We have investigated whether recombinant erythropoietin (r-Epo) elicits a change in intracellular free calcium (IFC) in purified Epo-responsive cells in spleens of mice treated with phenylhydrazine. Colony-forming units (CFU-E) were prepared by negative selection through immunologic panning. Anti-Forssman, Mac-1, Ia, and HSA antibodies were used to eliminate nonhematopoietic progenitors. After two pannings, 29 ± 1.5% (mean ± 1 SD) of the recovered cells were CFU-E. IFC was measured by labeling cells with the fluorescent dye Indo-1 and analyzing them on a flow cytometer from 15 seconds to 30 minutes after the addition of agonist. At each step of the panning procedure, there was no effect of r-Epo (0 to 10 U/mL) on IFC even in the larger cells that are predominantly CFU-E. As a positive control, calcium ionophore (A23187) significantly increased IFC in >90% of the spleen cells enriched in CFU-E. During growth of CFU-E in methylcellulose, the calcium ionophore did not affect the r-Epo–dependent formation of erythroid colonies. EGTA inhibited the formation of erythroid colonies. This inhibition appeared to be the result of a toxic effect of the chelator because the colony growth could not be restored when Ca2+ was added to the cultures in the presence of the EGTA. We conclude that the biologic action of Epo on responsive erythroid cells does not depend on acute changes in IFC.

ERYTHROPOIETIN (Epo) is a glycoprotein hormone required for the proliferation and differentiation of erythroid progenitor cells. There is very little information to date on the molecular events that follow the binding of Epo to its receptor. Alterations in transmembrane cation flux have a profound effect on the ability of cells to proliferate and differentiate. Calcium, in particular, has been implicated as an intracellular messenger for these processes in certain tissues. Several studies have suggested a role for calcium in the mediation of Epo’s action. However, the results in these reports are conflicting, perhaps because of the use of heterogeneous populations of hematopoietic cells. In this study we have used negative immunologic panning to partially purify colony-forming units (CFU)-erythroid (E) from spleens of normal mice treated with phenylhydrazine, and then determined the effect of Epo on intracellular free calcium (IFC) by FACS measurement of Indo-1 fluorescence. We also examined the effect of calcium ionophore and calcium chelation on the development of erythroid colonies.

MATERIALS AND METHODS

Enrichment of CFU-E. Phenylhydrazine hydrochloride (60 mg/kg of body weight per day) was injected into mice (C57BL × C3H, hybrids, Jackson Laboratory, Bar Harbor, ME) on days 0 and 1. Animals were killed on day 4. Immediately after resection, spleens were teased, chopped, and then passed through 18G, 21G, and 23G needles. After centrifuging the cells, red cell lysing buffer (NH4CI-Tris) was added to the pellets. The cells were resuspended, washed with cold PBS before use. The washed cells bound to the four rat anti-mouse monoclonal antibodies were spread and incubated for 30 minutes on ice with four rat anti-mouse monoclonal antibodies (Zymed Laboratories, San Francisco). Anti-Forssman antibody binds to post CFU-E erythroblasts and weakly to later stage myeloid cells; anti-Mac-1 antibody binds to polymorphonuclear cells and monocytes; anti-Ia M5/114 antibody binds weakly to B lymphocytes (but not T cells) and monocytes, and anti-HSA M1/75 antibody binds to late erythroblasts. Antibody-coated cells were then washed twice with a minimal essential medium (α-MEM) (GIBCO, Grand Island, NY)/2% fetal calf serum (FCS); heat-inactivated 56°C for 30 minutes; Hazelton Research Products, Denver, PA) and once with phosphate buffered saline (PBS)/5% FCS to remove excess antibody before panning. Anti-immunoglobulin (lg) (lg) plates were prepared by incubating culture plates with affinity-purified goat anti-rat lg (Zymed) overnight at 4°C followed by washing with cold PBS before use. The washed cells bound to the four rat anti-mouse monoclonal antibodies were spread and incubated overnight with goat anti-lg coated plates (2 mL of PBS/5% FCS/10 cm plate to be used: 25 × 106 cells/plate) and incubated for one hour at 4°C. The nonadherent antibody-negative cells were recovered by gentle pipetting without disrupting the antibody-coated cells bound to the plates, and washed twice with 4 mL PBS/5% FCS by gentle swirling, tilting, and decanting. A second incubation on another goat anti-lg coated plates was carried out to ensure removal of all antibody-labelled cells. At each stage of this enrichment procedure the cells were >90% viable as judged by exclusion of Trypan blue.

Cell culture. Cells were cultured in 0.9% methylcellulose (Fluka, Ronkonkoma, NY), 20% FCS, 1.3% deionized BSA (Sigma Chemical Corp, St Louis), penicillin and glutamine (GIBCO), and 1 × 10−4 mol/L 2-mercaptoethanol (Sigma). For the formation of CFU-E, 2 U/mL of pure r-Epo (gift from Genetics Institute, Inc, Cambridge, MA) were added to cultures along with 5% burst promoting activity (BPA). Pokeweed mitogen stimulated spleen cell supernatant (PWM-SCM) was used as a source of BPA. The cells were plated in 0.5-mL duplicates in flat-bottomed 24-well tissue culture plates (Linbro, Flow Laboratories Inc, McLean, VA) and incubated at 37°C in a high humidity 5% CO2, 95% air incubator. Cell densities of 5 × 105 cells/mL before panning and 106 cells/mL after panning were sufficiently low to clearly distinguish individual colonies. CFU-Es were counted on day 3. The number and morphology of erythroid colonies at day 3 did not differ significantly from those at day 2.

FACS analysis. After two pannings, nonadherent cells were collected and adjusted to 1 × 105 cells/mL by α-MEM (containing 1.8 mmol/L Ca2+). These cells were incubated with 2 μmol/L Indo-1 (AM) (Molecular Probes, Inc, Eugene, OR) for 30 minutes.
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quadrant delimiters were set on all histographs so as to divide cells with respect to volume were identified in the partially purified described.'7 Relative IFC is reported as the 405 nm/480 nm emission was measured at 405 nm and 480 nm as previously described.15 Relative IFC is reported as the 405 nm/480 nm fluorescence ratio. Data were collected as contour histograms of cell volume v fluorescence ratio and analyzed on a Consort 30 microcomputer (Becton-Dickinson). A significant increase in IFC levels was determined at 37°C using a mercury-arc lamp FACS Analyzer I (Becton-Dickinson, Mountain View, CA). Excitation was at 353 nm and fluorescence emission was measured at 405 nm and 480 nm as previously described.17 Relative IFC is reported as the 405 nm/480 nm fluorescence ratio. Data were collected as contour histograms of cell volume v fluorescence ratio and analyzed on a Consort 30 microcomputer (Becton-Dickinson). A significant increase in IFC was defined as an increase in the fluorescence ratio >2 SD above the baseline. No significant increase in IFC was noted following addition of r-Epo. Likewise no significant decrease in IFC was noted (data not shown).

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at 37°C. After the loading procedure, the cells were washed at room temperature and then placed in α-MEM at 4 × 10^6 cells/mL. After 30 to 60 minutes 250-μL aliquots of the cell suspension were incubated at 37°C with a chosen concentration of pure r-Epo diluted in PBS. In some experiments, calcium ionophore (A23187) (Sigma) was added to the cells at 37°C. IFC levels were determined at 37°C using a mercury-arc lamp FACS Analyzer I (Becton-Dickinson, Mountain View, CA). Excitation was at 353 nm and fluorescence emission was measured at 405 nm and 480 nm as previously described.15 Relative IFC is reported as the 405 nm/480 nm fluorescence ratio. Data were collected as contour histograms of cell volume v fluorescence ratio and analyzed on a Consort 30 microcomputer (Becton-Dickinson). A significant increase in IFC was defined as an increase in the fluorescence ratio >2 SD above the baseline. No significant increase in IFC was noted following addition of r-Epo. Likewise no significant decrease in IFC was noted (data not shown).

RESULTS

After phenylhydrazine treatment but before red-cell lysis, 9.2 ± 2.1% (mean ± 1 SD) of the nucleated cells were detected as CFU-E. After the first panning step, 26 ± 4.5% (mean ± 1 SD) of the cells were CFU-E, whereas after the second panning steps, 29 ± 1.5% were CFU-E. Eighty-eight percent of the CFU-E in the crude cell preparation were recovered after the second panning. The proportion of large cells measured by FACS before enrichment was 20%, whereas after two panning steps the proportion of large cells increased to 53% (Fig 2, A and C). Cells sampled at each step of the enrichment were incubated for three minutes at 37°C with 0.5 U/mL of r-Epo. No change in IFC was noted in either the large cells or the small cells (Fig 2, B and D). Moreover, as shown in Fig 1, serial measurements over a six-minute time span after adding r-Epo (0.5 U/mL) revealed no significant change in IFC in either the crude cell population or the enriched population, including the subset of large cells that are most likely to include CFU-E. The addition of higher concentrations of r-Epo (1.5 and 10 U/mL) to the enriched cells also failed to affect the IFC over a six-minute period (Fig 1). Moreover, no significant change in IFC was noted over a 30-minute period of incubation. The same results were obtained on cells that had been preserved in serum-free medium for eight hours prior to the loading of dye. As a positive control, the addition of the calcium ionophore A23187 at a concentration of 200 nmol/L to both the crude cell population and the enriched population resulted in the expected marked increase in IFC. Within five minutes the ratio of 405 nm/480 nm increased tenfold in >90% of the spleen cells enriched in CFU-E (Fig 2, E and F).

Fig 1. Time course of the change in IFC of unfractionated cells and CFU-E-enriched cells at different concentrations of r-Epo. The ordinate is the percentage of cells with a fluorescence signal (405 nm/480 nm) >2 SD above the baseline. No significant increase in IFC was noted following addition of r-Epo. Likewise no significant decrease in IFC was noted (data not shown).
Thus, the Indo-1 effectively entered the cells and was adequately de-esterified, allowing a change in $[\text{Ca}^{2+}]$ to be monitored in virtually all of the cells.

In order to explore the possible contribution of relatively long-term alterations in IFC on in vitro erythropoiesis, we examined the effect of calcium ionophore and EGTA on the maturation of enriched Epo-responsive cells in methylcellulose culture. As shown in Fig 3, there was no significant effect of calcium ionophore between 1 and 1,000 nmol/L in the presence of 2 U/mL r-Epo; 5% PWM-SCM; or both. The formation of CFU-E in vitro was inhibited by 5 mmol/L EGTA (pH 7.4) both in the presence and absence of r-Epo and PWM-SCM (Fig 3). In order to determine whether the inhibition of the formation of CFU-E by EGTA was due to lowering calcium by chelator, calcium chloride was added to the culture in the presence of EGTA (Fig 4). EGTA (5 mmol/L) inhibited the CFU-E formation in the presence of either r-Epo (2 U/mL) alone or in combination with 5% PWM-SCM. The formation of CFU-E could not be restored when up to 5 mmol/L calcium chloride was added to the culture medium, containing 1.8 mmol/L Ca$^{2+}$. Therefore the inhibition by EGTA of colony formation appears to be due to an effect independent of its chelation of calcium.

**DISCUSSION**

A large body of published work documents that striking alterations in IFC accompany functional perturbations of peripheral blood cells. Examples include the mitogenic stimulation of lymphocytes,4,6,17,19 the response of neutrophils to chemoattractants,20 and the activation of platelets.21,22 In contrast there is little information on the role of IFC in the growth and maturation of hematopoietic cells, owing in part...
to the difficulty in obtaining pure populations of progenitor cells and, until recently, the lack of well-characterized and purified hematopoietic growth factors.

There are conflicting reports in the literature about the role of IFC in erythropoiesis and the response to Epo. In 1979, Misiti and Spivak presented evidence that the response to Epo is affected by alterations in extracellular and intracellular calcium. These investigators used unfraccionated bone marrow from normal mice and of necessity an impure source of Epo. Epo-induced erythroid colony formation was increased by 10 nmol/L calcium ionophore (A23187) whereas 5 mmol/L EGTA inhibited Epo-induced erythroid colony formation. The inhibition of erythroid colony formation by EGTA could be reversed by 2 to 4 mmol/L Ca2+. We examined the effect of calcium ionophore and EGTA using a cell population enriched in CFU-E. In our hands, 1 to 1,000 nmol/L A23187 did not alter the formation of CFU-E. EGTA (5 mmol/L) inhibited the formation of CFU-E. However, this inhibition by EGTA could not be reversed by the addition of 1 to 5 mmol/L Ca2+. Therefore this inhibition of the formation of CFU-E by EGTA is unrelated to calcium binding.

The potential importance of IFC in response to Epo was strengthened by experiments of Sawyer and Krantz who showed that Epo stimulates 45Ca2+ uptake into Friend virus-infected erythroid cells. Their measurements were performed on bursts of erythroid cells plucked from plates after five days of culture. Thus, they used relatively mature cells that would be expected to have limited Epo responsiveness. Furthermore, they examined the effect of Epo on transmembrane calcium flux rather than IFC. Subsequently Epo was shown to inhibit membrane Ca2+-ATPase activity of reticulocytes, an even later stage in erythroid maturation with presumably an even lower number of Epo receptors.

**Fig 3.** The effect of calcium ionophore (A23187) and EGTA on the formation of CFU-E. Nonadherent cells after two panning steps were cultured with 1 to 1,000 nmol/L calcium ionophore (A23187) or 1 to 10 mmol/L EGTA. Values are means ± 1 SD.

**Fig 4.** The effect of EGTA and CaCl2 on the formation of CFU-E. Nonadherent cells after two panning steps were cultured with 2 U/mL r-Epo (CT1) or with 2 U/mL r-Epo + 5% PWM-SCM (CT2). One to 5 mmol/L CaCl2 was added to the cultures in the presence of 5 mmol/L EGTA. The culture medium contained 1.8 mmol/L Ca2+. Values are means ± 1 SD.
The studies cited above all deal with relatively long-term observations after the addition of Epo to erythroid precursor cells. The more focused question is whether IFC is altered as part of the signal transduction that attend the binding of Epo to its receptor. Choi et al observed an alteration in the pattern of phosphorylated proteins within 30 minutes after exposure of membranes of mouse erythroid cells and Rauscher erythroleukemia cells to Epo. This result was unaffected by the presence or absence of calcium. To pursue the role of acute Epo-dependent changes in IFC, it is necessary to have an accurate and sensitive method for measuring rapid changes in IFC as well as a uniform population of cells that are highly Epo responsive. In this report we show that recombinant Epo has no significant effect on IFC in a population of mouse erythroid cells highly enriched in CFU-E. Cells at this stage of differentiation are likely to have maximal Epo responsiveness.

We selected the fluorescent dye Indo 1 for measurements of short-term changes in IFC. This agent, along with Fura 2, has a much higher molar fluorescence intensity than its predecessor Quin 2 and therefore is sensitive to very small changes in IFC. Because of rapid rates of binding to and dissociation from Ca²⁺, there is no significant time lag between changes in IFC and the fluorescence signal. Moreover, owing to the shift in emission upon Ca²⁺ binding, the 405 nm/480 nm ratio allows a measurement of IFC that is unaffected by the presence or absence of calcium. To pursue the role of acute Epo-dependent changes in IFC, it is necessary to have a reproducible signal. A possible though unlikely confounding variable prevents detection of the absolute concentration of free calcium [Ca²⁺]. Therefore we report arbitrary fluorescence units rather than calculations of [Ca²⁺].

Ideally measurements of the molecular events in response to the binding of Epo should be performed on pure Epo-responsive cells. In agreement with our findings, recent reports suggest that Epo does not change IFC or calcium influx when added to pure Epo-responsive cells. In contrast, both Mladenovic et al and Miller et al have recently reported that Epo elicits an increase in IFC. The former study used normal unfractionated bone marrow and therefore a heterogenous population of normal hematopoietic cells. In contrast, Miller et al plucked erythroid colonies grown from human umbilical cord blood cells, which means that they used cells that were considerably more mature than CFU-E.

We analyzed a population of cells containing 29 ± 2% CFU-E. Since the CFU-E are predominantly in the larger cell population, the subset of cells > 15 arbitrary volume units (Fig 2, C and D) should comprise at least 50% CFU-E. If the binding of Epo to its receptor on CFU-E were to induce a significant increase in IFC, the use of this highly sensitive Ca²⁺ probe on this cell population should have enabled us to detect a reproducible signal. A possible though unlikely explanation for the negative results in this report is heterogeneity in response to Epo within the CFU-E. This problem could be addressed only by measuring IFC on individual cells as reported by Miller et al.

The inconsistent data on the role of intracellular calcium in response to Epo may reflect differences in the signals induced by Epo during erythroid maturation. Studies demonstrating significant alterations in IFC or calcium flux in response to Epo have used relatively mature erythroid precursors. Notwithstanding the caveats discussed in the above paragraph, our results indicate that in mouse CFU-E, where number of Epo receptors is likely to be maximal, the hormone's action is not accompanied by an acute alteration in IFC.

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