Action of Erythropoietin In Vitro on Rabbit Reticulocyte Membrane Ca^{2+}-ATPase Activity

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Abstract

The mechanism of action of erythropoietin is thought to require specific interaction with the target cell surface and involve alteration of cellular calcium metabolism. Using the rabbit reticulocyte membrane as a model of the immature red cell membrane, we investigated the effects of human recombinant erythropoietin on membrane Ca^{2+}-ATPase (calcium pump) activity in vitro. Erythropoietin in a concentration range of 0.025 to 3.0 U/ml progressively decreased membrane Ca^{2+}-ATPase activity by up to 64% ($P < 0.01$). These concentrations have been shown by others to stimulate in vitro erythroid growth. The action of erythropoietin on reticulocyte Ca^{2+}-ATPase required an incubation time of 1 h before enzyme assay for maximum effect and was neutralized by antierythropoietin antiserum. Other nonhemopoietic growth factors (epidermal growth factor, insulin) had no effect in this assay. Ca^{2+}-ATPase activity of membranes prepared from rabbit mature red blood cells was not inhibited by erythropoietin. The novel effect of erythropoietin on reticulocyte membrane Ca^{2+}-ATPase activity is a mechanism by which erythropoietin can influence cellular Ca^{2+} metabolism.

Introduction

Alteration of cellular calcium metabolism appears to be an early event in the proliferative and differentiation response of red cell progenitors to the glycoprotein hormone, erythropoietin. Chelation of extracellular calcium prevents erythropoietin-induced proliferation of rat bone marrow-derived progenitors in a plasma-clot culture system (1). Erythropoietin-sensitive, Friend virus-infected mouse bone marrow cells display increases in calcium influx and efflux in response to erythropoietin (2). Human bone marrow-derived mononuclear cells on addition of erythropoietin show an immediate rise in intracellular calcium as detected by Quin-2 fluorescence (3).

The plasma membrane of red cell progenitor cells is believed to be a primary site of erythropoietin action because (*a*) trypsin treatment of marrow cells obliterates erythropoietin responsiveness (4); (*b*) erythropoietin bound to agarose beads and probably excluded from the interior of the cell is biologically active (5) and (*c*) specific high affinity binding sites have been demonstrated on the surface of erythropoietin-responsive cells (6).

We have previously characterized the effect of thyroid hormone on the Ca^{2+}-ATPase activity of the red blood cell membrane (7, 8). In the course of this work we investigated the action of thyroid hormone on rabbit reticulocyte membrane Ca^{2+}-ATPase and found it to be (unlike the rabbit mature red cell membrane) dependent upon the sex of the donor animal and modulated by sex steroids (9). Since sex steroids such as testosterone and etiocholanolone were active in this membrane system and were known to potentiate the effect of erythropoietin in intact stem cell assays (10, 11), we postulated the reticulocyte (more properly, the macroreticulocyte or "stress" reticulocyte) membrane to be a hormonally responsive model of the membrane of more immature red cell progenitors. For this reason, and the likely involvement of erythropoietin in alteration of the cellular calcium metabolism at the level of the cell membrane, we investigated the effect of human recombinant erythropoietin on rabbit reticulocyte membrane Ca^{2+}-ATPase activity and compared these findings with parallel studies in mature red cell membranes.

Methods

Animals. Mature New Zealand White bucks and does (4–5 kg) were obtained from a commercial source and maintained on laboratory chow ad lib.

Reagents. Phenylhydrazine, bovine serum albumin (BSA) and Na_2ATP were obtained from Sigma Chemical Co. (St. Louis, MO). Human recombinant erythropoietin (specific activity, > 160,000 U/mg) from a mammalian cell host was obtained from AMGen (Thousand Oaks, CA). Rabbit anti-human erythropoietin antiserum was kindly provided by Dr. Giesela Clemens (University of California at Berkeley). Erythropoietin purified from human urine (specific activity, > 80,000 U/mg) was obtained from Toyoba Co. Ltd. (New York). Epidermal growth factor purified from mouse submaxillary glands (receptor grade) was obtained from Calbiochem-Behring (La Jolla, CA). Porcine insulin was provided by Eli Lilly and Co. (Indianapolis, IN).

Induction of reticulocytosis. Induced according to previously described schedules (9), reticulocytosis averaged 67% in phenylhydrazine-treated rabbits and 59% in repeatedly bled rabbits. Orthochromatic normoblasts averaged < 0.3% of the total red cell population.

Ca^{2+}-ATPase activity. Intact red cells were washed twice with 10 mM Tris/140 mM NaCl (pH 7.4) at 4°C, the buffy coat removed by aspiration, and membranes prepared hypotonically in Tris buffer by our previously reported method (7). These faintly pink membranes were resuspended to a final concentration of 0.5 mg/ml and stored at −70°C until use within 24–72 h. Enzyme assay buffer contained EGTA (100 μM), Mg^{2+} (1 mM), ATP (1 mM), Na^+ (75 mM), and K^+ (25 mM). In

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the standard assay, Ca^{2+}-ATPase activity was measured as the difference in hydrolysis of ATP in the absence and presence of 150 μM Ca^{2+} (corresponding to 20 μM free Ca^{2+} measured by ion specific electrode [8]). When calcium levels were varied in certain experiments, free calcium was calculated by a computer program kindly provided to us by Dr. John T. Penniston (Mayo Clinic of Rochester, MN). Enzyme activity was determined in duplicate and each batch of membranes was studied in two assays. Activity was expressed as micromoles phosphate (P_i) liberated/milligram membrane protein/30 min. P_i generation was linear over the time of the assay and proportional to the amount of enzyme added. Statistical significance was established using Student's paired t test.

Incubation of membranes with erythropoietin. Erythropoietin was diluted in 10 mM Tris/BSA (1 mg/ml), pH 7.4 and added to membrane suspensions in a vol/vol ratio of 1:100. Parallel control incubations contained only diluent for erythropoietin. The pH of the assay buffer was unaffected by the addition of erythropoietin. Erythropoietin and membranes were routinely incubated for 1 h at 37°C in a shaking water bath before assay. Preliminary experiments indicated control enzyme activity over the 1-h preincubation was well preserved, sustaining a decline in activity of only 8±3% (mean±SEM, $n = 8$). In the experiments reported here the contribution of this small decline was factored out of the results by incubating all membranes for 1 h. The time course of the erythropoietin effect was determined by incubating all membranes for a total of 1 h before assay as usual; at specific times within this incubation period erythropoietin was added to an aliquot of membranes and the incubation continued. The resultant enzyme activity was expressed as a percentage of the activity of parallel control membranes not exposed to erythropoietin.

The effect of erythropoietin on stimulation by calmodulin of Ca^{2+}-ATPase was determined by incubating varying concentrations of calmodulin with reticulocyte membranes in the presence and absence of erythropoietin (0.1 U/ml) for 1 h at 37°C before enzyme assay. Calmodulin was purified from beef brain according to the method of Charbonneau et al. (12).

To assess specificity of the erythropoietin effect, neutralizing antiserum in anticipated 10-fold excess (unit/unit basis) was added to membranes 5 min before the addition of erythropoietin (0.05 U/ml) and then the standard 1-h preincubation was continued. Parallel control incubations contained erythropoietin and the equivalent amount of serum from a nonimmunized rabbit. The effects of growth factors (epidermal growth factor, insulin) on Ca^{2+}-ATPase activity were similarly studied (1 h incubations before enzyme assay).

Results

Time course of the erythropoietin effect on membrane Ca^{2+}-ATPase activity. Inhibition of reticulocyte membrane Ca^{2+}-ATPase was induced by erythropoietin in a time-dependent manner (duration of preincubation) (Fig. 1). No effect was seen if erythropoietin was added immediately before enzyme assay. A progressively greater effect was seen up to 1 h of preincubation. Preincubation times were not extended beyond 1 h since control enzyme activity decayed significantly (results not shown). A preincubation time of 1 h was chosen as the standard for the experiments reported below.

Effect of neutralizing antiserum on erythropoietin action on Ca^{2+}-ATPase activity. Four experiments on reticulocyte membranes from different rabbits are summarized in Table I. Erythropoietin antiserum, alone, had a slight inhibitory effect on enzyme activity. Erythropoietin antiserum in 10-fold excess prevented 89% of the anticipated erythropoietin-induced inhibition compared to controls containing antierythropoietin, alone. Equivalent amounts of nonimmune serum had no effect on enzyme activity and did not prevent the erythropoietin-induced inhibition.

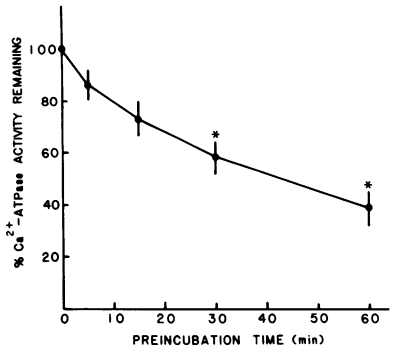

Figure 1. Effect of duration of exposure to erythropoietin on reticulocyte membrane Ca^{2+}-ATPase activity. Reticulocyte membranes were preincubated for 60 min at 37°C before enzyme assay. At various time points during this period, human recombinant erythropoietin (0.10 U/ml) was added to an aliquot of membranes and the incubation was continued. All samples were assayed for enzyme activity simultaneously at 60 min (time zero) and the results were expressed at each time point as percent of the untreated parallel control sample. Error bars represent SEM of four or more determinations. Asterisk denotes significant difference from control ($P < 0.05$, paired t test).

Concentration dependence of erythropoietin effect on Ca^{2+}-ATPase activity. A striking dose-dependent effect of erythropoietin on reticulocyte Ca^{2+}-ATPase activity is shown in Fig. 2. The value of enzyme activities corresponding to 100% were 0.598±0.077 (mean±SEM) and 0.343±0.029 μmol P_i/mg membrane protein/30 min for reticulocytes and mature red cells, respectively. Significant inhibition of activity was found at 0.05 U/ml of erythropoietin; progressive inhibition occurred at up to 3.0 U/ml (36% of original enzyme activity, $P < 0.01$). No significant effect could be seen on Ca^{2+}-ATPase activity from mature red cell membranes from untreated animals at any of the concentrations tested. Unlike the previously described thyroid hormone effect on rabbit reticulocyte Ca^{2+}-ATPase (9), the erythropoietin effect was comparable in cells from male and female donors (results not shown), so that the results from males and females were pooled in the data presented. If reticulocytes from bled, rather than phenylhydrazine treated rabbits were used, the progressive inhibition in response to erythropoietin was also observed (41% of original enzyme activity remained at 3.0 U/ml). The action of erythropoietin purified from human urine on reticulocyte membrane Ca^{2+}-ATPase activity was identical to that of recombinant erythropoietin (data not shown).

Table I. Change in Reticulocyte Ca^{2+}-ATPase Activity Resulting from Addition of Combinations of Antierythropoietin Antiserum, Nonimmune Serum, and Recombinant Erythropoietin (0.05 U/ml)

	Change in enzyme activity				
	$\mu mol\ P_i \cdot mg^{-1} \cdot 30\ min^{-1}$				
Mean	−0.009	−0.024	−0.214	−0.044	−0.227
±SEM	±0.006	±0.010	±0.038	±0.012	±0.038
Nonimmune serum	+	−	−	−	+
Antierythropoietin	−	+	−	+	−
Erythropoietin	−	−	+	+	+

Control enzyme activity was 0.697±0.135 μmol $P_i \cdot mg^{-1}$ membrane protein · 30 min^{-1} (mean±SEM). See Methods for details. Values are results of four experiments.

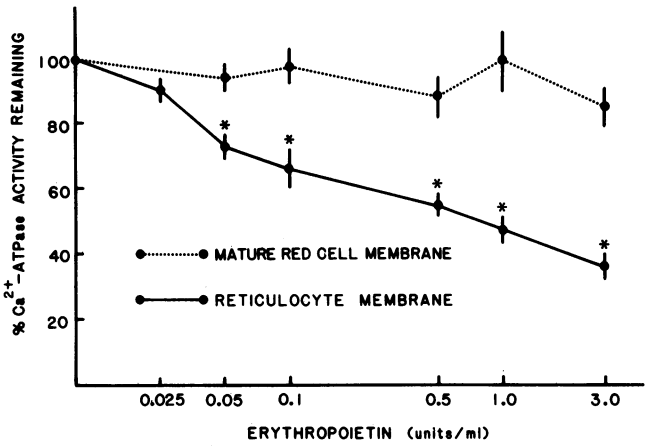

Figure 2. Effect of erythropoietin concentration on reticulocyte and mature red cell membrane Ca^{2+}-ATPase activity. Aliquots of membranes were incubated with the indicated concentration of human recombinant erythropoietin for 60 min at 37°C before enzyme assay. Enzyme activity is expressed as percent of the untreated parallel control. Error bars represent SEM of four determinations. Asterisks denote significant difference from untreated control ($P < 0.01$, paired t test).

Effect of calmodulin on erythropoietin-induced inhibition of enzyme activity. The effect of exogenous calmodulin on enzyme activity in the presence and absence of recombinant erythropoietin (0.1 U/ml) is illustrated in Fig. 3. In membranes unexposed to erythropoietin, calmodulin produced a concentration-dependent increase in activity at up to 100 ng calmodulin/mg membrane protein; a plateau in response occurred at 100 ng/mg. The response to calmodulin of erythropoietin-exposed membranes parallels that of the control membranes, but at a significantly lower level of enzyme activity. Comparing erythropoietin-treated to untreated membranes, full recovery of enzyme activity for a given concentration of calmodulin was incomplete at the highest calmodulin concentrations tested.

Effect of calcium concentration on erythropoietin-induced inhibition of enzyme activity. The effect of erythropoietin (0.1 U/ml) was present over a wide concentration range of free calcium (Fig. 4). There was no shift in the calcium sensitivity of the enzyme in the presence of erythropoietin.

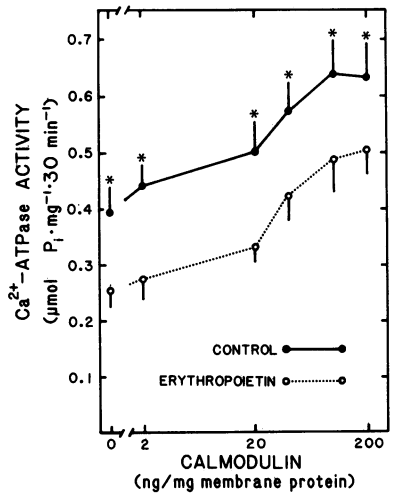

Figure 3. Effect of calmodulin on erythropoietin-induced inhibition of reticulocyte membrane Ca^{2+}-ATPase activity. Purified bovine brain calmodulin was added to membranes at the indicated concentration with or without recombinant erythropoietin (0.1 U/ml), incubated for 1 h at 37°C, then assayed for enzyme activity. Error bars represent the SEM of four experiments. Asterisks denote significant differences between control and erythropoietin-exposed membranes at a given calmodulin concentration ($P < 0.05$, paired t test).

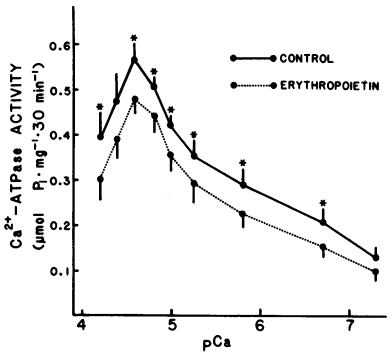

Figure 4. Effect of free calcium concentration of the ATPase assay on the inhibitory action of erythropoietin on reticulocyte membrane Ca^{2+}-ATPase activity. Free Ca^{2+} concentrations were computed (see Methods) and expressed as negative logarithms (pCa). Reticulocyte membranes were incubated with and without human recombinant erythropoietin (0.10 U/ml) for 60 min before enzyme assay conducted at the indicated free Ca^{2+} level. Each point is the mean SEM of four determinations. Asterisks denote significant differences between control samples and those containing erythropoietin ($P < 0.01$, paired t test).

Effect of growth factors other than erythropoietin on reticulocyte membrane Ca^{2+}-ATPase activity. Insulin (1 U/ml) and epidermal growth factor (100 ng/ml) had no inhibitory effect on enzyme activity (96±2% and 102±4%, respectively, of parallel controls [$n = 3$]).

Discussion

The erythropoietin effect on reticulocyte membrane Ca^{2+}-ATPase shown here is highly specific in that it is neutralized by antierythropoietin antibody and the effect is not mimicked by other growth factors tested. Biological relevance of our observation is supported by the similar erythropoietin dose range for Ca^{2+}-ATPase inhibition and for stimulation of CFU-E in vitro (13). Serum levels of erythropoietin measured by radioimmunoassay in anemia and secondary polycythemia are often in the concentration range used in the current studies (14, 15).

The function served by the inhibitory action of erythropoietin on membrane Ca^{2+}-ATPase activity, and presumably the calcium pump, is unknown. Such an action would foster in the intact cell an increase in free intracellular calcium, an erythropoietin effect demonstrated by Mladenovic et al. (3), on purified human progenitor cells. The increase in calcium uptake in response to erythropoietin noted by Sawyer and Krantz (2) indicates erythropoietin may have more than one effect on membrane function. It would be of interest to perform similar studies on the intact reticulocyte.

The biochemical mechanism of action of erythropoietin on reticulocyte membrane Ca^{2+}-ATPase is also a matter for speculation. Erythropoietin does not alter the calcium optimum of the enzyme (Fig. 4), indicating the effect is expressed in terms of the V_{max} of the enzyme. Erythropoietin is not acting simply as a competitive antagonist of calmodulin, the calcium-dependent regulatory protein for the Ca^{2+}-ATPase enzyme, since exogenous calmodulin does not completely overcome the inhibition induced by erythropoietin (Fig. 3). Erythropoietin is thought to act in the intact progenitor cell through a small number of high affinity receptor sites (6) on the cell surface. Studies of the cellular distribution (16) and mechanism of signal transduction

of this receptor have been limited. No studies address the issue of receptors on stress reticulocytes. Decreased phosphorylation of the plasma membrane has been noted to be an early finding in response to erythropoietin in the Rauscher leukemia cell line (17). This suggests erythropoietin is acting through a kinase or a phosphatase at the membrane level.

Acknowledgments

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