

Erythropoiesis In Vitro

ROLE OF CALCIUM

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ABSTRACT The in vitro plasma clot technique was employed to examine the role of calcium during the interaction of erythropoietin and mouse erythroid progenitor cells. Erythropoietin-induced erythroid colony formation was increased 24% by the carboxylic ionophore A23187 (10 nM), whereas a 35% increase was produced by the carboxylic ionophore Ro 2-2985/1 (1 nM). EGTA (3 mM) inhibited erythropoietin-induced erythroid colony formation. Inhibition of erythroid colony formation by EGTA could be reversed by Ca^{2+} , but not by Mn^{2+} , Mg^{2+} , Zn^{2+} , or Fe^{2+} . At least 30 min exposure of marrow cells to erythropoietin in vitro was required for production of erythroid colonies. EGTA substantially inhibited erythropoietin-induced erythroid colony formation even when the marrow cells were exposed to the hormone for up to 2 h before addition of the chelator. Marrow cells incubated first in calcium-free medium with erythropoietin and then cultured in the presence of calcium but not erythropoietin, failed to form erythroid colonies although colony formation occurred when erythropoietin was provided. Taken together, the data indicate that calcium is required for both extracellular and intracellular events during the interaction of erythropoietin with its target cells.

INTRODUCTION

Erythropoietin stimulates committed erythroid progenitor cells to differentiate but the mechanism involved is unknown (1). Experiments employing trypsin-treated marrow cells suggest that erythroid progenitor cells have surface receptors for erythropoietin (2). These cells also have β adrenergic receptors (3) and, under in vitro conditions, differentia-

tion and proliferation of erythroid precursor cells are enhanced by catecholamines (3) and other agents that interact with membrane-bound adenylate cyclase (4–6). It is unlikely, however, that activation of adenylate cyclase is the sole requirement for initiating erythroid cell differentiation. First, none of the β adrenergic agonists can initiate erythropoiesis in the absence of erythropoietin (3–6). Second, these agonists appear to act only on a subpopulation of erythroid progenitor cells which can be distinguished from the bulk of erythropoietin-sensitive cells by their behavior during gradient sedimentation (3, 5). Finally, no increase in cyclic AMP was observed when erythropoietin alone was incubated with its target cells (7). The alternative possibility that cyclic GMP is involved in the initiation of erythroid cell differentiation has not been substantiated (8).

Differentiation and proliferation of erythroid cells in vitro can be influenced by agents such as dimethyl sulfoxide (9) and amphotericin (9, 10) that alter cation flux at the level of the plasma membrane (11, 12). Alterations in transmembrane cation flux have a profound effect on the ability of cells to proliferate and differentiate (13), and calcium in particular has been implicated as an intracellular messenger for these processes in certain tissues (14). Because there is no compelling evidence supporting internalization of erythropoietin by its target cells (15) or implicating cyclic nucleotides as the sole intracellular messenger for the hormone, we investigated the role of divalent cations in the interaction of erythropoietin and its target cells. Our data indicate that one divalent cation, calcium, is required for the interaction of erythropoietin with erythroid progenitor cells.

METHODS

Preparation of marrow cell suspensions. Swiss-Webster female mice weighing 20–25 g (Buckberg Lab Animals, Tomkins Cove, N. Y.) were used in all experiments. The mice were killed by cervical dislocation and the femurs rapidly removed. Marrow cells were obtained by flushing the femoral marrow cavities with 2 ml of the alpha modification

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of Eagle's Minimal Essential Medium (MEM-alpha)¹ containing Hanks' balanced salt solution (16), and 50 U/ml penicillin and 50 µg/ml streptomycin. The cells were dispersed by repeated aspiration through a 23-gauge needle and adjusted to a concentration of 10⁷ cells/ml with MEM-alpha.

Erythroid colony assay. Erythroid colonies were grown in vitro employing the plasma clot technique described by McLeod et al. (16). To a mixture containing 0.2 ml fetal calf serum (Hem Research, Inc., Rockville, Md.), 0.1 ml deionized bovine serum albumin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), 1 U bovine thrombin (Parke, Davis & Co., Detroit, Mich.) in 0.1 ml NCTC 109 (Microbiological Associates, Walkersville, Md.), and 0.1 U erythropoietin (Sheep Plasma Step III 7 U/mg protein, Connaught Laboratories, Toronto, Canada) in 0.1 ml NCTC 109, were added 1 × 10⁶ mouse marrow cells in 0.1 ml MEM-alpha. Compounds to be tested were added to the cell-containing mixture in either NCTC 109 or 10 µl of absolute ethanol; control cultures received the vehicle alone. Additional NCTC 109 was used to bring the final volume to 0.9 ml. After the addition of 0.1 ml bovine citrated plasma (Grand Island Biological Co., Grand Island, N. Y.) and thorough mixing, 0.1-ml aliquots of the mixture were placed in the wells of a microtiter plate (Linbro Chemical Co., Hamden, Conn.) and allowed to clot. The plasma clots were incubated at 37°C in a tissue culture incubator (Napco Industries, Portland, Oreg.), with a high humidity, 5% CO₂-95% air atmosphere. After 48 h, a benzidine and hematoxylin-stained squash preparation of each plasma clot was obtained (16), and the number of benzidine-positive colonies containing eight or more cells were counted on three separate clots for each experimental point. Student's *t* test was used for analysis of the significance of differences.

Preliminary studies of in vitro erythroid colony formation revealed a linear relationship between the number of nucleated marrow cells cultured and the number of erythroid colonies present at 48 h. As previously described (16), the relationship between erythroid colony number and the concentration of added erythropoietin was sigmoidal with a plateau at 0.1 U/ml.

The carboxylic ionophore A23187 was a gift from Dr. Robert Hamill, Lilly Research Laboratories (Indianapolis, Ind.). Ro 2-2985/1, the sodium salt of the carboxylic ionophore X537A, was a gift from Dr. W. E. Scott, Hoffmann-La Roche, Inc. (Nutley, N. J.). EGTA was obtained from Sigma Chemical Co. (St. Louis, Mo.), dissolved in NCTC 109, and neutralized with NaOH before addition to the culture mixture. Polyethylene glycol (1,2-¹⁴C) (0.38 mCi/g sp act) was obtained from New England Nuclear (Boston, Mass.). All other chemicals employed were reagent grade.

RESULTS

To investigate the possibility that divalent cations are involved in the proliferation and differentiation of erythroid progenitor cells, we employed the carboxylic ionophores A23187 and Ro 2-2985/1. Incorporation of either ionophore in the plasma clot cultures caused a modest enhancement of erythroid colony formation (Table I). At a concentration of 10 nM, A23187 produced a 24% increase in erythroid colonies whereas 1 nM Ro 2-2985/1 increased colony number by 35% in

TABLE I
Effect of Carboxylic Ionophores on Erythroid Colony Formation

Ionophore concentration	Colonies/10 ⁶ cells*	
	A23187	Ro 2-2985/1
None	220±5	220±5
1 µM	41±6	65±8
0.1 µM	150±15	133±19
10 nM	272±5†	111±12
1 nM	190±10	298±18†
0.1 nM	—	200±17

Cultures contain 0.1 U/ml erythropoietin.

* Mean±SEM.

† *P* < 0.005.

the presence of a maximal stimulatory concentration of erythropoietin (0.1 U/ml). Neither ionophore supported erythroid colony formation in the absence of erythropoietin nor was a 30- to 60-min exposure to A23187 sufficient to potentiate the effect of erythropoietin (data not shown).

Enhancement of erythroid colony formation by A23187 and Ro 2-2985/1 suggested that divalent cations were involved in this process. Confirmation of a requirement for divalent cations was obtained by culturing the marrow cells in the presence of the chelating agent EGTA. Erythroid colony formation in vitro was inhibited by EGTA at a concentration of 3 mM although 1 mM EGTA had no effect on either colony number (Table II) or appearance. The inhibition was not a result of the influence of EGTA on other humoral regulators such as colony-stimulating factor or erythroid-enhancing factor (17) because colony growth with erythropoietin partially purified by sequential chromatography concanavalin A-agarose and Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.) was inhibited by EGTA to the same extent as crude erythropoietin (data not shown). Inhibition of colony formation by EGTA was also not the result of a toxic effect of the chelator for the reason that colony growth could be re-

TABLE II
Effect of EGTA on Erythroid Colony Formation

EGTA concentration	Colonies/10 ⁶ cells*
mM	
None	1,572±18.5
1	1,475±180
3	40±3.0
5	79±1.5
10	15±3.0

* Mean±SEM. Cultures contain 0.1 U/ml erythropoietin.

¹ Abbreviation used in this paper: MEM-alpha, Eagle's Minimal Essential Medium (alpha modification).

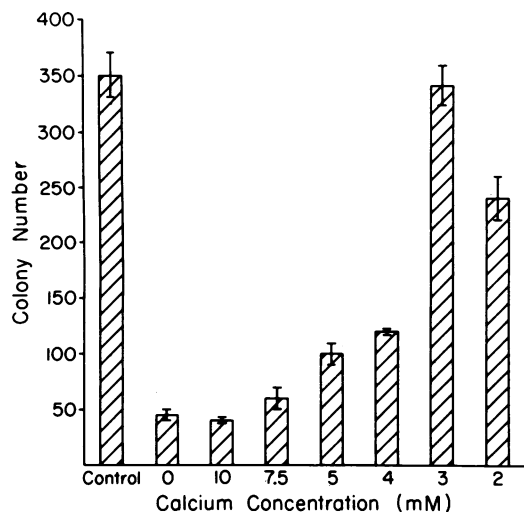


FIGURE 1 The effect of adding various concentrations of calcium chloride on erythroid colony formation in the presence of 5 mM EGTA. The control culture had a total calcium content of 1.8 mM and did not contain EGTA. The values represent the mean \pm SEM of three cultures.

stored when calcium chloride was added to the cultures in the presence of the EGTA (Fig. 1).

Prevention of EGTA-induced inhibition of erythroid colony formation was specific for calcium because it could not be prevented by $MgCl_2$, $ZnCl_2$, $MnCl_2$, or $FeSO_4$ at concentrations of 1–4 mM (Fig. 2). Prevention of inhibition of colony formation by EGTA was most satisfactory with 3 mM calcium chloride; further increments in calcium concentration failed to fully restore erythroid colony formation in the presence of 5 mM EGTA. This was most likely the result of a toxic effect of the high concentrations of calcium employed, because in the absence of EGTA, increasing

the calcium content of the culture medium from 1.8 to 4.8 mM suppressed colony formation by 47%.

To determine whether the requirement for calcium during erythroid colony formation was time dependent, mouse marrow cells were incubated *in vitro* with erythropoietin at 37°C in a high humidity 5% CO_2 -95% air atmosphere for selected time intervals before adding EGTA and bovine citrated plasma. As shown in Table III, when EGTA was added to the cultures at the time of exposure of the cells to erythropoietin or 15 min later, erythroid colony formation was inhibited by ~95%. Exposure of the cells to erythropoietin for periods of 30–120 min before adding EGTA resulted in an additional increment in erythroid colony formation. The number of colonies formed, however, was never >16% of the colonies formed in the absence of EGTA.

These results indicate that calcium is not only required for erythropoietin-induced colony formation but also that the requirement is time dependent and occurs at an early period during the interaction of erythropoietin and its target cells. The data do not indicate, however, whether calcium is required for binding erythropoietin to its target cells, whether it satisfies a metabolic need of the hormone-stimulated cells, or whether it is involved in both processes. To further define the role of calcium, it was necessary to determine the minimal *in vitro* exposure time to erythropoietin required by erythroid progenitor cells for erythroid colony formation in plasma clot cultures.

Marrow cells (3×10^6) suspended in 1 ml MEM-alpha containing 20% fetal calf serum and erythropoietin (0.1–0.2 U) were incubated at 37°C in a high humidity (5% CO_2 -95% air) atmosphere. At selected time intervals, an equal volume of cold MEM-alpha was added and the cell mixture was centrifuged at 1,000 g for 5 min at 4°C. The cells were resuspended in cold

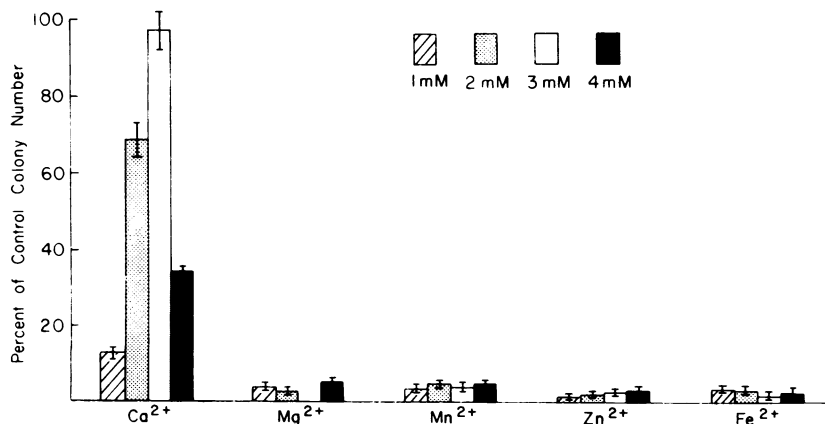


FIGURE 2 The ability of various concentrations of five divalent cations to prevent inhibition of erythroid colony formation by 5 mM EGTA. The data are expressed as the percent of control number achieved and represent the mean of three cultures.

TABLE III
Effect of Time of Addition of EGTA on
Erythroid Colony Formation

Time of addition of EGTA	Colonies/10 ⁶ cells*		Percent of control
	No EGTA	EGTA	
min			
0	203.5±24.6	12.0±1.0	5.8
	197.2±26.1	11.0±2.5	5.5
15	191.7±20.7	8.3±1.4	4.3
	260.0±18.5	14.0±2.8	5.3
30	187.0±28.0	17.0±1.9	9.0†
	245.0±27.7	32.3±3.8	13.1†
60	175.6±30.7	18.3±2.3	10.4†
	232.3±12.3	25.5±4.2	10.9†
120	173.0±16.5	28.0±8.4	16.1†
	234.6±23.9	33.2±5.9	14.1†

* Mean±SEM. Marrow cells (10⁶/ml) were incubated with erythropoietin (0.1 U/ml) for selected time intervals before addition of EGTA (5 mM).

† Erythroid colony formation at 30, 60, and 120 min in the presence of EGTA was significantly different from that at 0 and 15 minutes ($P < 0.05$).

MEM-alpha and washed twice. After the final wash, the cells were resuspended in 0.3 ml MEM-alpha, and 0.1-ml aliquots were cultured with and without erythropoietin.

Erythroid colony formation was never >5% of control values when marrow cells were exposed to the erythropoietin for time periods up to 15 min (Table IV). After 30 min of exposure to the hormone, erythroid colony formation increased to 18% of control values and no further increment was observed when exposure was continued for up to 1 h. These observations indicate that a minimum of 30 min of exposure to erythropoietin in vitro is required for induction of significant numbers

TABLE IV
Effect of Exposure Time to Erythropoietin In Vitro on
Erythroid Colony Formation

Duration of exposure to erythropoietin (0.1 U/ml)	Percent of 48 h colony number*
h	
48	100
0.08	3±1.7
0.25	4±1.4
0.50	18±5.8
1.0	19±1.1

* Mean±SEM.

of erythroid colonies in plasma clot cultures. Failure to obtain >20% of control colony formation when erythropoietin exposure was limited to 1 h or less suggests that within the population erythroid progenitor cells, there exist subpopulations that either differ with respect to their erythropoietin requirements or are not synchronous with regard to their ability to respond to the hormone.

To exclude the possibility that residual erythropoietin trapped within the cell pellet during washing was responsible for the induction of colony formation in these experiments, we employed ¹⁴C-labeled polyethylene glycol as a measure of trapped extracellular fluid (18). The labeled polyethylene glycol was added to the incubation medium, and the radioactivity of the mixture was determined. After the cells were washed according to the protocol, the radioactivity remaining in the cell pellet was determined. In repeated experiments, <0.08% of the added [¹⁴C]polyethylene glycol was recovered in the cell pellet. This quantity of [¹⁴C]polyethylene glycol represents ~1 μl of incubation medium containing 0.0001–0.0002 U of erythropoietin, an amount too small to stimulate erythroid colony formation in the plasma clot system.

For the reason that the growth of significant numbers of erythroid colonies could be obtained with only limited exposure of erythropoietin to its target cells, it was possible to determine whether calcium was required for binding erythropoietin to its target cells. The protocol for this experiment is shown in Table V. Marrow cells were incubated with or without EGTA in the presence of erythropoietin at 37°C in a 5% CO₂–95% air atmosphere. The concentration of erythropoietin was 0.2 U/ml in order to prevent hormone concentration from being a limiting factor. Based on the data in Table IV, the marrow cells were exposed to erythropoietin for 1 h to assure significant erythroid colony formation. After the incubation period, the cells were washed twice with calcium-free medium before cul-

TABLE V
Effect of EGTA on the Binding of Erythropoietin
to Bone Marrow Cells

Preculture treatment	Erythroid colony formation	
	Erythropoietin added (0.1 U/ml)	Erythropoietin not added
	Colonies/10 ⁶ cells*	
Incubation with 5 mM EGTA and erythropoietin (0.2 U/ml)	251±7	1±0.5
Incubation with erythropoietin (0.2 U/ml)	249±7	39±3

* Mean±SEM.

turing in medium containing calcium with or without erythropoietin (0.1 U/ml).

Marrow cells exposed to erythropoietin and EGTA failed to produce erythroid colonies when cultured in the absence of erythropoietin (Table V). By contrast, when marrow cells were exposed to erythropoietin alone for 1 h and cultured in its absence, erythroid colony formation was 16% of control values. This is within the range expected from our preliminary studies (Tables III and IV). Failure of marrow cells exposed to both erythropoietin and EGTA and subsequently cultured in the absence of erythropoietin to achieve this level of colony formation suggests that EGTA prevented the interaction of erythropoietin with its target cells. Because addition of erythropoietin restored colony formation to normal levels, neither EGTA toxicity, calcium deficiency, nor manipulation of the marrow cells can be invoked to explain the failure of colony formation when the cells were preincubated with both the hormone and the chelator and then cultured in their absence.

To examine the possibility that EGTA directly inactivates erythropoietin, we dissolved 2.0 U of erythropoietin in phosphate-buffered saline (pH 7.4) containing 5 mM EGTA and injected aliquots of this solution into exhypoxic polycythemic mice. After 48 h, the mice were injected with ^{59}Fe . Erythrocyte ^{59}Fe incorporation (mean \pm SEM) in mice injected with the erythropoietin-EGTA mixture ($11.8 \pm 3.1\%$) was identical to that in mice injected with erythropoietin alone ($10.4 \pm 3.1\%$). Incorporation of ^{59}Fe in control mice receiving EGTA alone was $2.6 \pm 0.7\%$ ($P < 0.025$).

DISCUSSION

The *in vitro* erythroid colony assay developed by Axelrad and his associates (16) provides a model system for studying the interaction between erythroid progenitor cells and erythropoietin or other agents that stimulate cell proliferation and differentiation. To date many such agents have been observed to enhance erythropoietin-induced erythroid colony formation *in vitro*. They include corticosteroids (19), androgens (20), growth and thyroid hormones (4–6, 21), catecholamines (3), cyclic nucleotides (22), cholera toxin (22), and prostaglandins (23). Considering the diverse nature of the substances employed, it is difficult to identify a common site on the erythroid progenitor cell at which they might act. Polypeptide hormones, catecholamines, and cholera toxin interact with membrane-bound adenylate cyclase. Steroid hormones influence cell function by interactions within the cell nucleus or by promoting the synthesis of cyclic AMP (24). Because none of these agents can stimulate erythropoiesis *in vitro* in the absence of erythropoietin, it is unlikely

that only the receptors at which they act govern erythroid cell differentiation. Furthermore, as a result of the complexity of the culture medium employed, it is possible that some of these agents may influence erythroid colony formation in a nonspecific fashion (25–27).

In the present study, we investigated the possibility that divalent cations are involved in the initiation of erythropoiesis by erythropoietin. Several types of evidence were obtained indicating that one divalent cation, calcium, has an integral role in erythropoietin-induced erythroid cell differentiation.

Addition of the carboxylic ionophores A23187 and Ro 2-2985/1 to the plasma clot cultures enhanced erythropoietin-induced erythroid colony formation. The ionophores failed to stimulate erythroid colony formation in the absence of erythropoietin, suggesting that the translocation of calcium across the plasma membrane which these agents promote is either a necessary but not sufficient signal for erythroid cell differentiation and proliferation or serves to recruit a subpopulation of erythroid progenitor cells to respond to the hormone. The modest degree of enhancement of colony formation may be a consequence of either of these possibilities or of the increased sensitivity of rodent cells to the toxic effects of the carboxylic ionophores (28).

The effect of the ionophores on *in vitro* erythroid colony formation was similar in several respects to their effect on phytohemagglutinin-induced lymphocyte transformation (28, 29). In each instance, the ionophores potentiated the effect of the inducing agent and did this within a narrow concentration range. In neither instance was a brief exposure to the ionophores sufficient to influence cell proliferation. In contrast to their effect on erythroid progenitor cells, however, the carboxylic ionophores stimulated lymphocyte transformation in the absence of an inducing agent (28, 29).

To further define the role of divalent cations in erythroid cell differentiation and proliferation, we employed the chelating agent EGTA, which in contrast to EDTA, has a high affinity for divalent cations (30). EGTA inhibited erythropoietin-induced erythroid colony formation and the inhibitory effect of the chelator could be prevented by the addition of calcium but not magnesium, zinc, manganese, or iron. Inhibition of phytohemagglutinin-induced lymphocyte transformation by EGTA is also reversed by calcium but not other divalent cations (31).

The time-course of EGTA-induced inhibition of erythroid colony formation and its reversal by calcium indicated that the initial requirement for the divalent cation occurred early in the course of erythropoietin-induced proliferation and differentiation. Our data suggest but do not prove that one function of calcium is to promote the binding of erythropoietin to its target

cells. Calcium also appears to be required for intracellular events associated with the initiation of erythropoiesis. This is supported by the effects of the carboxylic ionophores as well as the substantial suppression of colony formation by EGTA after binding of erythropoietin to its target cells should have occurred. It is noteworthy in this regard that adenylate and guanylate cyclases and phosphodiesterase are among the enzymes whose activity is influenced by calcium (14). Consequently, it is possible that calcium serves as a regulator of cyclic nucleotide metabolism within developing erythrocytes. This would explain the ability of β adrenergic agonists to potentiate the effect of erythropoietin, although failing to stimulate erythroid colony formation in the absence of the hormone (3–6). Alternatively, hormone-induced alterations in transmembrane calcium flux might serve to alter the translocation of other cations such as potassium (14) and thus influence cellular metabolism (32). In keeping with this, we have recently reported that ouabain enhances erythropoietin-induced erythroid colony formation in vitro (33). Further studies will be required to define the functions of calcium during the initiation of erythropoiesis and whether erythropoietin is directly responsible for alterations in the transmembrane flux of this divalent cation.

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