Cyclic AMP-binding and cyclic AMP-dependent protein kinase activities in the cytosol of differentiating bone marrow erythroblasts

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Cytosolic cyclic AMP-binding capacity and cyclic AMP-dependent protein kinase activity have been studied in relation to differentiation and maturation of rabbit bone marrow erythroblasts. Using cells fractionated by velocity sedimentation at unit gravity, it was found that both activities decreased in dividing cells when calculated in terms of cell number but remained constant per cell volume. After the final cell division, cyclic AMP-dependent protein kinase activity did not change further, whereas cyclic AMP-binding capacity declined. There were no qualitative, but only quantitative, changes in the cyclic AMP-binding proteins that are present in the cytosol of developing erythroblasts. In the immature cells, the apparent K_D for the interaction of binding proteins with cyclic AMP was 4×10^{-8} M. The data suggest that changes in cyclic AMP-binding activity during differentiation of erythroid cells are due both to changes in the amount of binding proteins and in their affinity for cyclic AMP. Plasma membranes of erythroblasts were also able to bind cyclic AMP but only in dividing cells.

Cyclic AMP is implicated in a wide variety of biological phenomena. There is convincing evidence that cyclic AMP exerts its influence by activating specific protein kinases in the cell (Greengard, 1978). Mammalian tissues contain cyclic AMP-dependent protein kinases which are composed of catalytic and regulatory subunits. Dissociation, and thus activation, of the enzyme occurs by binding of cyclic AMP to the regulatory subunit (for a review see Nimmo & Cohen, 1977). The existence of additional cyclic AMP-binding proteins in mammalian tissues (Chambaut *et al.*, 1971; Donovan & Oliver, 1972; Døskeland & Ueland, 1975; Sugden & Corbin, 1976; Talmadge *et al.*, 1977) and the increased level of cyclic AMP-binding proteins without concomitant increase in protein kinase activity (Prasad *et al.*, 1976) indicate that cyclic AMP-binding proteins other than the regulatory subunit of protein kinases may also be involved in cyclic AMP-mediated reactions. A cyclic AMP-binding protein not associated with a cyclic AMP-dependent protein kinase has been purified and characterized from rabbit erythrocytes (Yuh & Tao, 1974).

In earlier work we investigated changes in adenylate cyclase activity and cyclic AMP levels (Setchenska *et al.*, 1979) as well as plasma mem-

brane-associated protein kinase activity (Setchenska *et al.*, 1980) in differentiating bone marrow erythroid cells. The purpose of the present experiments was to obtain further information about the binding of cyclic AMP to specific proteins during the development and maturation of erythroblasts and to compare it with the changes in cytosolic cyclic AMP-dependent protein kinase activity.

The present results show that the amount and capacity of cytosolic AMP-binding proteins changes considerably during the development of erythroid cells. These changes correlate closely with the changes in cyclic AMP-dependent protein kinase activity, as well as with the intracellular cyclic AMP concentrations reported by us earlier (Setchenska *et al.*, 1979).

Experimental

Materials

The animals were white New Zealand rabbits. [8-³H]Adenosine 3':5'-phosphate (ammonium salt) and adenosine 5'-[γ -³²P]triphosphate (triethylammonium salt) were purchased from The Radiochemical Centre. Unlabelled cyclic AMP, cyclic GMP, AMP, ADP, ATP, adenine, adenosine, dithiothreitol and bovine serum albumin were

obtained from Boehringer. Sodium glycerophosphate, sodium fluoride, theophylline and EGTA were from Sigma. Protosol tissue and gel solubilizer was from New England Nuclear. All other reagents were AnalaR from BDH.

Assay of protein kinase activity

For measuring endogenous phosphorylation of cytosol proteins, standard assay mixtures (0.2 ml) contained 50 mM-sodium glycerophosphate buffer (pH 7.5), 10 mM-magnesium acetate, 2 mM-theophylline, 1 mM-sodium fluoride, 0.3 mM-EGTA, 10 nM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (180 c.p.m./pmol), 1 μM -cyclic AMP and cytosol protein. The assay mixture was incubated at 30°C for 10 min and the reaction was terminated by adding 4 ml of ice-cold 7.5% (w/v) trichloroacetic acid in 50 mM-potassium phosphate, followed by 0.2 ml of 0.6% bovine serum albumin. The mixture was allowed to stand for 5 min at 0°C. It was then centrifuged, the supernatant was discarded and the precipitate was dissolved in 0.1 ml of 1 M-NaOH and re-precipitated as above. This precipitation/dissolution procedure was repeated three times. After the final NaOH dissolution, the reaction mixture was added to 10 ml of scintillation fluid and the radioactivity was determined by liquid-scintillation spectrometry. Results are expressed as pmol of ^{32}P transferred from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /min per 10^6 cells. All assays were performed in duplicate.

Cyclic AMP-binding assay

Cyclic AMP-binding activity was determined using a reaction mixture (final volume 0.2 ml except where otherwise stated) containing 50 mM-Tris/HCl buffer, pH 7.5, 5 mM-EDTA, 5 mM-theophylline, 2 mM-dithiothreitol and 1 μM -cyclic $[\text{H}^3]\text{AMP}$ (30 Ci/mmol). The reaction was started by adding the sample containing cyclic AMP-binding protein. The mixture was kept at 4°C for 60 min and 0.1 ml of a 10% (w/v) charcoal suspension was then added to separate free and protein-bound nucleotide. After centrifugation at 10000 *g* for 5 min, 0.2 ml aliquots of the supernatant were counted in 10 ml of dioxan-based scintillation mixture. Blanks without cyclic AMP-binding protein were also included. Results are calculated as pmol of cyclic $[\text{H}^3]\text{AMP}$ bound/60 min by cytosol proteins corresponding to 10^6 cells.

Polyacrylamide disc gel electrophoresis

Electrophoretic analysis was performed by the procedure of Gabriel (1971). The buffer system used was Tris (3.0 g/l)/glycine (14.4 g/l), pH 8.3. A 7.5% (w/v) separating gel and 3% (w/v) stacking gel were polymerized in a glass column (5 mm $\times$ 7 cm). The samples were preincubated in cyclic AMP-binding assay mixture and a protein solution corresponding to 10^6 cells was applied to each gel.

Electrophoresis was performed in a cold room at 2 mA/gel for about 2 h or until the dye (Bromophenol Blue) front approached the bottom of the tube. The gels were stained with 1% Amido Black for 1 h and destained in 7% (v/v) acetic acid. Each band was sliced and dissolved in 0.5 ml of Protosol solution (Protosol/water/toluene, 9:1:10, by vol.) for 18 h at 60°C. Radioactivity was measured in low background scintillation vials.

Cell isolation and fractionation

Rabbits were made anaemic by five daily injections of phenylhydrazine [0.3 ml of a 2.5% (w/v) solution/kg body weight]. Bone marrow from the femur and humerus was suspended in ice-cold phosphate-buffered saline (0.145 M-NaCl/0.02 M-sodium phosphate, pH 7.4) by aspirating the marrow clumps through a 10 ml pipette. After filtration through a double layer of muslin, the cells were collected by centrifugation at 1000 *g* for 10 min and washed twice with phosphate-buffered saline. Erythroid cells were separated into 30 fractions by velocity sedimentation at unit gravity, as previously described (Denton & Arnstein, 1973; Clissold *et al.*, 1977). The number of cells was determined with a Coulter counter. Microscopic examination of cells stained with benzidine/Giemsa showed that fractions 1–5 contained mainly proerythroblasts, fractions 6–13 mainly basophilic cells, fractions 14–19 polychromatic cells, fractions 20–24 early orthochromatic cells, fractions 25–27 late orthochromatic cells and fractions 28–30 bone marrow reticulocytes. Appropriate fractions were combined to give six pooled fractions. Fractionated and total bone marrow cells were lysed in 10 mM-Tris/HCl buffer (pH 7.4) and cell debris was removed by centrifugation at 12000 *g* for 30 min.

Results

Cyclic AMP-dependent protein kinase activity decreased during cell differentiation up to the orthochromatic stage (Fig. 1a). In non-dividing cells the enzyme activity was low but remained constant. In dividing cells, however, cyclic AMP-dependent phosphorylation of endogenous proteins in proerythroblasts was twice as much as in basophilic cells. In turn the protein kinase activity of basophilic cells was twice that of polychromatic cells. This decrease in cyclic AMP-dependent protein kinase activity in dividing erythroblasts corresponds closely to the decrease in cell volume, which is approximately halved at each division. Thus, the enzyme activity per unit cell volume remains virtually constant probably throughout the whole differentiation process.

The cyclic AMP-binding capacity of cytosolic proteins declines sharply during erythroid cell

Fig. 1. Cyclic AMP-dependent protein kinase activity and cyclic AMP-binding capacity of differentiating bone marrow erythroid cells

The assay mixtures were as described in the Experimental section. The cell fractions were as follows: 1, proerythroblasts; 2, basophilic cells; 3, polychromatic cells; 4, early orthochromatic cells; 5, late orthochromatic cells. (a) Cyclic AMP-dependent protein kinase activity; (b) cyclic AMP binding capacity.

differentiation (Fig. 1b) when calculated per cell number, but remains almost constant per unit cell volume, as the binding capacity of basophilic cells is about half that of early proerythroblasts and about twice that of polychromatic cells.

Plasma membranes of differentiating bone marrow cells showed cyclic AMP-binding capacity only at the dividing stage of development (2.4, 1.5 and 0.8 pmol/ 10^6 cells in proerythroblasts, basophilic cells and polychromatic cells, respectively). After the last cell division, this activity was lost and plasma membrane proteins of early and late orthochromatic cells, as well as of reticulocytes and erythrocytes, did not bind cyclic AMP (results not shown).

In an attempt to identify the developmental changes of cytosolic cyclic AMP binding proteins, the 12000g supernatant of different cell fractions was subjected to polyacrylamide-gel electrophoresis. The separation profiles are presented in Fig. 2. Thirteen bands with relative mobilities of 0.07, 0.09, 0.12, 0.15, 0.22, 0.30, 0.37, 0.41, 0.45, 0.52, 0.62, 0.73 and 0.86 were obtained. There are considerable changes in the individual proteins during differentiation, but they will not be discussed in detail in the present paper. Haemoglobin is present in band 7 which increases as expected during differentiation, but band 6, with a relative mobility of 0.30, is of particular interest as labelled cyclic AMP was present only in this fraction. It can be seen that for equal numbers of cells the cytosolic proteins in band 6

Fig. 2. Electrophoretic separation of cytosol proteins of developing erythroblasts

Lysates from different bone marrow cells were subjected to polyacrylamide gel electrophoresis as described in the Experimental section: (a) proerythroblasts, (b) basophilic cells, (c) polychromatic cells, (d) early orthochromatic cells, (e) late orthochromatic cells. On each gel a solution of protein from 10^6 cells was applied. The origin is indicated by a solid arrow and the dye marker by an open arrow. The radioactivity of band 6 proteins is given.

decline during the differentiation process. Similarly, the bound radioactivity decreased. The present results show the occurrence of quantitative rather

Fig. 3. *Scatchard plot of cyclic AMP binding*

The standard assay system was as described in the Experimental section except that the final volume was 1 ml. The amount of free cyclic AMP was calculated from the total amount of cyclic AMP added to the reaction mixture less the amount bound.

Table 1. *Effect of non-radioactive cyclic AMP, cyclic GMP, adenine, adenosine, AMP, ADP and ATP on the cyclic [^3H]AMP binding activity*

The standard binding assay described in the Experimental section was used. Unlabelled nucleotides were present in $1\ \mu\text{M}$ concentration. The experiments were performed with total bone marrow lysates from anaemic rabbits.

Addition	Cyclic AMP bound	
	(pmol/ 10^6 cells)	(% of control)
Cyclic [^3H]AMP (control)	3.60	100
Cyclic AMP	0.37	10
Cyclic GMP	2.16	60
Adenine	3.10	85
Adenosine	1.44	40
AMP	3.42	95
ADP	3.53	98
ATP	3.50	98

than qualitative changes in cyclic AMP-binding proteins during erythroblast development.

With total bone marrow lysates it was found that the cyclic AMP-binding capacity increased linearly up to 30 min incubation time and in the range of 0.8 to 1.2×10^6 cells per assay. Cyclic AMP-binding activity was determined at eight different concen-

trations of cyclic AMP ranging from 1×10^{-8} to $1.2 \times 10^{-7}\text{M}$. The Scatchard plot (Fig. 3) was linear, suggesting homogeneity and non-cooperative binding. The apparent K_D was approx. $4 \times 10^{-8}\text{M}$.

The specificity of the binding protein was examined in competition experiments using labelled cyclic AMP with unlabelled nucleotides and related compounds (Table 1). Cyclic GMP competed with cyclic AMP, but was less effective. Adenosine was a potent inhibitor of cyclic AMP binding, whereas AMP, ADP and ATP were without any effect.

Discussion

Cyclic AMP-dependent protein kinase activity from rabbit erythrocyte cytosol has been resolved into three fractions possessing both phosphotransferase and cyclic AMP-binding activities (Tao & Hackett, 1973). The kinetic properties and substrate specificities of these kinases are remarkably similar. Traugh & Traut (1974) purified four fractions of protein kinase activity from the ribosome-free supernatant of rabbit reticulocytes and erythrocytes. Three of the resolved enzyme fractions were found to be cyclic AMP-regulated, whereas the fourth fraction was a cyclic AMP-independent protein kinase. The findings that cyclic AMP-dependent protein kinases are present in considerable amount in erythrocytes suggest that they may be important in red cell function. Although the cytosolic protein kinase activity per cell changes when cells divide during erythroblast development, our present results show that it remains constant per unit cell volume, unlike the membrane-associated cyclic AMP-dependent enzyme (Setchenska *et al.*, 1980). After the final cell division, cyclic AMP-dependent protein kinase activity does not change any more. In our previous work (Setchenska *et al.*, 1979) it was found that after the last cell division the intracellular levels of cyclic AMP also stay constant. The relatively constant levels of cytosolic cyclic AMP-dependent protein kinase in different erythroid cells suggest that this enzyme activity may have a general function in cellular metabolism rather than one specifically related to differentiation. One possibility is the activation of carbonic anhydrase. In the case of the bovine isoenzyme I it has been shown that phosphorylation by a cyclic AMP-dependent protein kinase from hog muscle or bovine brain is accompanied by a 4-fold increase in activity (Narumi & Miyamoto, 1974). A cyclic AMP-dependent protein kinase has also been suggested to function in the translational control of protein synthesis by haemin (Ochoa, 1979), although this view is not universally accepted (Austin & Clemens, 1980).

Two cyclic AMP-binding factors (receptors I and II) have been purified to homogeneity from rabbit erythrocytes. Neither receptor had any signi-

ficant effect on the activity of the catalytic moiety of rabbit erythrocyte cyclic AMP-dependent protein kinases. The molecular weight of each of these receptors is approx. 240000 and they are pentamers composed of monomeric units of identical mass and charge as estimated by gel filtration and polyacrylamide-gel electrophoresis (Yuh & Tao, 1974). Our results show no qualitative, but only quantitative, changes of cyclic AMP-binding proteins in the cytosol fractions of developing erythroblasts. The migration of band 6 proteins on polyacrylamide-gel electrophoresis in our experiments coincides with the mobility of the cyclic AMP-binding factors from rabbit erythrocytes when the same conditions of electrophoretic analysis were used (Yuh & Tao, 1974). In our experiments it was not possible to distinguish between radioactivity incorporated by cyclic AMP-binding factors and any which may have been bound by the regulatory subunits of protein kinases.

From the Scatchard plot shown in Fig. 3 we calculate an apparent K_D of approx. $4 \times 10^{-8} \text{ M}$ for the interaction of binding proteins with cyclic AMP. This value is much smaller than that reported for the K_m of this reaction in mature erythrocytes ($3 \times 10^{-7} \text{ M}$) by Yuh & Tao (1974), indicating possible changes in the affinity of cyclic AMP binding proteins during erythroid cell differentiation.

It is noteworthy that plasma membranes of erythroblasts showed cyclic AMP-binding activity only in dividing cells. Recently we have found that the phosphorylation of rabbit erythroblast plasma membrane proteins by membrane-associated protein kinase(s) is cyclic AMP-dependent also in dividing cells during the early stages of differentiation (Setchenska *et al.*, 1979). After the last cell division, the autophosphorylation of membrane ghosts was found to be no longer stimulated by cyclic AMP. It was suggested that in the early stages of rabbit erythroid cell differentiation both the catalytic and regulatory subunits of protein kinase are bound to the plasma membrane. When cell division ceases, conformational changes of the plasma membrane may occur which result in the regulatory subunit becoming detached and hence in the desensitization of protein kinase(s) to cyclic AMP. Although the function of plasma membrane

associated cyclic AMP-binding proteins in red blood cells is not known, the observed changes during erythroid cell differentiation and maturation may indicate a developmental role.

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