Ion channels in human erythroblasts. Modulation by erythropoietin.

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Research Article

To investigate the mechanism of intracellular Ca2+ ([Cai]) increase in human burst-forming unit-erythroid-derived erythroblasts by erythropoietin, we measured [Cai] with digital video imaging, cellular phosphoinositides with high performance liquid chromatography, and plasma membrane potential and currents with whole cell patch clamp. Chelation of extracellular free Ca2+ abolished [Cai] increase induced by erythropoietin. In addition, the levels of inositol-1,4,5-trisphosphate did not increase in erythropoietin-treated erythroblasts. These results indicate that in erythropoietin-stimulated cells, Ca2+ influx rather than intracellular Ca2+ mobilization was responsible for [Cai] rise. Both Ni2+ and moderately high doses of nifedipine blocked [Cai] increase, suggesting involvement of ion channels. Resting membrane potential in human erythroblasts was -10.9 +/- 1.0 mV and was not affected by erythropoietin, suggesting erythropoietin modulated a voltage-independent ion channel permeable to Ca2+. No voltage-dependent ion channel but a Ca(2+)-activated K+ channel was detected in human erythroblasts. The magnitude of erythropoietin-induced [Cai] increase, however, was insufficient to open Ca(2+)-activated K+ channels. Our data suggest erythropoietin modulated a voltage-independent ion channel permeable to Ca2+, resulting in sustained increases in [Cai].

Ion Channels in Human Erythroblasts

Modulation by Erythropoietin

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Abstract

To investigate the mechanism of intracellular Ca^{2+} ($[Ca_i]$) increase in human burst-forming unit–erythroid-derived erythroblasts by erythropoietin, we measured $[Ca_i]$ with digital video imaging, cellular phosphoinositides with high performance liquid chromatography, and plasma membrane potential and currents with whole cell patch clamp. Chelation of extracellular free Ca^{2+} abolished $[Ca_i]$ increase induced by erythropoietin. In addition, the levels of inositol-1,4,5-trisphosphate did not increase in erythropoietin-treated erythroblasts. These results indicate that in erythropoietin-stimulated cells, Ca^{2+} influx rather than intracellular Ca^{2+} mobilization was responsible for $[Ca_i]$ rise. Both Ni^{2+} and moderately high doses of nifedipine blocked $[Ca_i]$ increase, suggesting involvement of ion channels. Resting membrane potential in human erythroblasts was -10.9 ± 1.0 mV and was not affected by erythropoietin, suggesting erythropoietin modulated a voltage-independent ion channel permeable to Ca^{2+}. No voltage-dependent ion channel but a Ca^{2+}-activated K^+ channel was detected in human erythroblasts. The magnitude of erythropoietin-induced $[Ca_i]$ increase, however, was insufficient to open Ca^{2+}-activated K^+ channels. Our data suggest erythropoietin modulated a voltage-independent ion channel permeable to Ca^{2+}, resulting in sustained increases in $[Ca_i]$. (*J. Clin. Invest.* 1992. 90:1850–1856.) Key words: Fura-2 • digital video imaging • fluorescence microscopy • patch-clamp • Ca^{2+}-activated K^+ channels

Introduction

Signal transduction by erythropoietin (Epo)[1] involves a series of biochemical and ionic regulatory events. After erythropoietin stimulation of erythroid cells, increased intracellular free calcium (1–3), increased cAMP levels (4, 5), and activation of protein kinases (6) have been reported to occur. Evidence suggests that these may follow activation of a GTP-binding protein by the receptor (5, 7). Signals transmitted to the nucleus include increased nuclear calcium (8), activation of nuclear protein kinase C (9), and change in protooncogene expression (10–12). The precise sequence of events that follow hormone/receptor interaction and control erythroid proliferation and subsequent differentiation is undefined.

We have previously shown that Epo induces a dose-dependent increase in intracellular calcium ($[Ca_i]$) in single human burst-forming unit-erythroid (BFU-E)-derived erythroblasts (1), which is specific for stage of differentiation (2), and that Ca^{2+} increase is transmitted to the nucleus (8). The current study was undertaken to investigate the mechanism by which Epo stimulates the increase in $[Ca_i]$. Specifically, the influence of Epo on ionic permeabilities was evaluated. To better elucidate the regulation of membrane voltage and ion currents, we applied, for the first time, whole cell patch-clamp technique to human BFU-E–derived erythroblasts.

Methods

Preparation of BFU-E–derived erythroid precursors. Adult blood was obtained according to a protocol approved by the Milton S. Hershey Medical Center Committee on Clinical Investigation. Adult blood BFU-E were partially purified by 2-aminoethylisothiouronium bromide hydrobromide–treated sheep red blood cell (RBC) rosetting, adherence to plastic, and panning, as described previously (1, 2). Partially purified mononuclear cells from adult blood were cultured in humidified 4% CO_2 at 37°C in 0.9% methylcellulose media containing 30% FCS, 9.0 mg/ml deionized BSA (fraction V; Sigma Chemical Co., St. Louis, MO), 1.4×10^{-4} mol/liter β-mercaptoethanol, 2 U/ml recombinant Epo (> 10,000 U/mg) (Amgen Biologicals, Thousand Oaks, CA), and recombinant granulocyte macrophage colony-stimulating factor (gift of Dr. Steven Clark; Genetics Institutes, Cambridge, MA; 25 ng/ml final concentration). Concentrations of Epo and granulocyte macrophage–colony-stimulating factor were selected for plateau stimulation of growth (2).

To study erythroid precursors at specific stages of differentiation, cells from maturing BFU-E–derived colonies were plucked from culture on day 10. Day 10 cells are partially hemoglobinized and proliferative capacity is decreased since minimal additional increase in colony size occurs subsequently. Myeloid colonies represented < 1% of hematopoietic colonies cultured from partially purified PBMC prepared as described.

Measurement of $[Ca_i]$ in early erythroid precursor cells. BFU-E–derived cells were removed from culture on day 10 and labeled with plateau concentrations of anti-human β2-microglobulin (Chemicon International, Inc., Temecula, CA). Cells were then bound to anti-mouse Ig-coated glass coverslips by incubating at 4°C for 1 h. The cells were incubated in PBS (Table I) at 37°C for 20 min with 1 μmol/liter Fura-2 acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR). Total time lapse from removal of cells from culture to completion of Fura-2 loading was 3–5 h. Cell viability, as judged by trypan blue exclusion, was > 98%. Baseline $[Ca_i]$ and its changes in response to Epo (2

1. *Abbreviations used in this paper:* ANOVA, analysis of variance; BFU-E, burst-forming unit-erythroid; $[Ca_i]$, intracellular Ca^{2+}; Epo, erythropoietin; IP_3, inositol-1,4,5-triphosphate.

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Table I. Composition of Bath and Pipette Solutions

Solution	pH	NaCl	KCl	KASP	CsCl	$MgCl_2$	$CaCl_2$	EGTA	ATP	GTP	Hepes
Bath											
PBS	7.4	137	2.7			0.49	0.68				
EGTA	7.4	137	2.7			0.49	0.68	4			
CsCl	7.4	137			3.4	0.49	0.68				
KASP	7.2	5		140		2	2				10
Filling											
Normal	7.0	5	125			2		1	2		20
CsCl	7.0	5			125	2		1	2		20
KASP	7.2	5		140		2		0.2	1	0.02	10

All concentrations are millimolar. All bath solutions contained 200 mg/dl glucose. PBS and EGTA also contained 4.3 mM Na_2HPO_4 and 1.5 mM KH_2PO_4. Bath CsCl solution contained 7.2 mM Na_2HPO_4.

U/ml) in Fura-2–loaded cells incubated in either PBS with physiologic calcium (0.7 mM) or PBS without calcium (2 mM EGTA) were measured with the fluorescence microscopy–coupled digital video imaging system as described previously (1, 2, 5, 8). Cells were chosen based on visible Fura-2 fluorescence and normal cellular morphology (round cytoplasmic borders and absence of cytoplasmic vacuoles) by light microscopy (8). More than 90% of the cells on coverslips met these criteria. The dose of Epo was chosen to elicit maximal $[Ca_i]$ response both in terms of amplitude of $[Ca_i]$, as well as the percentage of cells with significant $[Ca_i]$ increase over baseline (1, 2). $[Ca_i]$ was calculated from ratiometric fluorescence images of Fura-2 loaded cells by the in vivo calibration method (2), using 224 nM as the K_d for Ca^{2+}–Fura-2 complex (13). It should be noted that using our Fura-2 loading protocol, we have previously shown that $[Ca_i]$ values derived from the Fura-2–free acid calibration curve (in vitro calibration) and from the in vivo calibration method are indistinguishable (2).

In some experiments, $CaCl_2$ (3 mM) or $NiCl_2$ (1 mM) was added at 10 min to PBS with or without Ca^{2+} (2 mM EGTA). Where stated, cells were pretreated with nifedipine (1, 10, or 50 μM) for 3 min before addition of Epo.

Measurement of cellular inositol phosphates. Day 10 human BFU-E–derived erythroblasts were removed from culture and incubated overnight at 1.4×10^6 cells/ml, 37°C, in inositol-free Iscove's modified Dulbecco's media (IMDM) containing 20% dialyzed FCS and [2-^{3}H]-myo-inositol (40 μCi) (American Radiolabeled Chemicals Inc., St. Louis, MO). Cells were washed, resuspended in inositol-free IMDM with 2% dialyzed FCS, and pretreated with lithium chloride (20 mM) for 15 min. The cell suspension was divided into two equal halves, and Epo (2 or 10 U/ml) was added to one of these halves. After 1, 5, or 10 min of incubation with Epo or its vehicle, equal volumes of 10% ice-cold TCA was added to quench the cell suspensions. After centrifugation (15,600 *g*), TCA was removed from the supernatant by four to five extractions with water-saturated diethylether. The supernatant was adjusted to pH 7–8 and stored frozen.

Inositol phosphates were separated on a 10 SAX column (Partisil; Whatman Inc., Clifton, NJ) with ammonium phosphate, pH 3–8, flow rate at 1 ml/min, as previously described (14, 15). Gradients used were 0.01–0.08 M over 30 min for [^{3}H]inositol-1-phosphate, 0.2–0.28 M over 30 min for [^{3}H]inositol-1,4-bisphosphate, 0.5–0.52 M over 30 min for [^{3}H]inositol-1,4,5-trisphosphate, and 1 M over 30 min for [^{3}H]-inositol-1,3,4,5-tetrakisphosphate. Standards for [^{3}H]inositol-mono-, bis-, and trisphosphates were purchased from Amersham Corp. (Arlington Heights, IL), and those for [^{3}H]inositol-tetrakisphosphate were purchased from New England Nuclear (Boston, MA). Gradients were controlled with the Maxima 820 program (Waters Assoc. Inc., Milford, MA). A mixture of the above standards could be separated by applying the gradients described, collecting 1-ml fractions, and determining radioactivity by liquid scintillation counting (Fig. 3 *A*).

Electrophysiological measurements. Whole cell patch-clamp recordings were performed at room temperature and as described by Hamill et al. (16). Micropipettes were fabricated from borosilicate glass capillaries (No. 1B150-4; World Precision Instruments, Sarasota, FL) with a two-stage needle/pipette puller (model 750; David Kopf Instruments, Tujunga, CA) and typically had diameters of 1–2 μm. Pipettes were fire polished as described by Leibowitz and Dionne (17), after which they showed resistances of 14±1 MΩ. For voltage clamping, current amplification, and current clamping, a patch-clamp amplifier (Axopatch-1C; Axon Instruments, Foster City, CA) with CV-4 1/100 headstage was used. For data acquisition and analysis, an IBM PC/AT computer interfaced with an IBX A/D and D/A converter (Indec Systems, Sunnyvale, CA) was used in conjunction with BASIC-FASTLAB software (Indec Systems).

After formation of GΩ seal (seal resistance 1.1–3.2 GΩ) by suction, pipette resistance was compensated by minimizing the duration of capacitive surge on the current trace. The patch membrane was then disrupted by applying 1.5 V DC to the patch for a controlled duration (ZAP function on Axopatch-1C). Reappearance of capacitive currents indicated that whole cell configuration had been attained. Cell capacitance and series resistance were compensated with analogue circuitry of the patch-clamp amplifier. Currents were filtered at 2 kHz and data acquired at 10 kHz. Voltages were referenced to the bath, i.e., positive voltage was depolarizing and negative voltage was hyperpolarizing. Outward current (positive current) represented flow of cations from cell to bath or anion flow from bath to cell.

Composition of different bath and pipette filling solutions are given in Table I. Pipette filling solutions were filtered with 0.22-μm filters (Millipore Corp., Bedford, MA) immediately before use. With PBS and normal filling solution, liquid junction potential (18) at the tip of electrode was 2.7±0.3 mV, the potential inside the electrode being negative. Since this potential level was taken as reference potential, membrane potential values measured in current-clamp experiments would be at least 2.7 mV more negative than those reported here. No corrections were made, since the junction potentials between pipette filling solution and cytoplasm, and that between PBS and reference electrode (PBS and 1 M KCl in 2% agar), were unknown.

Results

Influence of extracellular calcium on the $[Ca_i]$ increase in response to erythropoietin. To determine whether the increase in intracellular calcium measured in response to erythropoietin (1) originated from intracellular stores or external calcium, single day 10 BFU-E–derived erythroblasts loaded with Fura-2 were stimulated by Epo in the presence of physiologic calcium or its absence (2 mM EGTA). $[Ca_i]$ was measured over 20 min

Table II. Effect of External Calcium on [Ca_i] Increase in Day 10 BFU-E-derived Erythroblasts Stimulated with Erythropoietin

Stimulated with	External calcium	No. of cells	Basal [Ca_i]	Epo-induced peak [Ca_i]
			nM	*nM*
Epo	+	19	33±3	204±64*
Epo	−	12	26±4	30±5
PBS	+	16	22±3	25±4

Day 10 BFU-E-derived erythroblasts loaded with Fura-2 were stimulated with recombinant Epo (2 U/ml) or PBS (vehicle) in the presence of physiologic calcium (0.7 mM) or its absence (2 mM EGTA). [Ca_i] was measured over 20 min with fluorescence microscopy-coupled digital video imaging. Mean [Ca_i]±SEM at baseline and the peak after 20 min of Epo stimulation are shown. Number of cells shown were from four experiments on different blood donors. Differences among means were tested with one-way ANOVA. A priori comparisons of means of baseline vs. Epo-treated groups were then performed using F tests as tests of significance. * Significant increase above baseline ($P < 0.001$).

with fluorescence microscopy-coupled digital video imaging. Results are shown in Table II. [Ca_i] significantly increased in the presence of physiologic calcium ($P < 0.001$), but no increase was observed in the absence of external calcium. Calcium images of erythroblasts did not reveal redistribution of cell calcium in the absence of external Ca^{2+} (data not shown).

In another series of experiments, day 10 BFU-E-derived erythroblasts loaded with Fura-2 were stimulated with erythropoietin or PBS in the absence of external calcium (2 mM EGTA). Results are shown in Fig. 1. No change in [Ca_i] was observed over 20 min in the absence of extracellular free calcium. However, when calcium chloride (3 mM) was added at 10 min, there was a prompt increase in [Ca_i] from 28±7 to 131±49 nM in erythroblasts treated with erythropoietin at time 0. Addition of exogenous calcium to erythroblasts not primed with erythropoietin did not increase [Ca_i]. These results indicate that erythropoietin primed the Ca^{2+} influx pathway, which remained open so that when extracellular free Ca^{2+} was made available, [Ca_i] increased promptly.

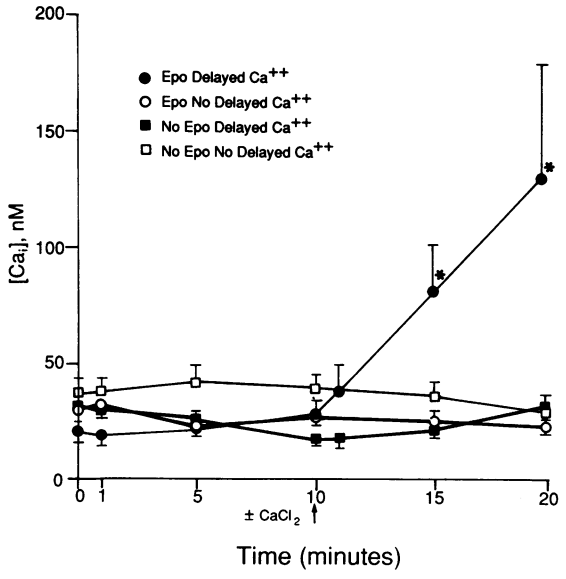

Figure 1. Effect of erythropoietin on Ca^{2+} permeability on human erythroblasts. Fura-2-loaded day 10 human BFU-E-derived erythroblasts were treated with Epo (2 U/ml) or PBS (vehicle) in 2 mM EGTA at 0 min. Exogenous calcium chloride (3 mM) was added to two groups at 10 min. Mean [Ca_i]±SEM was measured over 20 min by digital video imaging. 9 (— ○ —, — ■ —), 14 (— □ —), and 16 cells (— ● —) were studied. *Significant increase above baseline by one-way ANOVA ($P < 0.05$).

Influence of calcium channel blocking agents on the Epo-Induced Increase in [Ca_i]. To explore the mechanism of Ca^{2+} entry, Fura-2 loaded day 10 BFU-E-derived cells were pretreated in the presence of external calcium (0.7 mM) for 3 min with the L-type Ca^{2+} channel blocker nifedipine (19) at concentrations of 1, 10, and 50 μM. Cells were then stimulated with erythropoietin (2 U/ml) and [Ca_i] measured over 20 min. Results are shown in Table III. Nifedipine blocked the increase in [Ca_i] seen in response to erythropoietin, but only at doses higher than typically required to block voltage-sensitive L-type Ca^{2+} channels.

To examine if calcium influx is blockable after its activation by erythropoietin, day 10 BFU-E-derived erythroblasts were first treated with erythropoietin, and nickel chloride was added at 10 min after erythropoietin addition. As shown in Fig. 2, nickel chloride effectively reversed Epo-induced [Ca_i] increase even after the stimulatory effect of erythropoietin on Ca^{2+} influx was well established.

Effects of erythropoietin on cellular inositol-1,4,5-trisphosphate levels. BFU-E-derived erythroblasts labeled with [^{3}H]-myo-inositol were stimulated with Epo (2 or 10 U/ml) and cellular [^{3}H]inositol phosphates were measured. Because of the small number of BFU-E-derived erythroblasts, only control cells and one experimental group could be studied on any given day. Fig. 3 shows a representative experiment in which cells were stimulated by Epo (10 U/ml) for 5 min. No measurable increase in inositol-1,4,5-trisphosphate (IP_3) level was observed after 1, 5, and 10 min ($n = 2$ each) of Epo treatment. While the small [^{3}H]IP_3 signals (Fig. 3, *B* and *C*) may have precluded us from detecting a 20–30% increase in IP_3 in Epo-stimulated cells, it is unlikely that we would have missed a two- to three-

Table III. Nifedipine Blocks the Epo-induced [Ca_i] Increase Observed in the Presence of Physiologic Calcium

Pretreated with	Stimulated with	No. of cells	Basal [Ca_i]	Peak [Ca_i]
			nM	*nM*
IMDM	Epo	10	32±7	110±18*
Nif 1 μM	Epo	5	41±9	79±10*
Nif 10 μM	Epo	3	49±8	65±17
Nif 50 μM	Epo	7	33±8	44±12
Nif 50 μM	IMDM	3	18±5	16±2
IMDM	IMDM	5	29±6	49±20

Day 10 BFU-E-derived cells were pretreated with nifedipine (1, 10, or 50 μM) or IMDM for 3 min in the presence of physiologic calcium (0.7 mM). Baseline [Ca_i] measurements were obtained and the cells were then stimulated with Epo (2 U/ml) or IMDM. [Ca_i] was measured over 20 min. Mean peak [Ca_i]±SEM after stimulation is shown. * Significant increase above baseline by one-way ANOVA ($P < 0.05$).

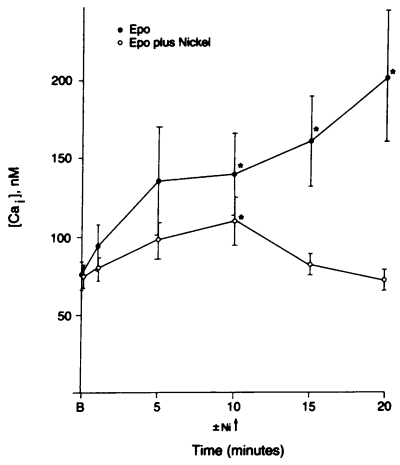

Figure 2. Nickel blocks erythropoietin-stimulated Ca^{2+} influx pathway. Fura-2–loaded day 10 human BFU-E–derived erythroblasts in PBS containing 0.67 mM Ca^{2+} were treated with Epo (2 U/ml) at 0 min. At 10 min, nickel chloride (1 mM) was added to one group of cells. Mean [Ca_i]±SEM was measured over 20 min by digital video imaging. 18 (— • —) and 19 (— ○ —) erythroblasts from six different donors were studied. *Significant increase above baseline ($P < 0.05$).

fold increase commonly observed in hormone-stimulated cells using this signaling pathway (14, 15).

Effects of erythropoietin on membrane potential in human BFU-E derived erythroblasts. One possible mechanism of erythropoietin-activated Ca^{2+} influx is via a voltage-dependent Ca^{2+} channel (19). To examine the mechanism of Epo-induced Ca^{2+} entry, we evaluated the effects of erythropoietin on membrane potential of human erythroblasts under current-clamp. The mean resting membrane potential was −10.9±1.0 mV (n = 43), with a range of −26 to +1 mV. Addition of erythropoietin (2 U/ml) did not cause significant depolarization (maximal potential change 8.8±1.4 mV, n = 8) when compared to Iscove's modified Dulbecco's medium control (maximal potential change 5.4±1.8 mV, n = 5).

Voltage-dependent whole cell currents in human BFU-E derived erythroblasts. To further evaluate whether erythropoietin modulates the activity of a voltage-dependent channel, current-voltage relationship of human erythroblasts in PBS was studied with depolarizing or hyperpolarizing 100-ms pulses from a holding potential of −60 mV under voltage clamp. With depolarization, we did not observe inward current at the beginning of the pulse and no time-dependent outward current in the late stage (Fig. 4 *A*). In addition, after repolarization to the holding potential, no tail current was observed. Addition of erythropoietin at doses (2 U/ml) that maximize [Ca_i] increase (1, 2) and Ca^{2+} influx did not reveal any voltage-dependent channel activity (data not shown).

Ionomycin-induced whole cell currents. Since erythropoietin increased [Ca_i] in day 10 human erythroblasts (2, 5, Table

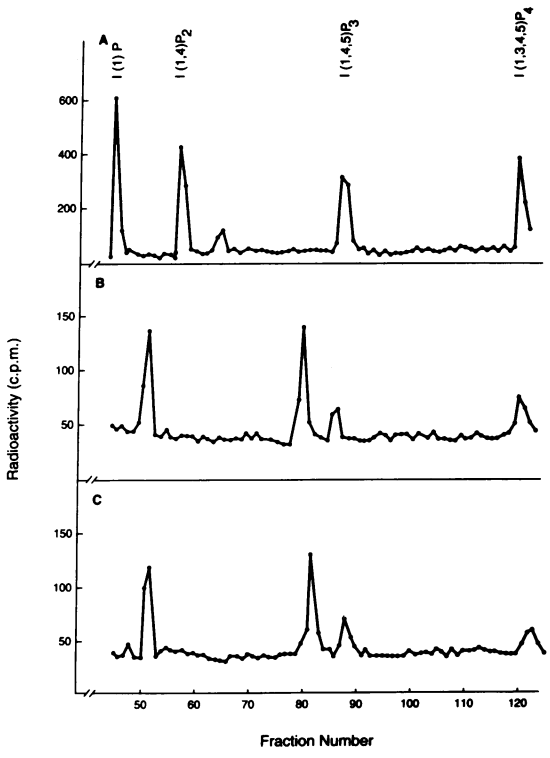

Figure 3. Effect of erythropoietin on levels of phosphoinositides in human erythroblasts. [^{3}H]myo-inositol labeled, day 10 human BFU-E–derived erythroblasts were treated with Epo (10 U/ml) for 5 min and cellular [^{3}H]inositol phosphates separated by HPLC as described in Methods. (*A*) Separation of [^{3}H]inositol phosphates standards. (*B*) Control. (*C*) Epo-stimulated cells. Fractions 86–89 eluted with [^{3}H]-inositol-1,4,5-trisphosphate and fractions 120–124 eluted with [^{3}H]-inositol-1,3,4,5-tetrakisphosphate. Fractions 80–83 eluted in the region expected for [^{3}H]inositol-1,3,4-trisphosphate (15). The peak in fractions 50–53 is unidentified but eluted between [^{3}H]inositol-1-phosphate and [^{3}H]inositol-1,4-bisphosphate.

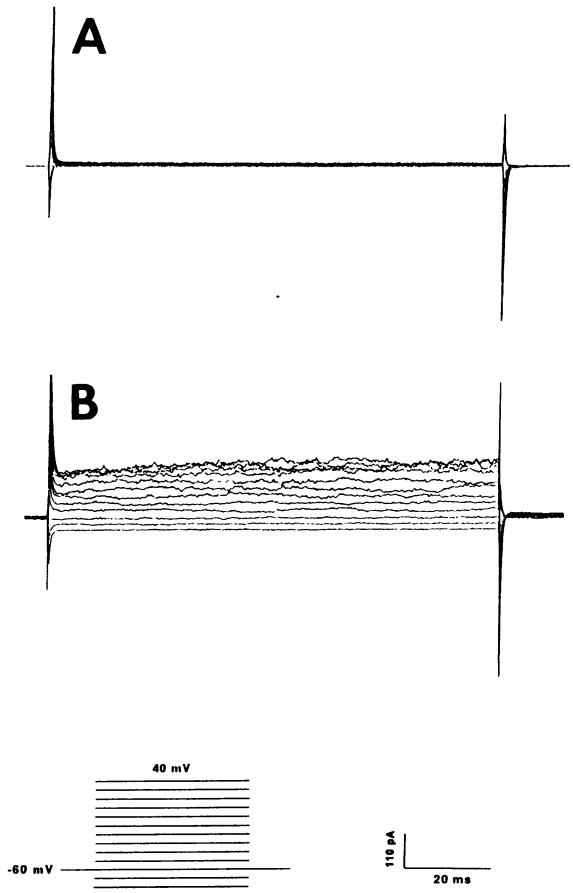

Figure 4. Membrane currents in a human BFU-E–derived erythroblast. (*A*) Whole cell currents in response to 100 ms voltage steps (−80–40 mV) every 5 s form holding potential of −60 mV. Bath was PBS and normal electrode filling solution was used in these experiments. (*B*) Same cell after addition of ionomycin (1 μM). For comparison, the gain was identical in both current traces. (*Inset*) Stimulus protocol and current and time scale.

II), and since Ca^{2+}-activated K^+ channels have been described in human RBCs (20), we attempted to detect Ca^{2+}-activated ion channels in human erythroblasts. To acutely increase $[Ca_i]$, ionomycin (1 μM) was added to cells in PBS containing 0.7 mM Ca^{2+}. Addition of ionomycin produced a large increase in outward current (Fig. 4 *B*) with a shift of reversal potential from 20 to −70 mV (Fig. 5).

The shift of reversal potential towards the estimated K^+ equilibrium potential (E_K = −86 mV; assuming 125 mM $[K_i]$ and 4.2 mM $[K_0]$ in PBS) suggests that the ionomycin-induced membrane current was mediated by K^+ efflux rather than Cl^- influx (assuming E_{Cl} = −9 mV as in human RBCs). Indeed, substitution of K^+ with Cs^+ in bath and pipette filling solutions abolished the ionomycin-induced membrane current (data not shown). On the other hand, replacement of Cl^- with aspartate (KASP) (Table I) had little to no effect on ionomycin-induced membrane currents (Fig. 6). These ion substitution experiments strongly suggest that the ionomycin-induced membrane current was carried by K^+ rather than Cl^- ions. In support of this, when membrane potential was monitored under current-clamp conditions, addition of ionomycin (1 μM) to human erythroblasts in PBS rapidly hyperpolarized cells from −2.8±1.9 to −71.5±4.2 mV (n = 4), which is close to E_K. In contrast, membrane potential of control cells changed from −7.0±2.7 to −1.6±1.9 mV (n = 4) during the same period of observation. Typical time courses of membrane potential changes are shown in Fig. 7.

When both extra- and intracellular Ca^{2+} were chelated with excess EGTA, addition of ionomycin to human erythroblasts could no longer elicit an increase in membrane current (data not shown), suggesting the ionomycin-induced K^+ current was Ca^{2+} activated. Reversibility of Ca^{2+} activation of whole cell current is demonstrated in Fig. 8. In this experiment, Ca^{2+} activation of K^+ channel was well established in a human erythroblast bathed in PBS. Subsequent addition of 4 mM EGTA to the same cell abolished the Ca^{2+}-activated membrane current.

Erythropoietin-induced $[Ca_i]$ increase and Ca^{2+}-activated K^+ channel. The maximal increase in $[Ca_i]$ of human erythroblasts treated with erythropoietin (2 U/ml) was ~ 200–300 nM (1, 2, 5; Table II). To assess whether this magnitude of $[Ca_i]$ increase was sufficient to activate Ca^{2+}-dependent K^+ channels, extracellular free Ca^{2+} was set to 300–400 nM (PBS with no added Ca^{2+} and containing 5 μM Fura-2 free acid; 363 nM as measured by Fura-2 fluorescence). Ionomycin (1 μM) was then added to erythroblasts to equilibrate extra- and intracellular free Ca^{2+} and membrane currents measured under voltage clamp. With extracellular free Ca^{2+} at 363 nM rather than 0.7 mM, ionomycin treatment did not increase whole cell currents in erythroblasts (n = 5). In parallel experiments using our digital video imaging system and under similar low extracellular free Ca^{2+} conditions, ionomycin treatment increased $[Ca_i]$ in Fura-2–loaded erythroblasts (from 99±10 to 416±27 nM; n = 7). This result suggests that at the low extracellular Ca^{2+} used in this series of electrophysiologic measurements, ionomycin

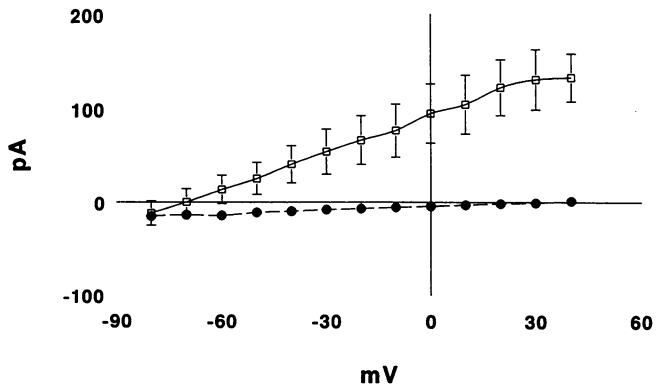

Figure 5. Effects of increased $[Ca_i]$ on membrane currents. Experiments were performed as in Fig. 4. Data represent mean±SEM from five cells before (●) and after (□) addition of ionomycin (1 μM). Note the shift of reversal potential from 20 to −70 mV after ionomycin treatment. Average linear slope conductance increased from 133 to 1,250 pS.

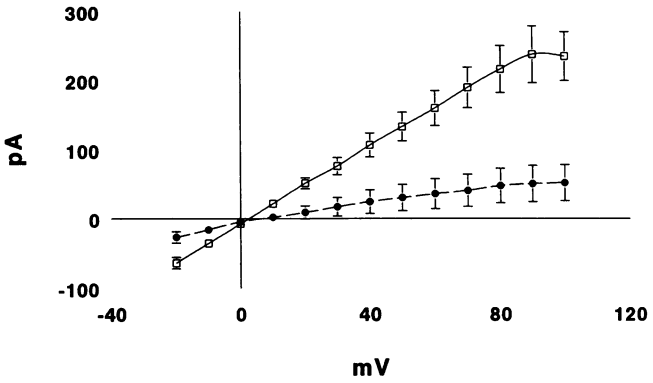

Figure 6. Whole cell currents in symmetrical KASP solution. Symmetrical KASP solutions (Table I) were used to minimize Cl^- concentrations both inside and outside the cells. Since the expected reversal potential was 0 mV, the stimulus protocol (*inset*, Fig. 4) was modified so that holding potential was 0 mV and command voltage pulses were stepped from −20 to 100 mV. Data represent mean±SEM from eight cells before (●) and after (□) addition of ionomycin (1 μM). Note preservation of Ca^{2+}-activated membrane current even when Cl^- was severely reduced.

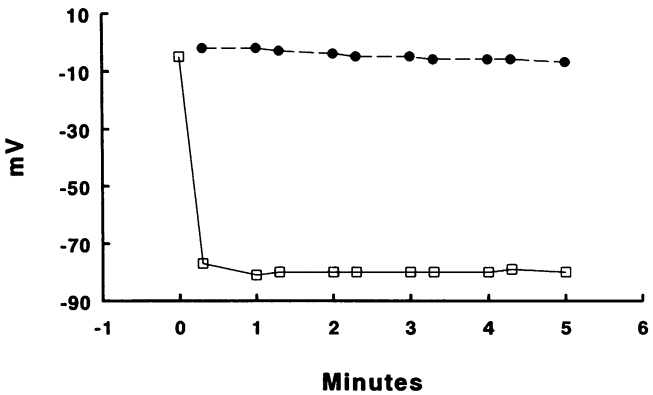

Figure 7. Ionomycin shifts membrane potential toward E_k. Bath solution was PBS and normal pipette filling solution was used. After rupturing the membrane patch under the electrode tip, holding potential was set to −20 mV and the cell was allowed to stabilize for 2–3 min. The Axopatch-1C amplifier was then switched to the current-clamp mode. After steady-state membrane potential was attained, ionomycin (1 μM) was added at time 0 to an erythroblast (□). Note immediate shift of membrane potential towards the K^+ equilibrium potential. Dimethylsulfoxide (ionomycin carrier) was added to another cell as a control (●), and no significant membrane potential change was observed.

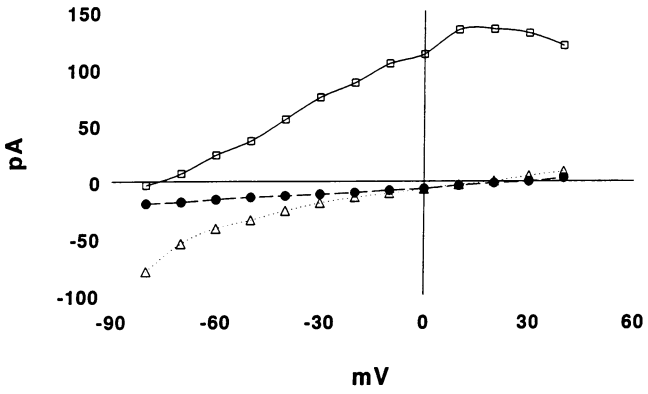

Figure 8. Reversibility of Ca^{2+}-activated whole cell currents. Experiment was performed as in Fig. 4. After baseline (●) current-voltage relationship was obtained for a human erythroblast in PBS, 1 μM of ionomycin (□) was added. Ca^{2+}-activated membrane currents similar to those observed in Fig. 5 were clearly evident. Subsequent addition of 4 mM EGTA (△) to the same cell completely abolished the Ca^{2+}-induced increase in membrane currents.

treatment should have increased $[Ca_i]$ to levels similar to those after erythropoietin treatment. We conclude that the magnitude of Epo-induced $[Ca_i]$ increase was insufficient to activate Ca^{2+}-activated K^+ channels.

Discussion

Previous studies by us (1, 2) and others (3) have demonstrated an increase in $[Ca_i]$ in human erythroblasts treated with erythropoietin. The increase in $[Ca_i]$ was mediated by a pertussis toxin–sensitive G-protein (5), and the Ca^{2+} signal was transmitted to the nucleus (8). The relevance of growth factor induced nuclear Ca^{2+} increase to cell growth and differentiation is highlighted by the recent finding that the cAMP response element-binding protein is phosphorylated by Ca^{2+}-calmodulin–dependent protein kinases I and II and thus functions as a Ca^{2+}-regulated transcription factor (21). The mechanism by which $[Ca_i]$ increases in response to Epo, however, is not clear.

Two generally accepted mechanisms for $[Ca_i]$ increase after receptor occupation include hydrolysis of membrane phosphoinositides (22) and activation of ion channels (19, 23). With respect to Epo-induced $[Ca_i]$ increase, the results of our current study indicate that the source of Ca^{2+} was entirely derived from extracellular medium, since EGTA obliterated the $[Ca_i]$ rise (Fig. 1 and Table II). It could be argued that the lack of $[Ca_i]$ increase in Epo-stimulated human erythroblasts incubated in zero Ca^{2+} media was caused by the redistribution of intracellular Ca^{2+} domains, such that despite a significant increase in Ca^{2+} near the submembranous region, the increase was "averaged" out to undetectability when whole cell $[Ca_i]$ was calculated. While we cannot categorically rule out this theoretical possibility, the homogeneous appearance of Ca^{2+} images taken at cell center plane makes this explanation unlikely, given the spatial resolution limit (0.5 μm) of our digital video imaging system with its ability to detect subcellular Ca^{2+} gradients (8). It is also unlikely that we would have missed the initial Ca^{2+} transient due to IP_3, given the temporal resolution (267 ms) of our digital video imaging system. Indeed, the observation that there was no detectable increase in IP_3 levels in Epo-treated cells (Fig. 3) lends further support to our interpretation that $[Ca_i]$ increase was caused by Ca^{2+} influx rather than intracellular Ca^{2+} release.

Ca^{2+} influx into Epo-treated erythroblasts was blocked by Ni^{2+} (Fig. 2) and moderately high doses of nifedipine (Table III), suggesting that the putative Ca^{2+} influx pathway involved in signal transduction of erythropoietin was an ion channel but unlikely to be a voltage-dependent L-type Ca^{2+} channel (19). Indeed, addition of Epo did not cause a significant change in membrane potential (Results). Taken together, our results suggest that Epo-induced Ca^{2+} influx was mediated via a voltage-insensitive ion channel permeable to Ca^{2+}.

The resting membrane potential (E_m) of day 10 human BFU-E–derived erythroblasts of −11 mV (Results) is similar to −9 mV measured in human RBCs with potential-sensitive fluorescent probes (24), and −8.5 mV estimate based on intracellular pH measurements (25). The close agreement between the measured E_m and the theoretical Cl^- equilibrium potential (E_{Cl} = −9 mV) (24, 25) is consistent with but does not unequivocally prove the hypothesis that in human erythroblasts, permeability to Cl^- is much higher than that to Na^+ or K^+. Over the range of voltage clamp applied (−80 to +40 mV), we did not detect the existence of any voltage-dependent channel activity (Fig. 4). Absence of voltage-dependent channels has been reported in some nonexcitable cells such as rat hepatocytes (26), but not in others such as human neutrophils (27) and lymphocytes (28). With respect to hematopoietic cells of red cell lineage, Hamill (29) reported K^+ selective channels that were not voltage-dependent in human RBCs.

Since erythropoietin increases $[Ca_i]$ from a baseline of 20–40 nM to 200–300 nM (1, 2, 5, 8; Table II), we investigated if ion channels would be activated by increases in $[Ca_i]$. Using ionomycin to "equilibrate" extra- and intracellular Ca^{2+}, we detected Ca^{2+}-activated K^+ channels in human erythroblasts. Ca^{2+}-activated K^+ channels have been described in a wide variety of cells (30) including human RBCs (20, 25) and are responsible for the Gardos effect (31). In human red blood cells, Ca^{2+}-activated K^+ channels are voltage independent, charybdotoxin sensitive, and typically of small (20–40 pS) single channel conductances (20, 25, 30). The number of Ca^{2+}-activated K^+ channels per human RBC has been estimated to be 1–55, based on unitary conductance, Rb^+ efflux measurements, and assuming "mean" channel open probability of 0.5 (20); 0.6–2 channels per cell based on V_{max} values of charybdotoxin-sensitive, Ca^{2+}-K^+ efflux (25); to 100–200 channels per RBC based on $^{86}Rb^+$ release from inside-out plasma membrane vesicles (32). While we did not measure directly the number of Ca^{2+}-activated K^+ channels on a human erythroblast, we have demonstrated clearly that when fully activated, the number and/or conductance of these channels were sufficient to move E_m towards E_K (Fig. 7). This is in contrast to the conclusion of Wolff et al. (25), based on their observations with human RBCs. The physiological role of Ca^{2+}-activated K^+ channels in human RBCs or human erythroblasts, however, remains undefined. More recently, Ca^{2+}-activated K^+ channels have been used as experimental markers for receptor-induced $[Ca_i]$ increase (33, 34). Indeed, in rat pituitary gonadotropes, stimulation with gonadotropin-releasing hormone increased $[Ca_i]$ rhythmically with corresponding periodic opening of Ca^{2+}-activated K^+ channels, resulting in oscillations in membrane current and rhythmic hyperpolarization (33). An important finding of the current study, therefore, is that at $[Ca_i]$ levels typically found in Epo-treated erythroblasts, Ca^{2+}-activated K^+ channels were not activated.

In the current series of experiments, we did not attempt to detect the Epo-regulatable Ca^{2+} channel in human erythroblasts using the whole cell patch-clamp configuration. Based on an estimated erythroblast volume of 0.5 pl (assuming a perfect sphere with a diameter of 10 μm) (8), and a $[Ca_i]$ increase rate of 100 nM/min (based on Fura-2 measurements; 2), the calculated Ca^{2+} current/cell would have been 1.7×10^{-4} pA. This current magnitude is too low to be detectable by the whole cell patch-clamp configuration. Even allowing for the differences between $^{45}Ca^{2+}$ influx rate (unidirectional flux) and rate of $[Ca_i]$ increase as detected by Fura-2 fluorescence (net Ca^{2+} flux–intracellular organelle sequestration–intracellular Ca^{2+} binding), the resultant estimated Ca^{2+} current/cell would still be at the lower limit of detection using the current whole cell configuration. Single channel recording techniques (16) may offer the requisite sensitivity to detect the activity of Epo-regulatable ion channels in the future. Indeed, recent application of single channel recording methodology revealed a voltage-insensitive, receptor-mediated Ca^{2+}-entry pathway in cultured endothelial cells (35).

In summary, we have demonstrated by single cell Ca^{2+} and electrophysiologic measurements that erythropoietin modulates a voltage-independent Ca^{2+} channel in human erythroblasts. Opening of this Ca^{2+} channel, rather than intracellular Ca^{2+} mobilization by IP_3, virtually accounts for the observed increase in $[Ca_i]$ in Epo-treated erythroblasts. The magnitude of Epo-induced $[Ca_i]$ increase, however, was not sufficient to open Ca^{2+}-activated K^+ channels in erythroblasts. Finally, no voltage-dependent channel activity was detected in human erythroblasts.

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