TRPC3 Is the Erythropoietin-regulated Calcium Channel in Human Erythroid Cells*

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Erythropoietin (Epo) stimulates a significant increase in the intracellular calcium concentration ([Ca2+]i) through activation of the murine transient receptor potential channel TRPC2, but TRPC2 is a pseudogene in humans. TRPC3 expression increases on normal human erythroid progenitors during differentiation. Here, we determined that erythropoietin regulates calcium influx through TRPC3. Epo stimulation of HEK 293T cells transfected with Epo receptor and TRPC3 resulted in a dose-dependent increase in [Ca2+]i, which required extracellular calcium influx. Treatment with the phospholipase C (PLC) inhibitor U-73122 or down-regulation of PLCγ1 by RNA interference inhibited the Epo-stimulated increase in [Ca2+]i, in TRPC3-transfected HEK 293T cells and in primary human erythroid precursors, demonstrating a requirement for PLC. TRPC3 associated with PLCγ, and substitution of predicted PLCγ Src homology 2 binding sites (Y226F, Y555F, Y648F, and Y674F) on TRPC3 reduced the interaction of TRPC3 with PLCγ and inhibited the rise in [Ca2+]i. Substitution of Tyr226 alone with phenylalanine significantly reduced the Epo-stimulated increase in [Ca2+]i, but not the association of PLCγ with TRPC3. PLC activation results in production of inositol 1,4,5-trisphosphate (IP3). To determine whether IP3 is involved in Epo activation of TRPC3, TRPC3 mutants were prepared with substitution or deletion of COOH-terminal IP3 receptor (IP3R) binding domains. In cells expressing TRPC3 with mutant IP3R binding sites and Epo receptor, interaction of IP3R with TRPC3 was abolished, and Epo-modulated increase in [Ca2+]i, was reduced. Our data demonstrate that Epo modulates TRPC3 activation through a PLCγ-mediated process that requires interaction of PLCγ and IP3R with TRPC3. They also show that TRPC3 Tyr226 is critical in Epo-dependent activation of TRPC3. These data demonstrate a redundancy of TRPC channel activation mechanisms by widely different agonists.

Erythropoietin (Epo)2 is a glycoprotein that is required for proliferation and differentiation of erythroid cells (1, 2). The erythropoietin receptor (Epo-R) is a member of the cytokine receptor superfamily, members of which share many signal transduction pathways (3). Epo has been shown to stimulate a dose-dependent increase in [Ca2+]i, that is mediated through a voltage-independent ion channel (4–6). In electrophysiological studies of normal human erythroid progenitor-derived cells, Epo stimulation increased calcium channel mean open time 2.5-fold and open probability 10-fold (5). To identify specific channels activated by erythropoietin, members of the transient receptor potential (TRP) protein superfamily were studied, because these channels have characteristics similar to those observed in electrophysiological studies of human erythroblasts (5). We determined that TRPC2, TRPC3, and TRPC6 are expressed on primary erythroid cells and that erythropoietin stimulated calcium influx through murine TRPC2 but not TRPC6 (7–9). Erythropoietin modulated calcium influx through TRPC2 through signaling mechanisms dependent on complex formation between TRPC2, Epo-R, phospholipase Cγ (PLCγ), and the inositol 1,4,5-trisphosphate receptor (IP3R), activation of PLCγ, and interaction of TRPC2 with IP3R (10). However, because TRPC2 is a pseudogene in humans (11), we hypothesized that the function of TRPC2 is provided by a different calcium-permeable channel in human erythroid cells.

Calcium is a universal intracellular second messenger that influences many cell functions and in erythroid cells has an important role in colony growth and in terminal stages of differentiation (6, 12–14). The erythropoietin receptor also has been shown to activate Ca2+ influx in other cell types. In myoblasts, Epo stimulated expansion of the progenitor population during differentiation and an increase in [Ca2+]i, dependent on extracellular calcium influx (15). In neuronal cell lines, Epo stimulated an increase in cell viability and an increase in 45Ca2+ uptake (16, 17). Determination of the mechanisms through which the erythropoietin receptor modulates Ca2+ influx is important in understanding regulation of erythroid proliferation and differentiation as well as the role of Epo-R expression in nonerythroid tissues and is likely to be applicable to other cytokine receptor pathways.

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2 The abbreviations used are: Epo, erythropoietin; Epo-R, Epo receptor; TRP, transient receptor potential; PLCγ, phospholipase Cγ; IP3, inositol 1,4,5-trisphosphate; IP3R, IP3 receptor(s); SH2, Src homology 2; HEK, human embryonic kidney; siRNA, small interfering RNA; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; CMV, cytomegalovirus.
**Epo Regulates TRPC3 in Human Erythroid Cells**

The TRP protein superfamily is a diverse group of voltage-independent Ca\(^{2+}\) permeable cation channels expressed on nonexcitable mammalian cells that are related to the archetypal *Drosophila* TRP (18–21). The TRP superfamily has been divided into six subfamilies. Many members of the TRPC subfamily are activated after stimulation of receptors, and most of these receptors activate different isoforms of PLC (20, 22). Activation of PLC results in hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol. Several mechanisms of TRPC regulation through PLC-mediated pathways have been proposed. One mechanism is through IP\(_3\) interaction with its receptor (IP\(_3\)R), resulting in release of Ca\(^{2+}\) from the endoplasmic reticulum and depletion of calcium from internal stores, triggering calcium entry across the plasma membrane through TRPC (23). Alternatively, high concentrations of IP\(_3\) in the vicinity of IP\(_3\)R, resulting from close association of IP\(_3\)R with PLC-coupled receptors, may directly activate IP\(_3\)R and the associated TRPC, whereas calmodulin binding inhibits TRPC activation (24). Epo stimulation of its receptor induces activation of both PLC\(_{\gamma1}\) and PLC\(_{\gamma2}\) (25–27). Because the TRPC subfamily shares a number of activation mechanisms and PLC\(_{\gamma}\) has previously been shown to be involved in modulation of cell surface expression of TRPC3 (28), we explored whether Epo could modulate Ca\(^{2+}\) influx in human erythroid cells through the TRPC family member TRPC3. We determined that TRPC3 is expressed on primary human erythroblasts, that Epo modulates extracellular calcium influx through TRPC3 in a dose-dependent manner, and that PLC activation and interaction with TRPC3 are required. TRPC3 with substitutions of predicted PLC\(_{\gamma}\) SH2 binding sites (Tyr\(^{226}\), Tyr\(^{555}\), Tyr\(^{648}\), and Tyr\(^{674}\)) showed decreased association with PLC\(_{\gamma}\). In contrast, substitution of TRPC3 Tyr\(^{226}\) was sufficient to reduce Epo-modulated calcium influx but not PLC\(_{\gamma}/\text{TRPC3}\) interaction. Epo failed to stimulate a significant increase in [Ca\(^{2+}\)]\(_i\) through TRPC3 with mutations of IP\(_3\)R binding sites, demonstrating that downstream of PLC\(_{\gamma}\) activation, IP\(_3\)R interaction with TRPC3 is required in Epo-induced calcium influx.

**EXPERIMENTAL PROCEDURES**

**Tissues and Cell Lines**—Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. UT-7 cells were cultured in minimal essential medium with 10% fetal calf serum and 0.5 units/ml erythropoietin (Amgen, Thousand Oaks, CA). TF-1 cells were cultured in RPMI 1640 medium with 10% fetal calf serum with 5 units/ml Epo (Amgen) or 1–2 ng/ml granulocyte-macrophage colony-stimulating factor (29). Peripheral blood from volunteer donors was obtained under protocols approved by the institution’s institutional review board. Human erythroid precursors were obtained from cultures of peripheral blood progenitors (BFU-E) using two methods. 1) Human BFU-E-derived erythroblasts were harvested from methylcellulose culture at days 10 and 14 as previously described (30). 2) Human erythroid progenitors/precursors were cultured using a two-phase liquid culture system (31, 32). Cells harvested at day 8 of Phase II were predominantly proerythroblasts and basophil normoblasts. CD34\(^+\) cells were purchased from AllCells, LLC (Emeryville, CA).

**Transfection of Human TRPC3 and Epo-R into HEK 293T Cells**—Human TRPC3 (gift of Dr. Lutz Birnbaumer) and human TRPC3 with mutations of the PLC\(_{\gamma}\) SH2 or IP\(_3\)R binding site were subcloned into pQBI50 (Qiagen, Carlsbad, CA) or pcDNA 3.1/V5-His (Invitrogen). HEK 293T cells at 50–70% confluence were transfected with these vectors and/or pTracer-CMV expressing Epo-R using Lipofectamine Plus (Invitrogen) or Lipofectamine 2000 in accordance with the manufacturer’s recommended protocols. HEK 293T cells were routinely studied 48 h after transfection.

**Measurement of [Ca\(^{2+}\)]\(_i\) with Digital Video Imaging**—HEK 293T cells were transfected with empty pQBI50 vector, pQBI50 vector expressing wild type or mutant TRPC3, and pTracer-CMV expressing Epo-R. In some experiments, PLC\(_{\gamma}\) was down-modulated with small interfering RNA (siRNA) (see below). Successful transfection of individual HEK 293T cells with pQBI50 vectors was verified by detection of BFP (excitation, 380 nm; emission, 435 nm) and transfection of pTracer-CMV by detection of green fluorescent protein (excitation, 478 nm; emission, 535 nm) with our fluorescence microscopy-coupled video digital imaging system (5, 33). To study changes in [Ca\(^{2+}\)]\(_i\) in transfected cells, we were not able to use Fura-2 as the detection fluorophore, because its excitation and emission wavelengths overlap with green fluorescent protein. Instead, we used the fluorescent indicator Fura Red (excitation, 440 and 490 nm; emission, 505 nm long pass), a dual wavelength excitation probe (34, 35). At 48 h post-transfection, HEK 293T cells were loaded with 5 \(\mu\)M Fura Red-AM (Molecular Probes, Inc., Eugene, OR) for 20–25 min at 37 \(^\circ\)C in the presence of Pluronic F-127. The extracellular buffer routinely contained 0.68 mM CaCl\(_2\). Experiments to look at the role of external calcium depletion were performed with the addition of 2 mM EGTA to the extracellular buffer. In some experiments, calcium (3 mM) was added to the medium at 10 min. In other experiments, cells were pretreated during Fura Red loading with active (U-73122, 5 \(\mu\)M; Sigma) or inactive (U-73343) PLC\(_{\gamma}\) inhibitors. HEK 293T cells were then treated with 0–40 units/ml Epo. [Ca\(^{2+}\)]\(_i\) was measured in individual cells at base line and at 5 s to 2 min intervals for 20 min by determining the fluorescence intensity ratio \(R(F_{440}\)/\(F_{380}\)\). The constants \(S_{\text{min}}\) and \(S_{\text{max}}\) and the \(K_D\) of Fura Red were calibrated, and \(R_{\text{min}}\) and \(R_{\text{max}}\) were measured for Fura Red as described previously (8). [Ca\(^{2+}\)]\(_i\), was calculated using the formula, \([\text{Ca}^{2+}] = K_D(R - R_{\text{min}})/(R_{\text{max}} - R_{\text{min}})(S_{\text{max}}/S_{\text{min}})\). Primary human erythroblasts were removed from methylcellulose culture of peripheral blood BFU-E at day 10, adhered to fibronectin-coated glass coverslips, and loaded with Fura Red for experiments to measure [Ca\(^{2+}\)]\(_i\).

**Immunoblotting and Immunoprecipitation**—For Western blotting, whole cell lysates or immunoprecipitates were separated on 8% polyacrylamide gels, followed by transfer to Hybond-C Extra membranes (Amersham Biosciences). Western blotting was performed as previously described (8). Blots were incubated with anti-V5-horseradish peroxidase (HRP) (1:10,000; Invitrogen), anti-Epo-R (SC697; diluted 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-PLC\(_{\gamma}\) (1:1000; SC-81; Santa Cruz Biotechnology), anti-
IP₃R II (1:500; SC 7278; Santa Cruz Biotechnology), anti-actin (1:10,000; Sigma), anti-tubulin (1:10,000; Sigma), or anti-TRPC3 (1:400; Alomone Laboratories, Jerusalem, Israel) antibodies. Blots were washed and incubated with the appropriate HRP-conjugated antibodies (1:2000). ECL was used for detection of signal.

To examine the interaction of TRPC3 with PLCγ, IP₃R, or Epo-R, immunoprecipitation was performed. Cells were washed in ice-cold Hanks’ balanced salt solution and lysed in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with Complete protease inhibitor mixture (Roche Applied Science). To determine whether TRPC3 associates with Epo-R, PLCγ, or IP₃R, HEK 293T cells were transfected with human TRPC3 (in pcDNA3.1/V5-His), hEpo-R (in pcDNA3), rat PLCγ1 (in pcDNA3), rat IP₃R type II (in pcDNA3, gift of G. Mignery) (36), or combinations of these vectors. Protein lysates were incubated with preimmune rabbit serum, anti-V5 (Invitrogen), anti-Epo-R, anti-PLCγ1, or anti-IP₃R type II antibodies for 4–6 h at 4 °C. For immunoprecipitation of TRPC3 in human primary cells, a rabbit polyclonal antibody was raised to a human TRPC3 C-terminal peptide, as previously described (37). Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were then added for 1–2 h at 4 °C with mixing, and immunoprecipitates were washed three times. Sample buffer (3×) was added to the pellets, and the samples were heated at 60 °C for 30 min. Western blotting was performed as described above, and blots were probed with anti-V5-HRP or anti-Epo-R, anti-PLCγ1, or anti-IP₃R type II antibodies, or anti-actin antibodies, followed by the appropriate HRP-conjugated secondary antibodies and ECL.

**RESULTS**

**Erythropoietin Stimulates Calcium Influx through TRPC3**—To examine whether human erythroid cells express TRPC3 channel protein, we performed Western blotting on lysates from the human Epo-responsive cell lines UT-7 and TF-1. Primary human erythroid cells were also studied at different stages of differentiation, including CD34⁺ cells and from day 10 and 14 BFU-E-derived erythroblasts. Equivalent amounts of protein were loaded in each lane. A, immunoblotting with anti-TRPC3 antibody demonstrated increased expression of TRPC3 in primary human erythroid cells during erythroid differentiation. Blots were probed with anti-actin antibody to compare loading of lanes. Representative results of four experiments are shown. B, densitometry was used to quantitate TRPC3 and actin bands from four experiments of lysates from CD34⁺ cells and day 10 and 14 BFU-E-derived erythroblasts. The TRPC3/actin ratio was calculated and normalized to CD34⁺ cells to allow comparison between experiments, and the mean normalized ratio ± S.E. was determined. TRPC3 expression was significantly less in CD34⁺ cells than in day 10 erythroblasts (p < 0.02). C, immunoblotting with anti-Epo-R antibody demonstrated greatest Epo-R expression in day 10 primary erythroblasts. Representative results of three experiments are shown.
**TABLE 1**

| Transfection | Stimulation | Base Line | Peak | % Inc | n |
|---------------|-------------|-----------|------|-------|---|
| BFP-TRPC3 + Epo-R | PBS | 37 ± 3 | 51 ± 8 | 39 ± 24⁎ | 8 |
| BFP-TRPC3 | Epo | 33 ± 2 | 60 ± 5 | 82 ± 11 | 22 |
| BFP-TRPC6 | Epo | 33 ± 2 | 56 ± 4 | 73 ± 10 | 31 |
| Epo-R | Epo | 32 ± 2 | 65 ± 4 | 107 ± 12 | 18 |
| BFP-TRPC3 + Epo-R | Epo | 35 ± 1 | 116 ± 3 | 242 ± 10 | 45 |
| BFP-TRPC6 + Epo-R | Epo | 32 ± 1 | 67 ± 3 | 125 ± 14 | 41 |

⁎ A significant difference from Epo-stimulated cells expressing Epo-R (p < 0.001).

Epo Regulates TRPC3 in Human Erythroid Cells

Fura red-loaded HEK 293T cells transfected with BFP-TRPC3 or BFP-TRPC6 and Epo-R were treated with 40 units/ml Epo. [Ca²⁺], mean ± S.E. in nM) was measured at base line, and the peak measurement was observed after monitoring over 20 min of Epo stimulation (40 units/ml). Percentage increase (Inc) above base line (mean ± S.E.) = peak [Ca²⁺]/base-line [Ca²⁺] × 100%, minus 100% (base line). n, number of individual cells studied.

To examine erythropoietin regulation of [Ca²⁺]i, through TRPC channels expressed on human erythroid cells, HEK 293T cells were transfected with Epo-R subcloned into pTracer-CMV and human TRPC3 or TRPC6 subcloned into pQBI50. pTracer-CMV contains a CMV promoter utilized to drive expression of Epo-R and an SV40 promoter driving expression of green fluorescent protein. The pQBI50 vector contains a CMV promoter to drive expression of BFP fused to TRPC3 or TRPC6 was confirmed by detection of BFP in the cotransfected with Epo-R and TRPC3, Epo stimulated a large and sustained increase in [Ca²⁺], above base line (242 ± 10%; Table 1), which peaked at 10–20 min. This was significantly greater that none observed in cells transfected with Epo-R alone (107 ± 12%) or in cells cotransfected with Epo-R and TRPC6 (125 ± 14%). The increase in [Ca²⁺]i in cells expressing Epo-R alone is probably due to Epo-R activation of endogenous channels, which include low levels of TRPC3. The increase in [Ca²⁺]i in Epo-treated cells cotransfected with Epo-R and TRPC6 was not statistically different from that in cells expressing Epo-R alone, consistent with previous studies (8). These results demonstrate that Epo-R modulates [Ca²⁺]i through TRPC3 but not TRPC6.

To examine erythropoietin regulation of [Ca²⁺]i through TRPC3 and TRPC6 (Table 1), an Epo concentration of 40 units/ml was utilized, which is on the plateau for the Epo-stimulated rise in [Ca²⁺]i in cells expressing TRPC2. To characterize the increase in [Ca²⁺]i, stimulated by Epo through TRPC3, an Epo dose-response curve was generated using HEK 293T cells heterologously expressing TRPC3 and Epo-R. At an Epo concentration of 0.25 units/ml, the peak increase in [Ca²⁺]i above base line was significantly greater than that of cells treated with diluent (PBS) (Fig. 2A; p < 0.0001). The peak increase in [Ca²⁺]i plateaued at an Epo dose of 10 units/ml or greater (Fig. 2A). The time course of the rise in [Ca²⁺]i was then characterized in HEK 293T cells transfected with TRPC3 and Epo-R and stimulated with 40 units/ml Epo or diluent (PBS). [Ca²⁺]i was measured at base line, at 5-s intervals for the first 30 s, at 15-s intervals for the next minute, and then at 2-min intervals to 20 min to minimize photobleaching. [Ca²⁺]i did not significantly increase above base line for the first 90 s after Epo stimulation. [Ca²⁺]i became significantly greater in cells stimulated with Epo compared with PBS starting at 8 min (Fig. 2B; p < 0.0001).
To examine whether the rise in \([Ca^{2+}]\) in the presence (0.68 mM) or absence (2 mM EGTA) of extracellular calcium.

The absence of a significant increase in \([Ca^{2+}]\), during the first minutes of Epo stimulation (Fig. 2B) suggested that the increase in \([Ca^{2+}]\) is not due to intracellular calcium release. To examine whether the rise in \([Ca^{2+}]\), in response to Epo originated primarily from external calcium influx or internal \(Ca^{2+}\) store release, HEK 293T cells transfected with Epo-R and TRPC3 were stimulated by Epo in the presence of extracellular calcium (0.68 mM) or its absence (2 mM EGTA). \([Ca^{2+}]\) was measured over 20 min in Fura Red-loaded cells (Fig. 3A). A significant increase in \([Ca^{2+}]\), in Epo-treated cells was not observed in the absence of extracellular calcium, in contrast to the significant increase observed in Epo-treated cells in the presence of extracellular calcium (Fig. 3A, \(p < 0.01\)). When \(CaCl_2\) (3 mM) was added at 10 min to cells treated with Epo at time 0 in the presence of EGTA, there was a prompt and significant increase in \([Ca^{2+}]\), \(p < 0.02\) (Fig. 3B).

The specific role of PLC\(\gamma\) in Epo activation of TRPC3 was examined using RNA interference targeted to PLC\(\gamma\). HEK 293T cells were transfected with siRNA targeted to PLC\(\gamma\) or nonspecific control siRNA as well as TRPC3 and Epo-R. The effectiveness of siRNA interference in reducing PLC\(\gamma\) expression was demonstrated by Western blotting. Transfection of HEK 293T cells treated with Epo compared with cells not treated with Epo (Fig. 3B, \(p < 0.0001\)). These results suggest that Epo activated TRPC3, which remained open so that when extracellular free calcium became available, \([Ca^{2+}]\), promptly increased.

The Epo-modulated Increase in \([Ca^{2+}]\) through TRPC3 Requires PLC\(\gamma\) Activation—Stimulation of primary human BFU-E-derived erythroblasts with Epo results in a significant increase in \([Ca^{2+}]\) (5, 40, 41). Epo stimulation of erythroid cells also results in activation of PLC\(\gamma\)1 and -2 (25, 27, 42). To determine whether PLC is involved in the Epo-stimulated increase in \([Ca^{2+}]\), primary human erythroid cells, BFU-E-derived erythroblasts were removed from methyl-cellulose culture at day 10, pretreated with the active PLC inhibitor U-73122 or the inactive analog U-73343, and stimulated with Epo. Epo stimulation of erythroid cells was not observed in activation of PLC\(\gamma\)1 and -2 (25, 27, 42). To determine whether PLC is involved in the Epo-stimulated increase in \([Ca^{2+}]\), primary human erythroid cells, BFU-E-derived erythroblasts were removed from methyl-cellulose culture at day 10, pretreated with the active PLC inhibitor U-73122 or the inactive analog U-73343, and stimulated with Epo. Epo stimulation of erythroid cells was not observed in activation of PLC\(\gamma\)1 and -2 (25, 27, 42). To determine whether PLC is involved in the Epo-stimulated increase in \([Ca^{2+}]\), primary human erythroid cells, BFU-E-derived erythroblasts were removed from methyl-cellulose culture at day 10, pretreated with the active PLC inhibitor U-73122 or the inactive analog U-73343, and stimulated with Epo.

The active PLC inhibitor U-73122 significantly blocked the Epo-stimulated increase in \([Ca^{2+}]\), modulated through TRPC3 (Fig. 4B; \(p < 0.0001\)), whereas the inactive analog U-73343 did not, suggesting that Epo regulation of TRPC3 is PLC-dependent.

The specific role of PLC\(\gamma\) in Epo activation of TRPC3 was examined using RNA interference targeted to PLC\(\gamma\). HEK 293T cells were transfected with siRNA targeted to PLC\(\gamma\) or nonspecific control siRNA as well as TRPC3 and Epo-R. The effectiveness of siRNA interference in reducing PLC\(\gamma\) expression was demonstrated by Western blotting. Transfection of HEK 293T
cells with siRNA directed to PLC\(\gamma\) resulted in significant suppression of endogenous PLC\(\gamma\) protein, compared with cells transfected with control siRNA (Fig. 5), confirming previous results (10, 38). Expression of TRPC3, Epo-R, and tubulin was not affected in cells transfected with PLC\(\gamma\) siRNA or control siRNA, demonstrating the specificity of the siRNA directed to PLC\(\gamma\) (Fig. 5). In these experiments, the higher molecular mass of TRPC3 (∼125 kDa) compared with endogenous TRPC3 is secondary to linkage of TRPC3 to BFP. The functional consequences of suppression of endogenous PLC\(\gamma\) expression on Epo-induced [Ca\(^{2+}\)]\(_i\) increase through TRPC3 by siRNA targeted to PLC\(\gamma\).

**TABLE 2**

| RNA interference | Stimulation | [Ca\(^{2+}\)]\(_i\) | % Inc | n |
|------------------|------------|---------------------|------|---|
| Control          | PBS        | 37 ± 2              | 55 ± 4 | 11 |
| PLC\(\gamma\) 1  | Epo        | 34 ± 1              | 122 ± 4 | 19 |
|                  | PBS        | 34 ± 3              | 57 ± 5 | 10 |
|                  | Epo        | 37 ± 1              | 92 ± 3 | 21 |

* A significant difference from Epo-stimulated cells expressing nonspecific control siRNA (\(p < 0.0001\)).

**FIGURE 5.** Western blot of HEK 293 T cells transfected with siRNA targeted to PLC\(\gamma\). Lysates were prepared from HEK 293 T cells transfected (Tx'd) with or without BFP-TRPC3 and Epo-R, and siRNA was targeted to PLC\(\gamma\) or control siRNA. Blots were probed with anti-PLC\(\gamma\), anti-TRPC3, anti-Epo-R, and anti-tubulin antibodies, followed by ECL.

**FIGURE 6.** Association of TRPC3 and TRPC3-F4 with PLC\(\gamma\) or Epo-R. A, PLC\(\gamma\) and V5-TRPC3 or V5-TRPC3-F4 were expressed in HEK 293 T cells. Immunoprecipitation (IP) was performed on lysates with anti-PLC\(\gamma\) or anti-V5 antibodies or normal rabbit serum (NRS). Western blotting (WB) was performed after immunoprecipitation with anti-PLC\(\gamma\) or anti-V5 antibodies. Representative results of five experiments are shown. B, immunoprecipitation was performed on lysates from primary human erythroid cells at Phase II day 8 of liquid culture with anti-PLC\(\gamma\) or anti-TRPC3 antibodies or normal rabbit serum. Western blotting of eluates was performed with anti-PLC\(\gamma\) or anti-TRPC3 antibodies. C, Epo-R and V5-TRPC3 or V5-TRPC3-F4 were expressed in HEK 293 T cells. Immunoprecipitation was performed on lysates with anti-Epo-R or anti-V5 antibodies or normal rabbit serum. Western blotting was performed with anti-Epo-R or anti-V5 antibodies. Representative results of three experiments are shown. Tx'd, transfected.
In control studies with normal rabbit serum, neither PLCγ nor TRPC3 precipitated (Fig. 6B).

To determine whether PLCγ and TRPC3 interact through PLCγ SH2 binding sites on TRPC3, four tyrosines (Tyr226, Tyr555, Tyr648, and Tyr674) were mutated to phenylalanine (TRPC3-F4). V5-TRPC3-F4 and PLCγ were expressed in HEK 293T cells, and immunoprecipitation was performed with anti-PLCγ or anti-V5 antibodies. These experiments demonstrated that the interaction of TRPC3 and PLCγ was significantly reduced with the TRPC3-F4 mutant (Figs. 6A and Fig. 7). These data show that PLCγ SH2 binding sites on TRPC3 are important in TRPC3 and PLCγ interaction. We previously showed that Epo-R associated with TRPC2 (10). To determine whether Epo-R also interacts with TRPC3, HEK 293T cells were transfected with Epo-R and V5-TRPC3 or V5-TRPC3-F4. Immunoprecipitation was performed with anti-Epo-R or anti-V5 antibodies or normal rabbit serum. Western blotting of precipitates demonstrated that Epo-R and TRPC3 precipitated (Fig. 6C). Neither Epo-R nor TRPC3 precipitated nonspecifically with normal rabbit serum. TRPC3-F4 also precipitated with Epo-R, demonstrating that elimination of TRPC3 PLCγ SH2 binding sites affected TRPC3/PLCγ interaction but not the association of TRPC3 with Epo-R.

To determine whether interaction with PLCγ is important in Epo activation of TRPC3, HEK 293T cells were cotransfected with Epo-R and BFP-TRPC3 or BFP-TRPC3-F4. In cells transfected with Epo-R and TRPC3-F4, the increase in [Ca2+]i observed in response to Epo was significantly reduced (Table 3; mean ± S.E. in n=6) (peak [Ca2+]i/base-line [Ca2+]i × 100%, minus 100% (base line). n, number of individual cells studied).

**TABLE 3**

| Transfection | Stimulation | Base line [% Inc | n
| --- | --- | --- | --- |
| --- | --- | --- | --- |
| TRPC3 + Epo-R | PBS | 33 ± 2 | 47 ± 2 | 43 ± 5 | 16 |
| TRPC3-F4 + Epo-R | PBS | 36 ± 2 | 52 ± 2 | 44 ± 5 | 16 |
| TRPC3-Y674F + Epo-R | PBS | 30 ± 1 | 43 ± 1 | 43 ± 6 | 6 |
| TRPC3-Y674F + Epo-R | PBS | 31 ± 1 | 105 ± 2 | 238 ± 10 | 9 |
| TRPC3-Y555F Y648F + Epo-R | PBS | 30 ± 1 | 41 ± 2 | 37 ± 3 | 6 |
| TRPC3-Y226F + Epo-R | PBS | 32 ± 1 | 102 ± 3 | 215 ± 10 | 8 |
| TRPC3-Y226F + Epo-R | PBS | 32 ± 1 | 45 ± 3 | 41 ± 6 | 6 |
| TRPC3-Y226F + Epo-R | PBS | 34 ± 2 | 62 ± 2 | 86 ± 7 | 9 |

* A significant difference from Epo-stimulated cells expressing wild type TRPC3 (p < 0.0001).

Transfection Stimulation

IP3 Receptors Are Involved in Epo Activation of TRPC3

Inhibition of Epo-stimulated increase in [Ca2+]i, through TRPC3-F4

HEK 293T cells were transfected with BFP-TRPC3, BFP-TRPC3-F4 (Y226F/Y555F/Y648F/Y674F), BFP-TRPC3-Y674F, BFP-TRPC3-Y555F/Y648F, or BFP-TRPC3-Y226F and Epo-R. Fura Red-loaded cells were treated with 40 units/ml Epo. [Ca2+]i was measured at base line and by monitoring over 20 min after Epo stimulation. Percentage increase (% Inc) above base line (mean ± S.E. in n=6) = peak [Ca2+]i/base-line [Ca2+]i × 100%, minus 100% (base line). n, number of individual cells studied.

IP3 Receptors Are Involved in Epo Activation of TRPC3

- TRPC3, BFP-TRPC3-Y674F, BFP-TRPC3-Y555F/Y648F, or BFP-TRPC3-Y226F cells transfected with Epo-R and BFP-TRPC3-Y226F, the increase in [Ca2+]i observed in response to Epo was significantly reduced (Table 3; p < 0.0001). In contrast, the Epo-stimulated rise in [Ca2+]i in cells expressing BFP-TRPC3-Y674F or BFP-TRPC3-Y555F/Y648F was not different from wild type BFP-TRPC3. We performed Western blotting on cells transfected to express Epo-R and BFP-TRPC3, BFP-TRPC3-Y674F, BFP-TRPC3-Y555F/Y648F, or BFP-TRPC3-Y226F. Expression of all constructs was similar (not shown).

To determine if TRPC3 binding to PLCγ was reduced in TRPC3 with the single substitution of Y226F, HEK 293T cells were transfected with V5-TRPC3, V5-TRPC3-F4, or V5-TRPC3-Y226F and PLCγ. Immunoprecipitation was performed with anti-PLCγ and anti-V5 antibodies. These experiments demonstrated that the interaction of TRPC3 and PLCγ was significantly reduced with V5-TRPC3-F4 but not with the V5-TRPC3-Y226F mutant (Fig. 7). These data show that TRPC3 Y226 is critical for TRPC3 activation by Epo but not for TRPC3 and PLCγ interaction.

**IP3 Receptors Are Involved in Epo Activation of TRPC3—PLC activation results in the production of IP3, and direct interaction of TRPCs with IP3-R is a common activation mechanism for TRP channels (24, 44).** TRPC3 has a conserved calmodulin/IP3 binding domain (amino acids 761–795), which binds to all IP3Rs (24). To examine the requirement for IP3-R binding to TRPC3 in Epo-modulated Ca2+ influx, we prepared 1) a deletion mutant of the TRPC3 IP3-R binding site from amino acids 761–795 (TRPC3-DEL) and 2) a substitution mutant of amino acids 775–787, replacing the sequence YQQIMKRKLKRYV with AQQIARAARKAA (TRPC3-SUB) (Fig. 8). To demonstrate that IP3-R binding to TRPC3 is abolished with these two mutants, immunoprecipitation was performed on lysates from HEK 293T cells transfected with V5-TRPC3, V5-TRPC3-DEL, or V5-TRPC3-SUB and IP3-R type II. Immunoprecipitation was performed with antibodies to V5 or IP3-R. Western blotting demonstrated that in HEK cells transfected with wild type...
V5-TRPC3 and IP$_3$R, TRPC3 and IP$_3$R precipitated reciprocally (Fig. 9). In contrast, IP$_3$R was not precipitated with anti-V5 antibody in cells expressing V5-TRPC3-DEL or V5-TRPC3-SUB; nor was V5-TRPC3-DEL or V5-TRPC3-SUB precipitated with anti-IP$_3$R antibody. The absence of precipitation with normal rabbit serum demonstrated the specificity of results (Fig. 9). These studies confirmed that direct association of IP$_3$R with TRPC3 was abolished in these two mutants.

To determine whether the association of IP$_3$R with TRPC3 is important in Epo-stimulated Ca$^{2+}$ influx through TRPC3, HEK 293T cells were transfected with Epo-R and BFP-TRPC3, BFP-TRPC3-DEL, BFP-TRPC3-SUB, or empty pQB150 vector. In cells transfected with Epo-R and BFP-TRPC3-DEL, the mean percentage increase in [Ca$^{2+}$]$_i$ above base line was 127 ± 5% after Epo stimulation, significantly less than in cells transfected with Epo-R and wild type TRPC3 (percentage increase = 266 ± 12%; Table 4; $p < 0.0001$). Similarly, the increase in [Ca$^{2+}$]$_i$, seen with BFP-TRPC3-SUB (150 ± 6%) was also significantly less than wild type TRPC3 ($p < 0.0001$). The [Ca$^{2+}$]$_i$ increase in Epo-stimulated cells expressing Epo-R and BFP-TRPC3-DEL or BFP-TRPC3-SUB was not significantly different from cells expressing empty pQB150 vector. These data demonstrate a requirement for the TRPC3 IP$_3$R binding domain in the Epo-modulated increase in [Ca$^{2+}$]$_i$.

To confirm that mutation of TRPC3 IP$_3$R binding sites did not affect TRPC3 insertion into the plasma membrane, HEK 293T cells were transfected with wild type V5-TRPC3, V5-TRPC3-DEL, or V5-TRPC3-SUB. Externalization of TRPC3 was assayed by biotinylation of cell surface proteins.

No significant difference in the cell surface expression of TRPC3 was detectable with biotinylation of TRPC3 IP$_3$R binding site mutants (Fig. 10). The lower molecular weight of V5-TRPC3-DEL is a result of the deletion of 35 amino acids, and the reduced weight of V5-TRPC3-SUB may result from differences in charge densities in the substituted protein. Also, no difference in cell surface expression of TRPC3-F4 was observed (Fig. 10).

**DISCUSSION**

Erythropoietin has been reported to stimulate an increase in [Ca$^{2+}$]$_i$ in normal human erythroid cells through a voltage-independent ion channel (4, 5). The identity of the Epo-regulated channel in human erythroid cells was unknown. We previously showed by reverse transcription-PCR and Western blotting that TRPC2 and TRPC6 are expressed in murine erythroblasts and erythroleukemia cell lines and that Epo modulates [Ca$^{2+}$]$_i$, through murine TRPC2 (7, 8). However, TRPC2 is a pseudogene in humans (11). Here, we report the presence of TRPC3 and TRPC6 in primary human erythroid cells and cell lines by Western blotting and reverse transcription-PCR. The major finding of this report is that the TRPC3 channel is regu-
lared by Epo, through a mechanism requiring PLCγ activation and interaction of TRPC3 with PLCγ and IP3R.

The first finding is that TRPC3 is expressed on normal human erythroid precursors and that the level of TRPC3 expression increases during erythroid differentiation. Using HEK 293T cells heterologously expressing TRPC3, we demonstrated that Epo stimulates a dose-dependent increase in [Ca2+]i, with a time course similar to the rise in [Ca2+]i, observed after Epo stimulation of cells expressing Epo-R and murine TRPC2. This finding suggests that TRPC3 and TRPC2 have a redundant function in Epo signaling in mice and that in human erythroid cells, TRPC3 is a channel through which Epo regulates [Ca2+]i. The Epo-stimulated increase in [Ca2+]i, through TRPC3 was slow but sustained. Quantitative fluorescent imaging measures [Ca2+]i, and the rate of the increase in [Ca2+]i, is dependent on a number of factors, including rates of Ca2+ influx, rates of Ca2+ efflux (by the plasma membrane Ca2+-ATPase), rates of sequestration by intracellular organelles (endoplasmic reticulum with the Ca2+-ATPase and mitochondria with the Ca2+ uniporter), and rates of Ca2+ leak from intracellular organelles. The rates of [Ca2+]i rise measured by digital video imaging are often slower and the amplitudes smaller when compared with unidirectional Ca2+ influx measured with whole cell current patch clamp. The slow but sustained rise in [Ca2+]i, reported here is similar to that which we observed in Epo-stimulated BFU-E-derived cells (41) despite single channel measurements showing increased channel mean open times and open probability with Epo (5).

The mechanisms through which Epo regulates [Ca2+]i, through TRPC3 were explored in this study. We examined the importance of PLC activation in Epo-stimulated calcium influx through TRPC3 using three independent approaches: disruption of PLC activity with inhibitors, reduction in PLCγ expression levels with siRNA, and interference with PLCγ binding to TRPC3. All three fundamentally different approaches resulted in significant inhibition of the rise in [Ca2+]i, following Epo stimulation. Thus, the second major finding of this report is that PLCγ activity is essential in Epo-stimulated TRPC3 activation and that TRPC3 PLCγ SH2 binding sites are important. Although the lack of complete inhibition of the rise in [Ca2+]i, by PLCγ-targeted siRNA probably resulted from incomplete suppression of PLCγ expression (Fig. 5), we cannot eliminate the possibility that other PLC members also expressed on primary erythroid cells and inhibited by U-73122, such as PLCβ family members (45), have a role. The lack of complete elimination of TRPC3 and PLCγ binding with the TRPC3-F4 mutant is consistent with previous observations that other PLCγ binding sites exist on TRPC3 (28).

An important finding is that we identified Tyr226 as an essential tyrosine required in Epo-dependent calcium influx through TRPC3. Previous studies with the muscarinic M5 acetylcholine receptor and the type 1a vasopression receptor (46) have also identified Tyr226 as a critical tyrosine in TRPC3 agonist-dependent activation. Although the function of Tyr226 in TRPC3 activation by Epo is not known, our biotinylation experiments confirmed that Tyr226 is not required for TRPC3 cell surface externalization. We hypothesize that Epo stimulation results in phosphorylation of Tyr226 and potentially other tyrosine residues on TRPC3. The mechanism of TRPC3 phosphorylation by Epo and its functional significance in channel gating are currently under investigation.

PLCγ activation results in production of diacylglycerol and IP3. TRPC3 can be directly activated by diacylglycerol (47–49) or by several mechanisms involving IP3, including calcium store depletion (50–52) or a change in the conformational coupling between the TRPC channel and IP3R after IP3 binding (53, 54). We previously have shown that calcium store release does not appear to play a key role in modulation of [Ca2+]i, following Epo activation of TRPC2 (10). As observed with TRPC2, the Epo-stimulated rise in [Ca2+]i, in cells expressing TRPC3 did not become significant until after 2 min of Epo stimulation, and the sustained increase in [Ca2+]i, was dependent on extracellular calcium entry. Although we could not rule out a local increase of Ca2+ from calcium store release that was effectively buffered, our experiments strongly suggest that the plateau increase in [Ca2+]i, observed after Epo stimulation was mediated primarily through Ca2+ influx.

Epo modulates TRPC2 opening through a mechanism requiring PLCγ activation and involving a signaling complex, including Epo-R, TRPC2, PLCγ, and IP3R (10). We confirm here that TRPC3 interacts with IP3R and that this interaction is required in Epo modulation, because when IP3R binding sites on TRPC3 are mutated or deleted, Ca2+ influx in response to Epo is significantly reduced. Our laboratory (4) and others (55) were unable to detect a global rise in IP3 in human erythroid cells in response to erythropoietin stimulation. However, since IP3R and PLCγ both directly interact with TRPC3, a small localized increase in IP3 could be produced near the cell membrane that activates IP3R and TRPC3 but would be difficult to detect using currently available biochemical techniques. A number of mechanisms have been proposed through which IP3R may activate TRPC3, including displacement of inhibitory calmodulin from a common binding domain (24, 56) as well as through interaction with the scaffold protein Homer 1 (53). Our experiments do not rule out a role for diacylglycerol in Epo activation of TRPC3; for other receptors, whether diacylglycerol- or IP3R-dependent pathways are utilized is dependent on a number of factors, including the level of TRPC3 expression, the cell type, and the presence of interacting proteins (54, 57).

We previously showed that coexpression of TRPC6 with TRPC2 and Epo-R inhibited the increase in [Ca2+]i, observed after Epo stimulation of TRPC2 and that TRPC2 and TRPC6 coassociate (9). Here, we confirm that TRPC6 does not respond to Epo stimulation, unlike the homologous TRPC3. Since both TRPC3 and TRPC6 are expressed on normal human erythroid precursors and TRPC3 and TRPC6 are reported to form heterotetramers (37, 58), we hypothesize that another pathway of TRPC3 regulation is the ability of TRPC6 to inhibit TRPC3 activation by Epo when these channels are coexpressed endogenously. Experiments are currently under way to identify the sequence differences between TRPC3 and TRPC6 that explain the different responses and to understand the downstream mechanisms that result in the activation of TRPC3 but not TRPC6 by Epo. Of note, three of the four TRPC3 PLCγ SH2 binding sites were preserved on TRPC6, but the equivalent amino acid to TRPC3 Tyr674 on TRPC6 is substituted with phe-

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nylalanine (Phe

However, this is unlikely to explain the functional differences between TRPC3 and TRPC6, since the mutant channel TRPC3 Y674F showed an Epo-dependent rise in [Ca

Here, we demonstrate that Epo-R modulates TRPC3 activation through PLCγ utilizing a mechanism requiring complex formation between TRPC3, PLCγ, and Epo-R and TRPC3 and IP

Based on data presented here, we hypothesize that Epo stimulation results in activation of PLCγ, which interacts with TRPC3 at multiple sites, including PLCγ SH2 binding domains. Localized production of IP3 binds to IP3R, which associates with TRPC3, leading to a change in the conformation of TRPC3, contributing to channel pore opening. Activation of both TRPC2 and TRPC3 by Epo is an example of several TRP channels sharing similar function and activation by the same agonist, raising questions about how the specificity of response may be regulated. Similarly, Tyr

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