perrott-Yee, S. (1961) *J. Biol. Chem.* **236**, 1126–1129

- Hulea, S. A. & Arnstein, H. R. V. (1977) *Biochim. Biophys. Acta* **476**, 131–148
- Hulea, S. A., Denton, M. J. & Arnstein, H. R. V. (1975) *FEBS Lett.* **51**, 346–350
- Jost, J.-P. & Rickenberg, H. V. (1971) *Annu. Rev. Biochem.* **40**, 741–774
- Kakiuchi, S., Yamazaki, R., Teshima, Y. & Miyamoto, E. (1975) *Biochem. J.* **146**, 109–120
- Larsen, E. L. & Vincenzi, F. F. (1979) *Science* **204**, 306–308
- Methven, P., Lemon, M. & Bhoola, K. (1980) *Biochem. J.* **186**, 491–498
- Pastan, I. & Perlman, R. (1970) *Science* **169**, 339–344
- Patterson, W. D., Hardman, J. G. & Sutherland, E. W. (1974) *Endocrinology* **95**, 325–327
- Paul, J. (1976) *Br. Med. Bull.* **32**, 277–281
- Setchenska, M. S. & Arnstein, H. R. V. (1978) *Biochem. J.* **170**, 193–201
- Setchenska, M. S. & Arnstein, H. R. V. (1979) *Int. J. Biochem.* **10**, 817–821
- Setchenska, M. S., Clissold, P. M., Vassileva-Popova, J. G. & Arnstein, H. R. V. (1979) in *Biophysical and Biochemical Information Transfer in Recognition* (Vassileva-Popova, J. G. & Jensen, E. V., eds.), pp. 609–617, Plenum Press, New York
- Setchenska, M. S., Vassileva-Popova, J. G. & Arnstein, H. R. V. (1980) *Int. J. Biochem.* **11**, 393–399
- Setchenska, M. S., Vassileva-Popova, J. G. & Arnstein, H. R. V. (1981) *Biochem. J.* **196**, 893–897
- Smoake, J. A., Sing, S.-Y. & Cheung, W. Y. (1974) *Biochem. Biophys. Acta* **341**, 402–411
- Spencer, N. & Peller, S. (1976) *Biochem. Soc. Trans.* **4**, 1153–1155
- Sutherland, E. W. (1972) *Science* **177**, 401–408
- Vassileva-Popova, J. G. & Yanev, T. (1975) in *Physical and Chemical Bases of Biological Information Transfer* (Vassileva-Popova, J. G., ed.), pp. 63–95, Plenum Press, New York
- Wolf, D. J., Poirier, P. G., Brostrom, C. O. & Brostrom, M. A. (1977) *J. Biol. Chem.* **252**, 4108–4117