G-Protein α Subunit Giα2 Mediates Erythropoietin Signal Transduction in Human Erythroid Precursors

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Abstract

Erythropoietin induces a dose-dependent increase in cytosolic calcium in human erythroblasts that is mediated by a voltage-independent Ca^{2+} channel. Inhibition of this response to erythropoietin by pertussis toxin suggests involvement of guanine nucleotide-binding regulatory proteins (G-proteins). The role of G-proteins in regulation of the erythropoietin-modulated Ca^{2+} channel was delineated here by microinjection of G-protein modulators or subunits into human erythroid precursors. This is the first report on the use of microinjection to study erythropoietin signal transduction in normal precursor cells. Fura-2 loaded day-10 burst-forming units-erythroid–derived erythroblasts were used for microinjection and free intracellular calcium concentration ($[Ca_i]$) was measured with digital video imaging. BCECF (1,2′,7′-bis(2-carboxyethyl)-5-(and -6-)-carboxyfluorescein) was included in microinjectate, and an increase in BCECF fluorescence was evidence of successful microinjection. Cells were microinjected with nonhydrolyzable analogues of GTP, GTPγS or GDPβS, which maintain the α subunit in an activated or inactivated state, respectively. $[Ca_i]$ increased significantly in a dose-dependent manner after microinjection of GTPγS. However, injection of GDPβS blocked the erythropoietin-induced calcium increase, providing direct evidence that activation of a G-protein is required. To delineate which G-protein subunits are involved, α or βγ transducin subunits were purified and microinjected as a sink for βγ or α subunits in the erythroblast, respectively. Transducin βγ, but not α, subunits eliminated the calcium response to erythropoietin, demonstrating the primary role of the α subunit. Microinjected antibodies to Giα2, but not Giα1 or Giα3, blocked the erythropoietin-stimulated $[Ca_i]$ rise, identifying Giα2 as the subunit involved. This was confirmed by the ability of microinjected recombinant myristoylated Giα2, but not Giα1 or Giα3 subunits, to reconstitute the response of pertussis toxin–treated erythroblasts to erythropoietin. These data directly demonstrate a physiologic function of G-proteins in hematopoietic cells and show that Giα2 is required in erythropoietin modulation of $[Ca_i]$ via influx through calcium channels. (*J. Clin. Invest.* 1996. 98:1728–1736.) Key words: G-proteins • calcium channels • erythropoiesis • hematopoietic growth factors • guanosine trisophosphate analogues

Introduction

Hematopoietic growth factors responsible for the proliferation and differentiation of hematopoietic cells have recently been cloned and the mechanism of signaling through the cytokine receptor family is a major focus of study (1). Heterotrimeric guanine nucleotide–binding regulatory proteins (G-proteins)[1] are modulators of membrane-mediated signal transduction and are composed of α, β, and γ subunits (2). Multiple family members of each subunit are known. The α subunits have been organized into four major groups based on sequence homology: Gsα (Gsα, Golf); Giα (Gi1-3α, Go1-2α, Gt1-2α, Gzα); Gqα (Gqα, G11α, G14-16α), and the G12α group (G12α, G13α) (3, 4). Studies of the expression of G-protein α subunit genes in hematopoietic cells have revealed that certain subunits are ubiquitous, while others are lineage restricted or expression modulated during growth factor–induced differentiation (4–9). The function of many of these G-protein α subunits is largely undetermined.

Recent evidence supports an important role for heterotrimeric G-proteins in the signaling pathway of erythropoietin. Gsα and Giα1–3 subunits, but not Goα, have been identified in erythroid precursors or erythroleukemia cells (4, 9–11). In an erythropoietin-sensitive murine erythroleukemia cell line (RED-1), a marked loss of Giα3 and an increase in a truncated cystolic form of Giα2 were associated with erythroid differentiation (9). Pertussis toxin ADP ribosylates members of the Gi family, uncoupling Gi proteins from their receptors. Pertussis toxin has been shown to ADP ribosylate Giα subunits from human and rat fetal liver erythroid precursors and to block erythropoietin-stimulated large colony formation in precursors from rat liver and erythropoietin-dependent proliferation of an erythropoietin (Epo)-responsive cell line 32 D C123 (10, 12, 13). These data provide evidence for involvement of heterotrimeric G-proteins in erythropoiesis.

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1. *Abbreviations used in this paper:* 2-ME, 2-mercaptoethanol; BCECF, 1, 2′,7′-bis(2-carboxyethyl)-5-(and -6-)-carboxyfluorescein; BFU-E, burst-forming units-erythroid; DVI, digital video imaging; Epo, erythropoietin; G-protein, guanine nucleotide-binding regulatory protein; IMDM, Iscove's modified Dulbecco's media; IP3, inositol-1,4,5-trisphosphate; IP4, inositol-1,3,4,5-tetrakisphosphate; R, fluorescence intensity ratio; ROS, retinal outer segment(s); $[Ca_i]$, free intracellular calcium concentration.

Using digital video imaging (DVI) and whole cell patch clamp, we previously demonstrated that erythropoietin modulates a voltage-independent ion channel permeable to calcium on single human burst-forming units-erythroid (BFU-E)–derived erythroid precursors, resulting in a sustained increase in free intracellular calcium concentration (14). Voltage-independent, ligand-regulated ion channels represent a diverse family of proteins with alternative ion selectivity and regulation. G-proteins, inositol-1,4,5-trisphosphate (IP3), or inositol-1,3,4,5-tetrakisphosphate (IP4) have been demonstrated to directly regulate the activity of ion channels (15, 16). Focusing on erythropoietin signal transduction, IP3 and IP4 levels did not change in response to erythropoietin stimulation (14, 17). In contrast, pertussis toxin blocked the increase in free intracellular calcium concentration (Ca_i) stimulated by erythropoietin in day-10 BFU-E–derived erythroid precursors, suggesting that heterotrimeric G-proteins are involved in the pathway through which erythropoietin modulates calcium channels (10).

To directly explore the role of G-proteins in erythropoietin signal transduction, we developed a system to microinject normal erythroid cells, which remained growth factor responsive, while monitoring the success of microinjection with a photomultiplier-based system and [Ca_i] with digital video imaging. We microinjected single day 10 BFU-E–derived erythroblasts (10-μm diameter) with nonhydrolyzable guanine nucleotide analogues, GTPγS and GDPβS, and demonstrated that activation of a G-protein is capable of and required for modulation of the Epo-regulated calcium channel. Both Gα and Gβγ subunits can be effectors for ion channels (16). To determine whether an α or βγ subunit modulated the calcium channel on erythroblasts, we microinjected transducin α and βγ subunits to sequester endogenous Gβγ or Gα subunits, respectively, after their release from G-proteins by erythropoietin receptor stimulation. Microinjection of transducin βγ, but not α, subunits eliminated the increase in [Ca_i] in response to erythropoietin. Further experiments with anti–Giα 1–, 2–, and 3–specific antibodies and myristoylated recombinant Giα1, 2, or 3 subunits demonstrated the role of Giα2. Together with previous observations, these data provide direct evidence that the Giα2 subunit is required for the regulation of calcium channels by erythropoietin.

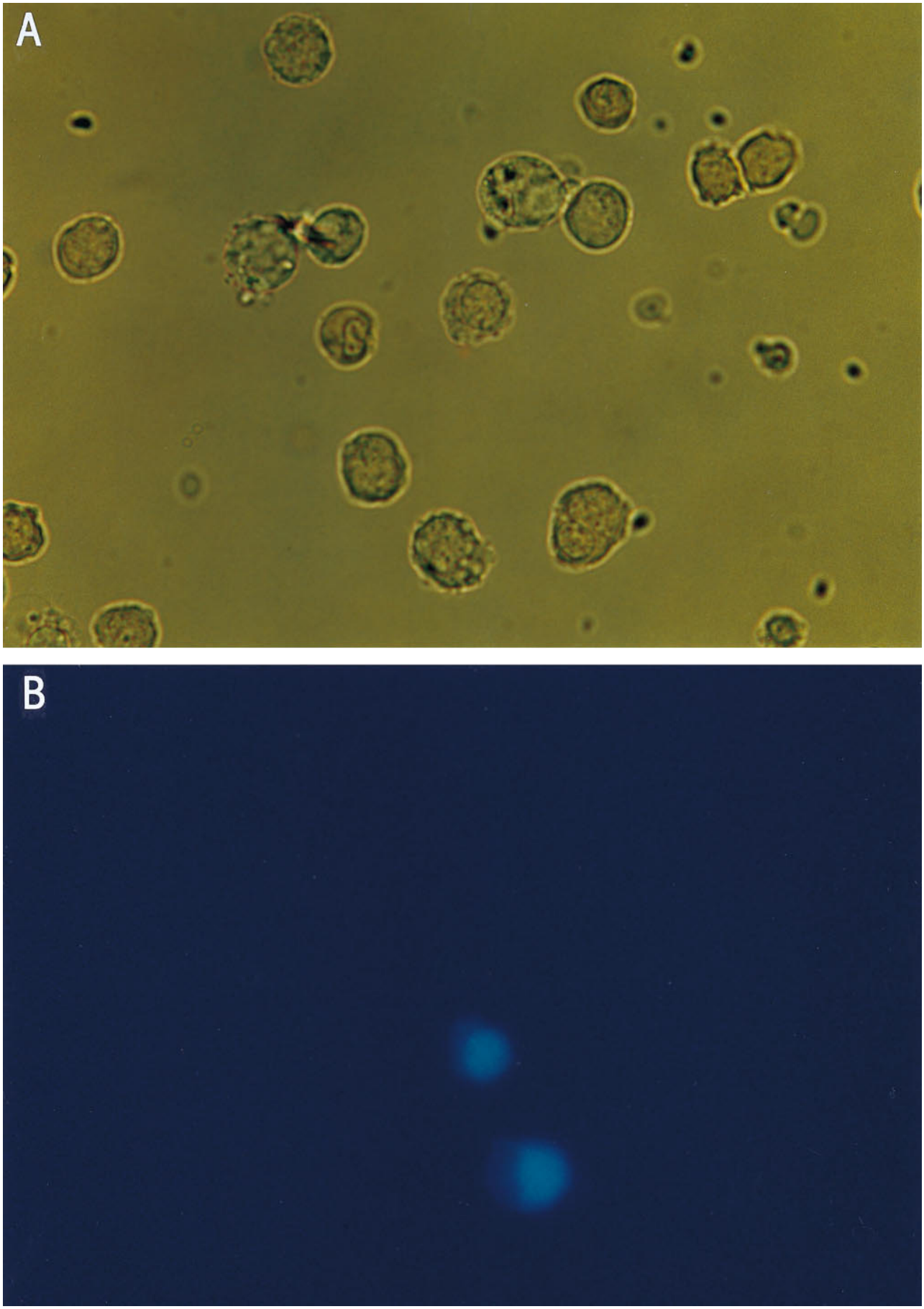
Methods

Preparation of BFU-derived erythroblasts. Peripheral blood was obtained from normal volunteer donors at The Milton S. Hershey Medical Center under protocols approved by the Institution's Clinical Investigation Committee. Peripheral blood mononuclear cells were separated on Ficoll-Paque (Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ) and cultured in 0.9% methylcellulose media containing 30% fetal calf serum, 9.0 mg/ml deionized bovine serum albumin (Cohn fraction V; Sigma Chemical Co., St. Louis, MO), 1.4×10^{-4} mol/liter β-mercaptoethanol, and 2 U/ml erythropoietin (recombinant Epo > 100,000 U/mg; R & D Systems, Inc., Minneapolis, MN). Single BFU-E, when cultured in methylcellulose, proliferate and differentiate over 14 d to large colonies containing $1–5 \times 10^4$ mature erythroblasts. These cells can be removed from culture at different days to study a well defined population of normal human cells at distinct stages of maturation. Cells from maturing BFU-E–derived colonies were plucked from culture on day 10. Day 10 cells are differentiating erythroblasts with minimal proliferative capacity and day 10 BFU-E–derived colonies can be easily identified by light microscopy since they are partially hemoglobinized. Cytocentrifuge preparations of aliquots of these day 10 BFU-E–derived cells routinely identified > 99.9% as erythroid precursors. The increase in [Ca_i] in day 10 cells in response to erythropoietin has been shown to be erythropoietin dose dependent and stage of erythroid differentiation specific (18). This response is specifically blocked by pretreatment of erythroblasts with purified antierythropoietin receptor antibody (Protein A affinity-purified mh2er/16.5.1; Genetics Institute, Cambridge, MA), but not by ImmunoPure mouse IgG (31204; Pierce Chemical Co., Rockford, IL) (data not shown).

Measurement of [Ca_i] with digital video imaging. BFU-E–derived cells were removed from culture on day 10 and labeled with anti–human β2 microglobulin (Boehringer Mannheim Corp., Indianapolis, IN) (18). Cells were bound to anti–mouse Ig-coated glass coverslips by incubating at 4°C for 40–60 min. The cells were then incubated in PBS at 37°C for 20 min with 2 μM 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%. Fura-2–loaded cells in PBS were visualized with the digital video imaging system previously described (10, 14, 18), except that the fluorescent light source was changed to a much more compact, filter-based (±10 nm bandpass) system (Ionoptix, Milton, MA). By quantitating the fluorescence intensity ratio (R) at 350/380, small changes in [Ca_i] can be detected independent of local variations in cell thickness or dye content. Baseline fluorescence intensity ratio (R) and the change in R of single cells after microinjection were measured with the computer-based digital video imaging system (18). Microinjection is described below. Some of the microinjected cells were treated with erythropoietin and R was measured. The dose of Epo used (2 U/ml; > 100,000 U/mg; R and D Systems, Inc.) was chosen to elicit maximal [Ca_i] response both in terms of amplitude of [Ca_i] increase as well as percentage of cells with significant [Ca_i] increase over baseline (18). Iscove's modified Dulbeco's media (IMDM) containing 1% FCS was used as control for nonspecific stimulation of [Ca_i] by protein. Previously, no increase in [Ca_i] was observed in erythroid precursors in response to a control hormone, insulin, chosen because insulin stimulates cell growth as well as causes an increase in [Ca_i] in nonerythroid cells (18). In some experiments, external calcium was depleted by adding 2 mM EGTA to PBS without calcium to the chamber immediately before Fura-2 measurements and microinjection.

Microinjection of erythroid precursors. Microinjection was performed using a micromanipulator 5171 and microinjector 5242 from Eppendorf (Hamburg, Germany) installed on our inverted microscope-based digital video imaging system (IM35; Carl Zeiss, Inc., Thornwood, NY) (18). Epifluorescence (either 505±20 nm for Fura-2, or 544±20 nm for 1,2′,7′-bis(2-carboxyethyl)-5-(and -6-)-carboxyfluorescein [BCECF]) collected by the UV fluor 100x/1.3 NA oil objective (Nikon Inc., Melville, NY) was directed either to an intensified CCD camera (3000 F; Fairchild Industrial Prods. Co., Winston-Salem, NC) or passed through a pinhole (1.6 mm) and captured by a photomultiplier (R928-07; Hamamatsu Phototonics, Hamamatsu City, Japan) (18–20). Photomultiplier output was routed through an amplifier/discriminator (C609; Thorn EMI, Middlesex, UK) before arrival at a counter/timer board (C660; Thorn EMI) situated in a 486 personal computer (20). We routinely used digital video imaging to measure Fura-2 fluorescence and the photomultiplier-based system

Figure 1. Microinjection of day 10 BFU-E–derived erythroblasts. (*A*) Day 10 BFU-E–derived cells after microinjection of two cells with 5 mM calcium green in the pipette. (*B*) Fluorescence of the same cells excited at 505 nm. Calcium green was not used routinely in experiments because of its potential to buffer calcium.


A
B

to monitor BCECF fluorescence introduced into the same cell by microinjection.

Femtotips purchased from Eppendorf (advertised diameter, 0.5±0.2 μm) were initially used for injections but, even with minimal injection pressure and time settings, these generally resulted in leaky cells that rapidly disintegrated. This was probably due to the fact that the femtotip diameter was ~ 5% of erythroblast diameter. Microinjection pipettes of smaller diameter were subsequently custom made with a microelectrode puller (P-97; Sutter Instruments Co., Novato, CA). Optimal settings for successful microinjection for these small cells were determined with alumina silica glass pipettes (o.d. 1.0 mm; i.d., 0.68 mm; Sutter Instruments Co.). Estimated tip diameter was 0.06 μm. The applied pressure of Eppendorf microinjector was adjusted to 20 hPa, back pressure 20 hPa, and the time of injection 0.1 s. Cells were injected with an internal buffer consisting of (in mM): KCl 125, NaCl 5, Hepes 20, $MgCl_2$ 2, and ATP 2. BCECF (100 μM; Molecular Probes, Inc., Eugene, OR) was included in the internal buffer to document successful injection. All data presented are from cells in which BCECF fluorescence significantly increased from cell autofluorescence before injection. To quantitate BCECF fluorescence, cells were excited at 478.5±10 nm and emission was measured at 540±20 nm with the photomultiplier-based system described above before and after microinjection (20). In early experiments to define parameters for successful microinjection, cells were microinjected with calcium green (5 mM; Molecular Probes, Inc.) for visual demonstration of dye retention (Fig. 1).

After injection, healthy cells defined by round cytoplasmic borders, absence of cytoplasmic vacuoles, and retention of strong Fura-2 fluorescence were selected for study. In some experiments, cells were allowed to recover 10–15 min after injection before stimulation with erythropoietin. We did not measure R_{min}/R_{max} of Fura-2 each day because of practical constraints associated with single-cell injection. Instead, our results are expressed as the fluorescence intensity ratio at 350/380. We have previously demonstrated by digital video imaging that R_{max}/R_{min} of Fura-2 in solution was similar to that for intracellular Fura-2. In addition, it can be mathematically shown that at the low $[Ca_i]$ concentration typically found in erythroid precursors (40–200 nM), the fluorescence intensity ratio (F350/F380) is directly proportional to $[Ca_i]$ (18).

Preparation of transducin α or βγ subunits. Transducin subunits were prepared from bovine retinas using the method of Bigjay and Chabre (21). In the dark, 100 frozen bovine retinas were thawed and shaken in 400 ml 45% sucrose in Buffer X ([in mM]: KCl 12, $MgCl_2$ 5, Hepes 20, PMSF 0.1, 2-mercaptoethanol 5, pH 7.4). Membranes were collected by centrifugation at 20,000 *g* for 20 min at 4°C. Floating retinal outer segment (ROS) membranes were diluted in Buffer X and then subjected to centrifugation at 20,000 *g* for 20 min at 4°C. The ROS membrane pellet was sedimented through a sucrose step gradient (density 1.105, 1.115, and 1.135) at 53,000 *g* for 45 min at 4°C, and the ROS membranes were collected at the 1.135/1.115 interface. The purified ROS were collected by centrifugation and suspended at 0.5 mg rhodopsin/ml in Iso buffer ([in mM]: KCl 120, $MgCl_2$ 0.1, Tris 20, PMSF 0.1, 2-mercaptoethanol 5, pH 7.5). This material was harvested by centrifugation at 31,000 *g* for 45 min to eliminate soluble proteins. The pellet was then fully illuminated, resuspended at 4 mg rhodopsin/ml in Iso buffer with 200 μM GTP, and sedimented immediately at 31,000 *g* to solubilize transducin α subunits. The transducin α subunit was concentrated in a filter (Amicon Corp. Danvers, MA), stored at −70°C, and used at a final concentration in the pipette of 5 μg/ml. Transducin α–depleted ROS membranes were resuspended in Hypo Buffer ([in mM]: $MgCl_2$ 0.1, Tris 5, PMSF 0.1, 2-mercaptoethanol 5, pH 7.5) to solubilize the transducin βγ subunit. The βγ subunit was injected at 5 μg/ml in the pipette. Purity of subunits was demonstrated by SDS–polyacrylamide gel electrophoresis (see Fig. 4).

Identification of Giα subunits. BFU-E–derived colonies were plucked from culture at day 10 until 0.5–1 × 10^7 erythroid precursors had been harvested and suspended in IMDM at 4°C. Cells were then washed with PBS once and collected by centrifugation at 600 *g* for 10 min. The pellet was resuspended in 5 mmol/liter $MgCl_2$, 1 mmol/liter EDTA, 4.4 mmol/liter Tris, 5 μg/ml aprotinin, and 5 μg/ml leupeptin at pH 8. The suspension was vortexed vigorously to effect hypotonic lysis, and nuclei and mitochondria were removed by centrifugation at 650 *g* for 15 min. The supernatant was subjected to centrifugation at 41,000 *g* for 20 min. The membrane pellet was resuspended in 20 ml of 20 mmol/liter Hepes, 2 mmol/liter $MgCl_2$, and 1 mmol/liter EDTA. Electrophoresis was performed on a 10% acrylamide gel. Blots were probed with purified IgG rabbit polyclonal antipeptide antibodies specific for Giα1 (to Giα1 159–168 peptide), Giα1 and Giα2 (to Gαi1,2 COOH terminal 345–354 peptide conserved sequence) or Giα3 (to Giα3 COOH terminal 345–354 peptide) from BIOMOL Res. Labs., Inc. (Plymouth Meeting, PA). Anti–Giα1 and –Giα3 were diluted 1/300 and anti–Giα1,2 1/1,000. Detection was with ECL (Amersham Corp., Life Sciences, Buckinghamshire, England).

Microinjection used the same three antibodies in the pipette, diluted 10% with BCECF. All antibodies were diluted in the same buffer. In some experiments, cells were microinjected with affinity-purified anti–Giα1,2, raised to the same peptide sequence, from Upstate Biotechnology Inc. (Lake Placid, NY). Results were identical with both anti–Giα1,2 antibodies.

Myristoylated recombinant Giα1, Giα2, and Giα3 were purified after coexpression in *Escherichia coli* with *Saccharomyces cerevisiae* *N*-myristoyltransferase (22). Activity of recombinant Giα subunit preparations was assessed by GTPγS binding (23). In some experiments Giα2 was heat inactivated by incubation at 90°C for 5 min. Final concentrations of Giα subunits in the microinjection pipette were Giα1 26 nM, Giα2 23 nM, and Giα3 31 nM. Giα subunits were injected into day 10 erythroblasts pretreated for 80 min with 5 μg/ml pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) (10).

Results

Microinjection of BFU-E–derived cells. There are no previous reports demonstrating successful microinjection of normal erythroid precursor cells, which are very small (10 μm) and fragile (19). Commercially available micropipettes (Eppendorf femtotips, 0.5-μm tip diameter) were too large for successful

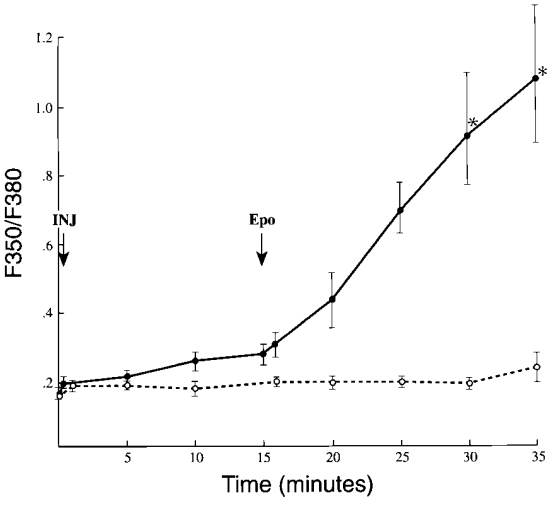


Figure 2. Time course of microinjected BFU-E–derived erythroblasts stimulated with erythropoietin. Fura-2–loaded day 10 erythroblasts were injected at time 0 with 100 μM BCECF in internal buffer, and then treated at 15 min with 2 U/ml erythropoietin (●, $n = 6$) or IMDM as a control (○, $n = 5$). Fura-2 measurements are expressed as fluorescence ratio (F350/F380). *A significant increase over the postinjection baseline at 15 min, $P \leq 0.05$.

injection. We first established injection pipette size and injection parameters. The microinjection apparatus was designed so that fluorescence of injected cells could be monitored with digital video imaging (18), the photomultiplier-based system (20), or both. BCECF fluorescence was used to assess microinjection success since BCECF provides a fluorescent signal independent of Fura-2 fluorescence. Approximately 80% of cells were successfully injected with BCECF as evidenced by the increase in BCECF fluorescence over autofluorescence of 250± 26% (mean±SEM). In Fig. 1, successful microinjection is demonstrated.

To determine whether microinjection, by itself, produced artifactual changes in [Ca_i], Fura-2–loaded day 10 erythroblasts were microinjected with BCECF. The time course of [Ca_i] changes after microinjection of five day 10 BFU-E–derived cells is shown in Fig. 2. The F350/F380 ratio remained stable during the 15-min recovery period after microinjection and after IMDM treatment, indicating that no significant increase in [Ca_i] was observed after microinjection with BCECF alone.

A dose-dependent increase in [Ca_i] after stimulation of day 10 BFU-E–derived cells with erythropoietin has previously been demonstrated (10, 18). To determine whether day 10 cells retained their ability to respond to erythropoietin after microinjection, Fura-2 fluorescence intensity ratios were measured after erythropoietin treatment of six cells injected with BCECF (Fig. 2). The Fura-2 fluorescence intensity ratio increased significantly after erythropoietin stimulation compared to cells treated with IMDM ($P < 0.05$). The time course of [Ca_i] rise in injected day 10 erythroblasts stimulated with erythropoietin was similar to that previously observed in noninjected cells (10, 18). Microinjection of cells did not adversely affect the ability of erythropoietin to modulate calcium influx.

Microinjection of nonhydrolyzable guanine nucleotide analogues demonstrates a requirement for G-protein activation in erythropoietin modulation of calcium channels. To establish the functional role of GTP-binding proteins in erythroblasts, we microinjected the nonhydrolyzable guanine nucleotide analogue GTPγS into day 10 BFU-E–derived erythroblasts. Microinjection of GTPγS, which maintains the α subunit in an active state, resulted in a dose-dependent increase in [Ca_i] in erythroid cells (Table I). This increase was measured within 30 s of the microinjection, and was sustained during the 20-min observation period after injection (Fig. 3). To ascertain that the GTPγS preparation did not introduce a significant amount of contaminant calcium to the internal buffer, the calcium concentration of 0, 5, 10, 25, and 50 mM solutions of GTPγS in internal buffer were measured independently with the Spex fluorimeter. No significant differences in the calcium content of solutions containing GTPγS compared to internal buffer alone were observed. To confirm that the rise in [Ca_i] was dependent on external calcium as previously observed (14), microinjection was done on cells incubated in PBS without calcium in the presence of 2 mM EGTA. The calcium rise in response to GTPγS injection was not observed (Table I) in the absence of external calcium. These results provide strong evidence that a calcium-permeable channel in erythroblasts is regulated by GTP-binding proteins. The increase in peak fluorescence of GTPγS-injected cells above that measured after erythropoietin stimulation may be due to activation by GTPγS of additional G-protein pathways that enhance free intracellular calcium.

Microinjection of 50 mM GDPβS did not result in any increase in [Ca_i], supporting the specificity of the response to GTPγS (Fig. 3). Of greater significance, microinjection of GDPβS, which maintains the α subunit in an inactive state, blocked the expected erythropoietin-induced [Ca_i] increase (Table II). These data demonstrate that erythropoietin regulation of calcium influx requires activation of a G-protein.

Microinjection of the transducin βγ, but not α, subunit blocks the Epo-stimulated calcium rise. To delineate which

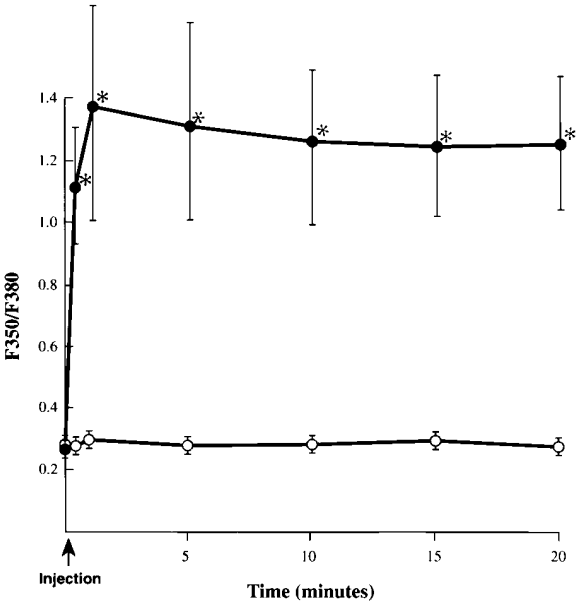


Figure 3. Microinjection of day 10 BFU-E–derived erythroblasts with GTPγS or GDPβS. Day 10 Fura-2–loaded BFU-E–derived cells were injected at time 0 with 50 mM GTPγS ($n = 7$, ●) or 50 mM GDPβS ($n = 4$, ○) in the pipette. Results are expressed as the fluorescence ratio (F350/F380). Mean±SEM is shown. *A significant increase in F350/F380 compared to baseline, $P < 0.05$.

Table I. Microinjection of GTPγS Stimulates Increased [Ca_i]

		F350/F380			
	GTPγS	Baseline	Peak	Percent increase	number
	mM				
+Ca	0	0.16±0.01	0.20±0.1	126±10	5
	5	0.17±0.05	0.29±0.09	176±3*	3
	25	0.16±0.04	1.04±0.35‡	850±366*	4
	50	0.26±0.50	1.84±0.26‡	1,000±271*	7
−Ca	50	0.31±0.10	0.30±0.06	111±16	4

*Significant increase compared to cells injected without GTPγS ($P < 0.05$). ‡Significant increase in F350/F380 compared to baseline ($P < 0.05$). Fura-2–loaded day 10 BFU-E–derived cells were microinjected with 0, 5, 25, 50 mM GTPγS and 100 μM BCECF in internal buffer. Fluorescence ratios (F350/F380) were obtained with DVI before and 1, 5, 10, 15, and 20 min after injection. Cells were injected in the presence of external calcium (0.7 mM) or in PBS without calcium (2 mM EGTA, −Ca). Results are expressed as the mean±SEM for maximal F350/F380 ratio or percent increase over preinjection baseline. number = number of cells studied.

Table II. Microinjection of GDPβS Inhibits the Erythropoietin-modulated Calcium Rise

Treatment		F350/F380			
GDPβS	Epo	Baseline	Peak	Percent increase	number
−	−	0.19±0.01	0.25±0.03	120±11	5
+	−	0.29±0.03	0.30±0.27	102±2	4
−	+	0.29±0.03	1.12±0.29*	370±81*	6
+	+	0.32±0.07	0.37±0.11	119±9	5

Fura-2–loaded day 10 BFU-E–derived cells were microinjected with a pipette containing 0 or 50 mM GDPβS and 100 μM BCECF in internal buffer. Fluorescence intensity ratios (F350/F380) were obtained with DVI before and 1, 5, and 10 min after injection. F350/F380 at 10 min was the postinjection baseline. Cells were then trated with or without Epo (2 U/ml) and F350/F380 followed for an additional 20 min. Results are expressed as the mean±SEM for F350/F380 at the postinjection baseline, the peak following Epo or IMDM treatment of injected cells, and the percent increase at peak compared with the baseline. *Significant increase compared to control (GDPβS-, Epo-), $P < 0.05$.

G-protein subunits are involved in erythropoietin regulation of the calcium channel, transducin α or βγ subunits were microinjected as a sink for Gβγ or Gα subunits, respectively, in erythroblasts. Transducin α or βγ subunits are not expressed in erythroblasts. By sequestering endogenous βγ or α subunits, as previously shown by Faure et al. (24), they can be used to determine the involvement of the bound subunit in a signaling pathway. Purity of transducin α and βγ subunits was demonstrated by SDS–polyacrylamide gel electrophoresis (Fig. 4). Concentration of each subunit in the pipette was 5 μg/ml, selected so that the subunits in their buffers (Iso, Hypo) were diluted 1/100 in internal buffer. Results are shown in Fig. 5. The Epo-stimulated increase in calcium was not affected upon microinjection of the α subunit ($P < 0.05$). However, microinjection of the transducin βγ subunit completely blocked the ability of erythropoietin to induce a rise in cytosolic free calcium. In control cells microinjected with Hypo or Iso buffer diluted 1/100, the rise in $[Ca_i]$ in response to erythropoietin followed the typical time course (data not shown). The simplest explanation of the mechanism of transducin βγ inhibition is that transducin βγ sequesters the endogenous Gα. This points to the importance of the G-protein α subunit rather than βγ in erythropoietin modulation of calcium channels.

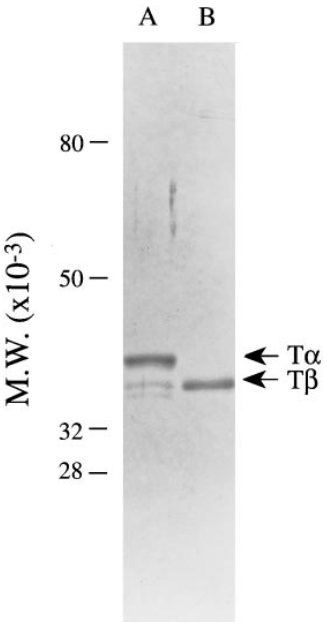


Figure 4. Purity of transducin α and βγ subunits. 2 μg of each purified subunit was applied to a 12.5% SDS–polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue. Lane *A* is the supernatant containing the α subunit after the addition of 200 μM GTP to Iso buffer, and *B* shows the supernatant containing the βγ subunit extracted in Hypo buffer. Tα and Tβ are indicated.

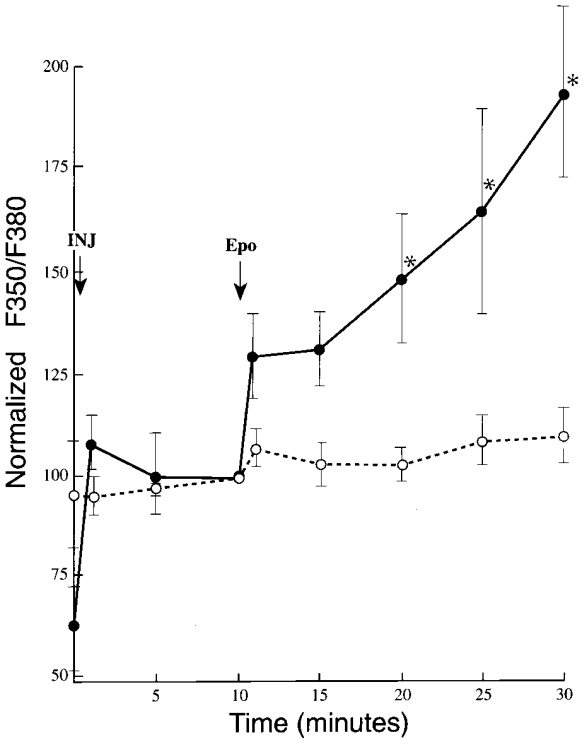


Figure 5. Microinjection of transducin α or βγ subunits. Fura-2–loaded day 10 BFU-E–derived erythroblasts were microinjected with a pipette containing 5 μg/ml α (●, $n = 6$) or βγ (○, $n = 7$) transducin diluted in internal buffer. Fluorescent ratio images (F350/F380) were obtained with DVI before injection, at 1, 5, and 10 min after injection, and at 1, 5, 10, 15, and 20 min after stimulation with erythropoietin at 2 U/ml. Results are normalized to the F350/F380 measured at 10 min, which was taken to be 100%. F350/F380 peak after erythropoietin stimulation was 1.22±0.41 after injection of the α subunit and 0.27±0.03 for the βγ. *A significant increase above the postinjection baseline, $P < 0.05$.

Giα2 is the pertussis toxin–sensitive subunit involved in regulation of calcium influx. We previously examined day 10 erythroblasts for the presence of pertussis toxin–sensitive G-proteins, and identified Giα1, Giα2, and Giα3 but not Goα (10). To determine which of the Giα subunits are involved in modulation of the erythropoietin-regulated calcium channel, we microinjected purified antibodies to Giα1, Giα1 and 2, or Giα3 into day 10 erythroblasts. Purified antibody to Giα2 was not available. Western blotting with these antibodies clearly demonstrated the presence of Giα2 and Giα3 in erythroblast membranes. Antibody against Giα1 also showed a weak band corresponding to Giα1 (Fig. 6). Microinjection of antibodies specific for Giα1 or Giα3 had no effect on the erythropoietin modulated rise in $[Ca_i]$, whereas this was blocked by microinjection of antibody to Giα1 and 2 (Table III). Since antibody to Giα1 failed to inhibit the Epo-induced increase in $[Ca_i]$, we conclude that inhibition of the calcium rise was secondary to

Figure 6. Giα subunits on day 10 erythroblast membranes. Membrane preparations were made from 0.5×10^7 (Giα1,2) or 1×10^7 (Giα1 and Giα3) day 10 erythroblasts and electrophoresed on a 10% SDS–polyacrylamide gel. Membranes were blotted with anti–Giα1 (*left*), anti–Giα1, 2 (*middle*), or anti–Giα3 antibody (*right*) and detection was performed with ECL.

inhibition of Giα2 and that Giα2 is required in erythropoietin stimulation of calcium entry.

To confirm the results obtained with antibody experiments, we sought to reconstitute the receptor-mediated response by injecting purified recombinant Giα subunits into pertussis toxin–treated erythroid precursors. Perfused Giα subunits have previously been used successfully to reconstitute the response of pertussis toxin–treated neurons to bradykinin (25). BFU-E derived erythroblasts were pretreated with 5 μg/ml pertussis toxin, which inhibited the increase in $[Ca_i]$ observed after erythropoietin stimulation (10). Microinjection of myrisolated recombinant Giα2 but not Giα1 or Giα3 into pertussis toxin–pretreated erythroblasts reconstituted the increase in $[Ca_i]$ in response to erythropoietin stimulation (Table IV). Recombinant heat-inactivated Giα2 had no activity (data not shown). By using several specific reagents for G-protein subunits, we have identified Giα2 as the subunit involved in erythropoietin modulation of calcium channels.

Discussion

Here, we microinjected normal erythroid precursors to study erythropoietin signal transduction. Digital video imaging, used to measure $[Ca_i]$ in Fura-2–loaded cells, was combined with a photomultiplier-based system, which measured BCECF fluorescence to document successful microinjection. We used this system to establish a functional role for G-proteins in the modulation of ligand-regulated calcium entry in erythroid cells. Furthermore, we identified the G-protein mediating this pathway as Giα2.

Voltage-independent calcium channels constitute a large family of proteins expressed in both excitable and nonexcitable cells with diverse properties. Calcium influx through voltage-independent calcium channels is regulated by several mechanisms, including calcium depletion, IP3 or IP4 activation, or via covalent modifications such as phosphorylation (15, 26). Some receptor-operated calcium channels are directly regulated by G-proteins and they respond to intracellular application of GTPγS (27). Erythropoietin has previously been demonstrated to modulate a voltage-independent ion channel permeable to calcium that is not regulated by IP3 or IP4 (14, 28, 29). Here, microinjection of GTPγS resulted in an increase in intracellular $[Ca_i]$ that was dependent on external calcium, demonstrating that the activation of a G-protein alone is capable of modulating calcium influx in erythroblasts. GDPβS blocked the erythropoietin–stimulated calcium rise, demonstrating that G-proteins are also required for erythropoietin-modulated calcium channel activation. The rapid GTPγS-induced rise in calcium and the higher peak fluorescence compared with the slower Epo-induced rise may be due to activation by GTPγS of other G-protein pathways that enhance $[Ca_i]$ in erythroid cells in addition to those regulated by erythropoietin. Other factors that may contribute are: (*a*) influx through the erythropoietin-regulated calcium channel is slow (14); and (*b*) maximal binding of erythropoietin to its receptor has been shown to take up to 15 min in SKT6 cells (30).

At least three of the four major G-protein α subgroups, Gsα, Gqα, and Giα subunits are expressed in hematopoietic cells (4, 10, 31, 32). The G-protein involved in regulation of Epo-induced calcium influx in erythroblasts is pertussis toxin sensitive (10). Of the known pertussis toxin-sensitive G-proteins, transducin (Gt), Gi, and Go, only Gi isoforms are present in day 10 erythroblasts (9, 10), pointing to Gi involvement in mediating calcium entry. Gi is composed of α and βγ subunits, both of which are released upon stimulation of the appropriate receptor and both of which are capable of regulating downstream effectors including adenylyl cyclase type II, mitogen-activated protein kinase, and phospholipase C (16, 24, 33). In fact, the first indication that the βγ subunits could directly regulate effectors was the demonstration of a βγ-acti-

Table III. Antibody to Giα2 Inhibits Epo Stimulation of $[Ca_i]$

	F350/F380			
Antibody	Baseline	Peak	Percent increase	number
Anti–Giα1	0.33±0.03	0.68±0.11*	210±33	6
Anti–Giα1, 2	0.32±0.03	0.36±0.04	110±4‡	8
Anti–Giα3	0.26±0.02	0.56±0.13*	202±27	6
Buffer	0.30±0.02	0.64±0.07*	220±27	15

Fura-2–loaded day 10 BFU-E–derived cells were microinjected with a pipette containing purified anti–Giα1, anti–Giα1, 2, and anti–Giα3 antibodies or internal buffer. Fluorescence intensity ratios (F350/F380) were obtained before and 1, 5, 10, and 15 min after injection. F350/F380 at 15 min was the postinjection baseline. Cells were then treated with Epo2 U/ml and F350/F380 measured for 20 additional min. Results are expressed as the mean±SEM for F350/F380 or percent increase over postinjection baseline. *Significant increase over baseline, $P < 0.05$. ‡Significant difference from buffer control.

Table IV. Microinjection of Myristoylated Giα Subunits into Pertussis Toxin–treated Erythroblasts

	F350/F380			
Microinjected Giα	Baseline	Peak	Percent increase	number
None	0.39±0.03	0.40±0.03	104±3	19
Giα1	0.44±0.04	0.43±0.02	98±4	6
Giα2	0.48±0.01	0.77±0.06*	159±10‡	7
Giα3	0.38±0.03	0.41±0.04	107±3	10
Epo control	0.32±0.02	0.62±0.05*	201±17‡	11

Fura-2–loaded day 10 BFU-E–derived cells pretreated with 5 μg/ml pertussis toxin were microinjected with a pipette containing recombinant myristoylated Giα1 (26 nM), Giα2 (23 nM), Giα3 (31 nM) or internal buffer. Fluorescence intensity ratios (F350/F380) were obtained before and 1, 5 and 10 min after injection. F350/F380 at 10 min was the postinjection baseline. Cells were then treated with Epo 2 U/ml and F350/F380 measured for 20 additional min. Results are expressed as the mean±SEM for F350/F380 or percent increase over postinjection baseline. *Significant increase over baseline, $P < 0.05$. ‡Significant difference from buffer control.

vated K^+ channel in the heart (16). We sought to determine whether the released α subunit or βγ subunit of Gi was responsible for the erythropoietin-mediated rise in [Ca_i]. Using microinjection instead of over expression as used by Faure et al. (24), the α subunit of Gt (transducin) was introduced to sequester endogenous βγ subunits released from Gi upon stimulation of the Epo receptor. Similarly, the βγ subunit of transducin was introduced to sequester Giα subunits. Microinjection of transducin α subunits had no effect, but microinjected transducin βγ subunits blocked the erythropoietin-stimulated calcium increase. Thus, regulation of the calcium rise by a Gi protein α subunit rather than βγ was specifically demonstrated. There are three isoforms of the α subunit of Gi, α1-3, all of which have been shown to be present on day 10 erythroblasts (4, 9–11). Here, we demonstrated the importance of Giα2 in regulation of a voltage-independent calcium channel by erythropoietin using two different types of specific reagents. Giα2 has been shown to be important in CSF-1–induced macrophage proliferation (8), regulation of differentiation of teratocarcinoma cells (34), and can function as an oncogene in the neoplastic transformation of Rat-1 cells (35). The mechanisms through which Giα2 mediates these effects are not established, but a rise in intracellular calcium is a mechanism that could influence many downstream effectors.

The importance of intracellular calcium concentration in regulation of cell proliferation/differentiation is becoming increasingly clear. In murine erythroleukemia cells undergoing differentiation, McMahon et al. (36) reported the appearance of a nuclear Ca^{++}, Mg^{++} dependent endonuclease capable of generating single strand breaks in chromosomal DNA. Calcium has been directly linked to expression of protooncogenes (37), and may influence transcription factor phosphorylation by activation of protein kinase C (38) or Ca^{++}–calmodulin-dependent protein kinases (39, 40). [Ca_i] has been shown to influence activation of the tyrosine kinase PYK2, which subsequently modulates MAP kinase activity (41). Ca^{2+}-loaded calmodulin can inhibit DNA binding of several basic-helix-loop-helix transcription factors including E2A and SCL (42, 43) and activate Ras through its influence on Ras–GRF exchange factor (44). The increase in [Ca_i] stimulated by erythropoietin is transmitted to the nucleus, where a nuclear to cytoplasmic calcium gradient has been demonstrated (19). The specific mechanisms through which the erythropoietin-stimulated calcium rise influences erythroid differentiation are not yet established, but may involve several of these pathways.

Receptors for the superfamily of hematopoietic cytokines (including Epo, GM-CSF, IL-3) are made of several chains, identical or distinct, with single membrane spanning domains (1). Although these receptors are different from the well described G-protein–coupled seven transmembrane domain receptor family, increasing evidence suggests involvement of G-proteins in their signal transduction pathways (4, 6–13). Here, we have clearly demonstrated an unconventional pathway of signaling for hematopoietic cytokine receptors involving Giα2. A role for Giα2 in signaling from two other receptors with single membrane spanning domains, CSF-1 (8) and insulin-like growth factor-II (45), has been shown. The molecular details of the pathway between erythropoietin receptor binding, Giα2 activation, and calcium channel modulation need to be pursued, as well as the relationship of this aspect of erythropoietin signaling to Jak and STAT activation (46).

In summary, we have demonstrated here for the first time successful microinjection of normal human hematopoietic cells to directly study signal transduction mechanisms. We have demonstrated a functional role for G-proteins in modulating the erythropoietin-dependent calcium channel during erythroid differentiation and have specifically identified Giα2 as the G-protein subunit involved.

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