Erythropoietin Modulates Calcium Influx through TRPC2*

Received for publication, June 4, 2002, and in revised form, July 9, 2002
Published, JBC Papers in Press, July 11, 2002, DOI 10.1074/jbc.M205541200

Xin Chu‡, Joseph Y. Cheung‡‡, Dwayne L. Barber¶¶, Lutz Birnbaumer**, Lawrence I. Rothblum‡, Kathleen Conrad‡, Virginia Abrasonis†, Yiu-mo Chan‡, Richard Stahl‡, David J. Carey‡, and Barbara A. Miller*

From ‡The Henry Hood Research Program, The Sigfried and Janet Weis Center for Research, and the Departments of $Medicine and ‡‡Pediatrics, the Geisinger Clinic, Danville, Pennsylvania 17822, the ¶Division of Cellular and Molecular Biology, Ontario Cancer Institute, Toronto, Ontario MSG 2M9, Canada, and the **Division of Intramural Research, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Mammalian isoforms of calcium-permeable Drosophila transient receptor potential channels (TRPC) are involved in the sustained phase of calcium entry in non-excitable cells. Erythropoietin (Epo) stimulates a rise in intracellular calcium ([Ca]i) via activation of voltage-independent calcium channel(s) in erythroid cells. Here, involvement of murine orthologs of classical TRPC in the Epo-modulated increase in [Ca]i was examined. RT-PCR of TRPC 1–6 revealed high expression of only TRPC2 in Epo-dependent cell lines HCD-57 and Ba/F3 Epo-R, in which Epo stimulates a rise in [Ca]i. Using RT-PCR, Western blotting, and immunolocalization, expression of the longest isoform of mTRPC2, clone 14, was demonstrated in HCD-57 cells, Ba/F3 Epo-R cells, and primary murine erythroblasts. To determine whether erythropoietin is capable of modulating calcium influx through TRPC2, CHO cells were cotransfected with Epo-R subcloned into pTracer-CMV and either murine TRPC2 clone 14 or TRPC6, a negative control, into pQBi50. Successful transfection of Epo-R was verified in single cells by detection of green fluorescent protein from pTracer-CMV using digital video imaging, and successful transfection of TRPC was confirmed by detection of blue fluorescent protein fused through a flexible linker to TRPC. [Ca]i changes were simultaneously monitored in cells loaded with Rhod-2 or Fura Red. Epo stimulation of CHO cells cotransfected with Epo-R and TRPC2 resulted in a rise in [Ca]i above base line (372 ± 71%), which was significantly greater (p ≤ 0.0007) than that seen in cells transfected with TRPC6 or empty pQBi50 vector. This rise in [Ca]i required Epo and extracellular calcium. These results identify a calcium-permeable channel, TRPC2, in erythroid cells and demonstrate modulation of calcium influx through this channel by erythropoietin.

Erythropoietin (Epo) is a hematopoietic growth factor that regulates proliferation, differentiation, and viability of erythroid progenitors and precursors (1–3). Regulation of intracellular calcium ([Ca]i) by erythropoietin is one of the signaling mechanisms controlling proliferation and differentiation of erythroid cells (4–10). Evidence implicating calcium in control of erythroid growth and differentiation includes: (a) enhancement of Epo-induced murine erythroid colony growth by the ionophore A23187 and inhibition by treatment with EGTA, a nonspecific chelator of calcium (7); (b) demonstration that an increase in Ca2+ influx is an early and necessary step in the commitment to differentiation of murine erythroleukemia cells (8–10); and (c) the significant rise in [Ca]i, stimulated by Epo observed at specific stages of human BFU-E differentiation (5).

Substantial evidence supports the conclusion that erythropoietin stimulates calcium influx in erythroid cells through voltage-independent calcium-permeable channel(s) (8, 10, 11–13). In patch clamp studies of human erythroid progenitor-derived cells, Epo stimulation increased calcium channel mean open time 2.5-fold and open probability 10-fold (13).

Recently, the ability of erythropoietin to activate calcium influx and influence cell proliferation and viability via stimulation of its receptor (Epo-R) in nonerythroid cells has also been demonstrated. Myoblasts have been shown to express Epo-R, and Epo stimulates myoblast proliferation to expand the progenitor population during differentiation (14). In these cells, Epo stimulated an increase in [Ca]i, that was entirely dependent on extracellular calcium influx. Erythropoietin receptors have also been identified on neuronal cell lines and Epo stimulated calcium influx in these cells as well (15). Other studies have demonstrated an important neuroprotective and neurotropic effect of erythropoietin on brain tissue (16–20). Epo stimulated an increase in cell viability in nerve growth factor-deprived cells, as well as increases in 45Ca2+ uptake and [Ca]i. These effects were inhibited by nicardipine, suggesting that Epo may stimulate neuronal function and viability through activation of calcium channels (19). These studies suggest a broader role for Epo as a growth factor capable of maintaining proliferation and preventing apoptosis during differentiation and emphasize the importance of understanding the mechanism of erythropoietin regulation of calcium influx.

A major impediment in determining the mechanisms through which erythropoietin modulates calcium entry and understanding the impact of this on cell growth and differentiation has been the difficulty in identifying and cloning the calcium-permeable channel(s) involved. Recently, a transient receptor potential; GFP, green fluorescent protein; BFP, blue fluorescent protein; FCS, fetal calf serum; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; CMV, cytomegalovirus; VNO, vomeronasal organ.

* This work was supported by National Institutes of Health Grants DK 46778 (to B. A. M.), HL 58672 (to J. Y. C.), GM 46991 (to L. I. R.), and NS 21925, NS 37716, and NS 41363 (to D. J. C.) and grants from the Geisinger Foundation (to B. A. M., J. Y. C., L. I. R., Y. C., and D. J. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Research Scientist of the National Cancer Institute of Canada.

‡‡ To whom correspondence should be addressed: The Henry Hood Research Program, The Sigfried and Janet Weis Center for Research, Geisinger Clinic, 100 N. Academy Ave., Danville, PA 17822-2616. Tel.: 570-271-6675; Fax: 570-271-6701; E-Mail: bamiller1@geisinger.edu.

† The abbreviations used are: Epo, erythropoietin; TRP, transient

Published, JBC Papers in Press, July 11, 2002, DOI 10.1074/jbc.M205541200

Printed in U.S.A.
receptor potential (TRP) protein superfamily was identified, consisting of a diverse group of calcium-permeable cation channels expressed in nonexcitable cells, based on the archetypal TRP cloned in Drosophila (21, 22). Drosophila TRP is predominantly expressed in the visual system, is required for phototransduction, and is coupled via a G protein to phospholipase C. Based on sequences from Drosophila TRP, a large number of mammalian isoforms have been cloned, which have been divided into six subfamilies (22). All mammalian isoforms share six putative transmembrane domains similar to the core structure of many pore-forming subunits of voltage-gated channels except that they lack positive charged residues necessary for the voltage sensor. The classical (22) or short (21) TRP channels (TRPC) were selected for our initial studies, reported here, because these channels have many characteristics similar to the voltage-independent, Epo-modulated calcium-permeable channels identified in human erythroblasts. These TRPC proteins all contain ankyrin repeat domains, which may be important in protein/protein interactions, and amino acid sequence identity greater than 30% in their N terminus but high variability in their carboxyl-terminal region beyond the conserved TRP domain. This diverse family of channels has both store-operated and receptor-modulated members, which function through intracellular second messenger systems (21, 22).

The mechanism of regulation of [Ca2+]i by erythropoietin has previously been examined at the single cell level using fluorescence microscopy coupled to digital video imaging (4, 5, 12, 13, 23). In this report, we used RT-PCR to determine the expression pattern of TRP on murine erythroid cell lines HCD-57 and Ba/F3 Epo-R, in which Epo stimulates a rise in [Ca2+]i (23). TRPC2 was the only TRPC detected in these cells under conditions that identified the presence of all TRPC in bone marrow. Two of four TRPC splice variants were expressed in these hematopoietic cells. The expression and function of the longest isoform, TRPC2 clone 14 (24), was examined. Cell fractionation and immunolocalization using an antibody specific for TRPC2 were identified by detection of blue fluorescence (60 mg/kg) intraperitoneally on days 1 and 2. Mice were monitored daily for 7 days. Experiments were performed with at least 6 mice per group. Data were determined using a digital video imaging system in which images of single cells that expressed transfected Epo-R were identified by immunolocalization using an antibody specific for TRPC2 clone 14 (24), was examined. Cell fractionation and immunolocalization using an antibody specific for TRPC2 were identified by detection of blue fluorescence (60 mg/kg) intraperitoneally on days 1 and 2. Mice were monitored daily for 7 days. Experiments were performed with at least 6 mice per group. Data were determined using a digital video imaging system in which images of single cells that expressed transfected Epo-R were identified by detection of blue fluorescence (60 mg/kg) intraperitoneally on days 1 and 2. Mice were monitored daily for 7 days. Experiments were performed with at least 6 mice per group. Data were determined using a digital video imaging system in which images of single cells that expressed transfected Epo-R were identified by detection of blue fluorescence.

Epo Modulates Calcium Influx through TRPC2

**Tissue and Cell Lines—** Tissues were obtained from C57Bl/6 mice, frozen in liquid nitrogen, and kept at −80 °C until use. Ba/F3 cells stably transfected with wild type murine Epo-R were cultured in RPMI with 10% FCS, 1 mg/ml G418 (Invitrogen), and 500 µg/ml IL-3. HCD-57 cells (Dr. Sandra Ruscetti) were grown in Iscove’s modified Dulbecco’s medium with 30% FCS, 5 × 10−5 M 2-mercaptoethanol, and 0.4 units/ml Epo. CHO cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FCS.

Spleen erythroblasts were obtained by injecting mice with phenylhydrazine (60 mg/kg) intraperitoneally on days 1 and 2. Mice were sacrificed on day 5 by cervical dislocation, the spleen was removed, and a single cell suspension was prepared (25, 26). To isolate erythroid lineage cells (27), the spleen cell suspension was washed and labeled with Ter-119 Microbeads (10 µl/107 cells; Miltenyi Biotech, Auburn, CA). Ter-119− cells were selected by magnetic sorting with the VarioMACS (Miltenyi). Wright–Giemsa-stained smears of marrow aspirates from hematoietic cells with anti-mTRPC2 clone 14 antibody—HCD-57 cells (3 × 106 cells/chamber) were plated in each well of Lab-Tek Permanox Chamber Slides precoated with fibronectin. After 30 min, cells were washed twice with PBS, fixed in methanol at −20 °C for 10 min, and permeabilized in 0.5% Triton X-100 in PBS for 5 min. Incubation for 10 min in 20% normal goat serum preceded staining with primary antibody (anti-TRPC2 clone 14) for 20 min at room temperature followed by secondary antibody (FITC donkey anti-rabbit IgG, Jackson Laboratories, West Grove, PA) for 20 min in the dark. Slides were stained with 3,5-diaminobenzidine (Vector Laboratories, Burlingame, CA) to visualize DNA. Cells were viewed using a Nikon Optiphot-2 microscope equipped for epifluorescence. Images were acquired with an air-cooled CCD SenSys digital camera from Photometrics (Tucson, AZ) and processed using IPLab and Enhanced Photon Reassignment software programs obtained from Scanalytics, Inc. (Fairfax, VA).

**Immunofluorescent labeling with TRPC2 antibodies—** The pTracer vector contains a CMV promoter, which allowed expression of transfected genes in a variety of cell types. The expression pattern of TRPC on murine erythroid cell lines HCD-57 and Ba/F3 Epo-R, in which Epo stimulates a rise in [Ca2+]i (23). TRPC2 was the only TRPC detected in these cells under conditions that identified the presence of all TRPC in bone marrow. Two of four TRPC splice variants were expressed in these hematopoietic cells. The expression and function of the longest isoform, TRPC2 clone 14 (24), was examined. Cell fractionation and immunolocalization using an antibody specific for TRPC2 clone 14 demonstrated plasma membrane expression. The ability of erythropoietin to modulate calcium influx through TRPC2 was determined using a digital video imaging system in which single cells that expressed transfected Epo-R were identified by detection of green fluorescent protein (GFP), cells that express transfected TRPC2 were identified by detection of blue fluorescent protein (BFP), and [Ca2+]i changes were simultaneously measured by Rhod-2 or Fura Red fluorescence.

**Experimental Procedures**

**Tissue and Cell Lines—** Tissues were obtained from C57Bl/6 mice, frozen in liquid nitrogen, and kept at −80 °C until use. Ba/F3 cells stably transfected with wild type murine Epo-R were cultured in RPMI with 10% FCS, 1 mg/ml G418 (Invitrogen), and 500 µg/ml IL-3. HCD-57 cells (Dr. Sandra Ruscetti) were grown in Iscove’s modified Dulbecco’s medium with 30% FCS, 5 × 10−5 M 2-mercaptoethanol, and 0.4 units/ml Epo. CHO cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FCS.

Spleen erythroblasts were obtained by injecting mice with phenylhydrazine (60 mg/kg) intraperitoneally on days 1 and 2. Mice were sacrificed on day 5 by cervical dislocation, the spleen was removed, and a single cell suspension was prepared (25, 26). To isolate erythroid lineage cells (27), the spleen cell suspension was washed and labeled with Ter-119 Microbeads (10 µl/107 cells; Miltenyi Biotech, Auburn, CA). Ter-119− cells were selected by magnetic sorting with the Vario-MACS (Miltenyi). Wright–Giemsa-stained smears of marrow aspirates from hematoietic cells with anti-mTRPC2 clone 14 antibody—HCD-57 cells (3 × 106 cells/chamber) were plated in each well of Lab-Tek Permanox Chamber Slides precoated with fibronectin. After 30 min, cells were washed twice with PBS, fixed in methanol at −20 °C for 10 min, and permeabilized in 0.5% Triton X-100 in PBS for 5 min. Incubation for 10 min in 20% normal goat serum preceded staining with primary antibody (anti-TRPC2 clone 14) for 20 min at room temperature followed by secondary antibody (FITC donkey anti-rabbit IgG, Jackson Laboratories, West Grove, PA) for 20 min in the dark. Slides were stained with 3,5-diaminobenzidine (Vector Laboratories, Burlingame, CA) to visualize DNA. Cells were viewed using a Nikon Optiphot-2 microscope equipped for epifluorescence. Images were acquired with an air-cooled CCD SenSys digital camera from Photometrics (Tucson, AZ) and processed using IPLab and Enhanced Photon Reassignment software programs obtained from Scanalytics, Inc. (Fairfax, VA).

**Immunoblotting of Crude Membrane Preparations—** Cell pellets from CHO cells nontransfected or transfected with mTRPC2 clone 14 in pcDNA3 (see below), Ba/F3 Epo-R cells, HCD-57 cells, and Ter-119 splenic erythroblasts were removed from storage at −80 °C, and 1 ml of Buffer I (10 mM Tris-HCl, pH 7.4, 1× protease inhibitor mixture) was added. Fresh murine brain, heart, kidney, and spleen were homogenized with a Dounce homogenizer on ice to create a cell suspension retaining intact cells (confirmed by microscopy) and centrifuged, and Buffer I was added to each pellet. Lysates were sonicated, and an equal volume of Buffer II (10 mM Tris-HCl, pH 7.4, 300 mM KCl, 20% sucrose, 1× protease inhibitor mixture) was added. Cells were then centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was spun at 100,000 × g for 1 h at 4 °C. Crude membranes were solubilized in buffer containing 62 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol. Protein was quantified using the DC Bio-Rad Protein Assay to analyze diluted samples. Conditions for SDS-PAGE and Western blotting with ECL were as described previously (23, 29). Electrophoresis was performed on 8% polyacrylamide gels. After transfer, nitrocellulose membranes were incubated with anti-TRPC2 clone 14 (1:500) or anti-mEpo-R (sc697); diluted 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Donkey anti-rabbit horseradish peroxidase-conjugated antibody (1:2000) was used as the secondary antibody.

**Transfection of mEpo-R and mTRPC into CHO Cells—** The pTracer vector (Invitrogen) containing an SV40 promoter driving expression of a GFP gene and a CMV promoter driving expression of mEpo-R was reported previously (23). TRPC2 clone 14 (24), TRPC2 clone 17 (24), and mTRPC6 (28) in pcDNA3 were subcloned into pQBi50 (QbioGen, Carlsbad, CA). The pQBi50 vector contains a CMV promoter, which allows expression at a level higher than that of the endogenous mEpo-R.
drives expression of SuperGlo BFP fused through a flexible linker to TRPC2 or TRPC6. CHO cells at 50% confluence were transfected with pTracer-CMV vector (3 μg/ml), pQBI50 vector (5 μg/ml), and LipofectAMINE (8 μg/ml; Invitrogen) in Opti-MEM 1 for 5 h at 37 °C. One ml was added to each 35-mm dish. At 5 h, an equal volume of Dulbecco’s modified Eagle’s medium with 20% FCS was added, and 18 h later this medium was replaced with Dulbecco’s modified Eagle’s medium with 10% FCS. Successful transfection of CHO cells with Epo-R and TRPC was verified by detection of GFP (excitation, 478 nm; emission, 535 nm) and BFP (excitation, 380 nm; emission, 435 nm), respectively, in the cells with digital video imaging (4, 5, 12, 13, 23). The optimal time for expression of pTracer CMV Epo-R and pQBI50 TRPC2 was 48–72 h after transfection, and this time interval was selected to examine the response of transfected CHO cells to Epo. At this time, 20–40% of individual CHO cells expressed both GFP and BFP. Successful transfection was also confirmed by Western blotting using whole cell lysates of nontransfected and transfected CHO cells (23). Anti-TRPC6 was obtained from Alomone Laboratories (Jerusalem, Israel).

Measurement of [Ca\textsuperscript{i}] with Digital Video Imaging—A fluorescence microscopy-coupled digital video imaging system was used to measure [Ca\textsuperscript{i}], (50x544) microscope-channel digital video imaging system was used to measure [Ca\textsuperscript{i}], (50x544). To study changes in [Ca\textsuperscript{i}], in transfected cells, we were not able to use Fura-2 as the detection fluorophore because its excitation and emission wavelengths overlap with those of GFP. Instead, we used the fluorescent Ca\textsuperscript{2+} indicators Rhod-2 (Molecular Probes, EBO2, Eugene, OR) (23, 30, 31) and, in later experiments, Fura Red (32, 33). Rhod-2 is a single wavelength excitation Ca\textsuperscript{2+} fluorophore (excitation, 540 nm; emission, 600 nm), and its fluorescence intensity is proportional to [Ca\textsuperscript{i}], fluorophore concentration, optical path, and excitation light intensity. The ratio $F_r$ (fluorescence at time $r$) divided by $F_0$ (fluorescence at base line) was used to reflect changes in [Ca\textsuperscript{i}], in Rhod-2-loaded CHO cells. CHO cells were loaded with Rhod-2 ($2 \mu\text{M}, 20$ min, $37^\circ\text{C}$) and stimulated with recombinant erythropoietin (2000 units/ml; Amgen). Rhod-2 fluorescence was measured at base line and at 1, 5, 10, and 15, and 20 min after Epo stimulation. In later experiments, to minimize errors associated with fluorophore leakage and variation in lamp intensity, we used Fura Red (excitation, 460 and 490 nm; emission, 500 nm long pass) (32, 33), a dual wavelength excitation probe whose fluorescence intensity ratio is related to [Ca\textsuperscript{i}]. In these experiments, transfected CHO cells were loaded with $5 \mu\text{M}$ Fura Red-AM, in the presence of Pluronic F-127 to enhance loading, for 30 min at $37^\circ\text{C}$. Epifluorescence collected at 460-nm excitation was divided by that collected at 490-nm excitation to obtain the fluorescence intensity ratio, which was measured at base line and over a 20-min interval as described for Rhod-2. In some experiments, cells were incubated immediately prior to and during Epo stimulation with PBS containing $0.5 \mu\text{M}$ probenecid (Sigma) to block fluorophore exit from the cell. [Ca\textsuperscript{i}] measurements were performed in PBS either with (0.7 mM) or without external calcium (2 mM EGTA).

RESULTS

Expression of TRPC in Murine Tissues and Erythroid Cell Lines Using RT-PCR—The ability of erythropoietin to stimulate calcium influx in murine erythroid cells and erythroblast cell lines has been demonstrated previously (7–11, 23). Here, to explore whether this influx occurred through the classical TRP channels, the expression of TRPC1 to -6 was examined in HCD-57 murine erythroleukemia cells and in Ba/F3 Epo-R cells, a hematopoietic cell line stably transfected with murine Epo-R and previously shown to respond to Epo with a late calcium influx in murine erythroid cells and erythroleukemia cell lines has been demonstrated previously (7–11, 23). Expression was compared with that found in several other murine tissues, and brain was used as a positive control, since most TRPC are expressed in the brain. RT-PCR was performed using RNA isolated from murine brain, heart, kidney, spleen, Ba/F3 Epo-R, and HCD-57 cells. Results are shown in Fig. 1. TRPC2 mRNA was expressed in Ba/F3 Epo-R cells and HCD-57 cells. No TRPC transcript bands were observed when PCR was performed without the reverse transcriptase step, demonstrating that these products did not result from contaminating DNA. The identity of PCR bands was confirmed by sequencing. In contrast, no expression of other classical TRPC was detected in these hematopoietic cell lines.

Four splice variants of murine TRPC2 have been cloned: mTRPC2 clone 14 (24) (GenBank\textsuperscript{TM} accession number AF111108), mTRPC2 clone 17 (24) (accession number AF111107), mTRPC2 α (34) (accession number AF230802), and mTRPC2 β (34) (accession number AF230803). All of these isoforms would be detected by the mTRPC2 primer set used for RT-PCR in Fig. 1. To determine which of these four isoforms are expressed in Ba/F3 and HCD-57 cells, we performed RT-PCR on RNA from brain, Ba/F3 Epo-R, and HCD-57 cells using primers that are capable of distinguishing them based on sequence differences or size of the PCR product. A schema comparing the cDNAs of the different isoforms and illustrating the strategy of primer selection is shown in Fig. 2A. The primer nucleotide sequences are provided under “Experimental Procedures.” Results of RT-PCR are shown in Fig. 2B. RNA for mTRPC2 α and for mTRPC2 clone 14 were found in these hematopoietic cell lines. The identity of PCR bands was confirmed by DNA sequencing. In contrast, mTRPC2 β and mTRPC2 clone 17 were not detected. An appropriately sized PCR product for TRPC2 clone 17 was observed with the clone 17 primers when cDNA for TRPC2 clone 17 (24) was used as the template, demonstrating the ability of this primer set to produce a product when template was available.

Generation of Antibody Specific to mTRPC2 Clone 14—To further study the expression and function of the longest mTRPC2 isoform, clone 14, an affinity-purified antibody was generated to an epitope unique to the N terminus of mTRPC2 clone 14. To characterize antibody specificity, in vitro translation was performed using cDNAs for mTRPC2 clone 14, mTRPC2 clone 17, and mTRPC6 cloned into pcDNA3. Expression of the appropriate proteins was first documented with \textsuperscript{35}S incorporation. These results are shown in Fig. 3A. Despite several attempts, translation of mTRPC2 clone 17 could not be improved, possibly because of the location of the translation start site of this isoform (Fig. 2A) and/or the absence of a perfect Kozak sequence. In vitro translation reactions were then prepared without \textsuperscript{35}S. Western blotting was performed with each of these in vitro translation products with antibody generated to mTRPC2 clone 14 (Fig. 3B) or with antibody to mTRPC6 (Fig. 3C). These results demonstrate the specificity of antibody generated to clone 14.

Membrane Localization of mTRPC2 Clone 14 in Hematopoietic Cell Lines—To determine whether mTRPC2 is expressed in the plasma membrane of hematopoietic cell lines, immunolocalization studies were performed with HCD-57 cells using anti-mTRPC2 clone 14. DAPI staining was used to localize DNA. Nonimmune rabbit serum was used as a control for
specificity. Cell staining was visualized by fluorescence microscopy (Fig. 4). Images at different planes through the cell were deconvolved (Scanalytics software) to remove out-of-focus contaminating light to generate high resolution images. Representative results (Fig. 4, c and d) shown here demonstrate that endogenous mTRPC2 clone 14 protein is localized at or in close proximity to the plasma membrane in these cells.

To confirm the localization of mTRPC2 to the plasma membrane, crude membrane preparations were prepared from non-transfected CHO cells, mTRPC2 clone 14-transfected CHO cells, and mTRPC6 in pcDNA3. A, in vitro translation products were prepared using 35S-labeled methionine, and equivalent amounts of each reaction were loaded in each lane. B, Western blot of in vitro translation products from the same three cDNAs. These were prepared as in A, except nonlabeled methionine was used. Blots were probed with anti-mTRPC2 clone 14 and demonstrate the specificity of this antibody. C, Western blots probed as described in B were stripped and reprobed with antibody to mTRPC6. Representative results of two experiments are shown.

**FIG. 2.** RT-PCR of mTRPC2 isoforms in murine hematopoietic cells. A, schema of cDNA for the four mTRPC2 splice variants α, β, clone 14, and clone 17. Primer locations (see “Experimental Procedures”) that distinguish each isoform are indicated by the arrows. Hatched regions indicate DNA sequences unique to mTRPC2 α or clone 17. Presumed ATG start sites, calcium pore regions, and the conserved TRP motif are indicated. B, RT-PCR was performed on RNA isolated from murine brain, Ba/F3 Epo-R cells, and HCD-57 cells using primers specific for each of the four mTRPC2 splice variants. RT-PCR with β-actin primers was performed as a control for RNA quality.

**FIG. 3.** Specificity of mTRPC2 clone 14 antibody. In vitro translated proteins were prepared using cDNA of mTRPC2 clone 14, mTRPC2 clone 17, and mTRPC6 in pcDNA3. A, in vitro translation products were prepared using 35S-labeled methionine, and equivalent amounts of each reaction were loaded in each lane. B, Western blot of in vitro translation products from the same three cDNAs. These were prepared as in A, except nonlabeled methionine was used. Blots were probed with anti-mTRPC2 clone 14 and demonstrate the specificity of this antibody. C, Western blots probed as described in B were stripped and reprobed with antibody to mTRPC6. Representative results of two experiments are shown.

**FIG. 4.** Immunofluorescence of HCD-57 cells stained with mTRPC2 clone 14 antibody. HCD-57 cells fixed to glass slides were stained with anti-mTRPC2 clone 14 antibody (a–d) or nonimmune rabbit serum (e–h) as primary antibody and with FITC-donkey antirabbit IgG as secondary antibody. DAPI was used to stain DNA. Fluorescent cell images for FITC (a and e), and FITC and DAPI merged (b and f) are shown. Images were taken at three representative planes for each cell for FITC fluorescence (c and g) or FITC and DAPI fluorescence merged (d and h). After deconvolution to remove out-of-focus contaminating light (Scanalytics software), images clearly demonstrate endogenous mTRPC2 expression at or in close proximity to the plasma membrane.
also showed BFP fluorescence, indicating successful transfection with mTRPC2 (Fig. 6C). [Ca], was measured in the same representative cells (indicated by the arrows) with Rhod-2 fluorescence (Fig. 6D). No interference by GFP/BFP was detected under conditions for Rhod-2 or Fura Red (see below) fluorescence measurements.

Expression of transfected mTRPC in CHO cells was further confirmed by immunoblotting. Cell lysates from nontransfected CHO cells or CHO cells transfected with mTRPC2 clone 14 or mTRPC6 were prepared. Western blotting was performed with antibody to mTRPC2 clone 14, and blots were stripped and reprobed with antibody to mTRPC6. Results confirming expression are shown in Fig. 7. The higher molecular weight of both proteins shown here compared with that in reticulocyte lysates (prepared from pcDNA3; Fig. 3) or crude membrane fractions (Fig. 5) is a result of linkage to BFP. These results also confirm that CHO cells express undetectable amounts of TRPC2 clone 14 or TRPC6 orthologs or that the TRPC antibodies fail to cross-react with the hamster TRPC proteins.
Epo Modulates Calcium Influx through TRPC2

Response of CHO Cells Transfected with Epo-R and mTRPC2

CHO cells were cotransfected with pTracer-CMV mEpo-R and with empty pQB150 vector, mTRPC2 clone 14 subcloned into pQB150, or mTRPC6 subcloned into pQB150. F0 in Rhod-2-loaded cells was measured before Epo stimulation, and Ff was measured at 1, 2.5, 5, 10, 15, and 20 min after Epo stimulation (10 units/ml). Mean Ff, peak Ff, and the percentage increase of Ff/F0 ± S.E. are shown. F0/Ff fluorescence at time 0/fluorescence at time 0, n, number of cells studied.

| TRPC    | Stimulation | F0    | Ff    | Ff/F0 | n  |
|---------|-------------|-------|-------|-------|----|
| BFP      | Epo         | 32 ± 6| 40 ± 7| 137 ± 18| 10 |
| BFP-TRPC2| PBS         | 36 ± 7| 37 ± 8| 101 ± 4  |  8 |
| Epo      |             | 23 ± 4| 79 ± 19| 372 ± 71*  | 13 |
| BFP-TRPC6| PBS         | 35 ± 11| 37 ± 11| 108 ± 18|  3 |
| Epo      |             | 32 ± 5| 50 ± 8| 173 ± 32 | 14 |

*Significantly greater than other groups by one-way analysis of variance (p < 0.007).

Table II

Response to Epo of CHO cells transfected with Epo-R and mTRPC2 in the presence of probenecid

CHO cells were cotransfected with pTracer-CMV mEpo-R and with empty pQB150 vector, mTRPC2 clone 14 subcloned into pQB150, and mTRPC6 subcloned into pQB150. F0 in Rhod-2-loaded cells was measured before Epo stimulation and Ff at intervals over 20 min after Epo stimulation (10 units/ml) in the presence of 0.5 mM probenecid. Mean Ff, peak Ff, and percentage increase of Ff/F0 ± S.E. are shown. F0/Ff fluorescence at time 0/fluorescence at time 0, n, number of cells studied.

| TRPC        | Stimulation | F0 | Ff | Ff/F0 (%) | n   |
|-------------|-------------|----|----|-----------|-----|
| BFP-TRPC2   | PBS         | 39 ± 15| 43 ± 18| 104 ± 11|  5  |
| Epo         |             | 38 ± 6| 172 ± 25| 459 ± 108*| 15  |
| BFP-TRPC6   | PBS         | 24 ± 6| 30 ± 14| 108 ± 24|  4  |
| Epo         |             | 44 ± 6| 59 ± 7  | 130 ± 10|  9  |

*Significantly greater than other groups by one-way analyses of variance (p < 0.03).

FIG. 7. Western blot of transfected CHO cells. Lysates were prepared from nontransfected (-) CHO cells or CHO cells transfected with BFP-tagged TRPC2 clone 14 or TRPC6 in pQB150. Fifty μg of protein was loaded in each lane. Western blotting was performed with anti-mTRPC2 c14 or anti-mTRPC6 antibodies, followed by ECL.

FIG. 8. Requirement for Epo and external calcium in the Epo-stimulated calcium increase in transfected CHO cells. Fura Red-loaded CHO cells transfected with pTracer-CMV Epo-R and pQB150 mTRPC2 c14 in 2 mM EGTA were treated with Epo (10 units/ml) or PBS (vehicle) at 0 min. Exogenous calcium chloride (3 mM) was added where indicated at 10 min. The mean percentage change ± S.E. in the fluorescence intensity ratio at each time point compared with base line is shown.

Discussion

In this report, CHO cells were transfected with wild-type Epo-R and with specific TRPC channels to study erythropoietin signal transduction involving calcium channel activation. This is the first study of receptor-mediated calcium signaling in which successful transfection of both receptor and putative calcium channel were authenticated at the single cell level by fluorescence from different fluorophores, GFP and BFP, and [Ca], modulation was studied with a third fluorophore, Rhod-2.
Epo Modulates Calcium Influx through TRPC2

TRPC2 has been shown to be activated by both calcium store release (24, 36) and receptor-operated mechanisms, including activation by the M5 muscarinic receptor (24) and in sperm by the glycoprotein ZP3 in the egg’s extracellular matrix (36). TRPC2 has also been shown to have a very important function in rodent pheromone receptor activation in the VNO (37), through a mechanism postulated to involve phospholipase C but not calcium store release, since VNO lacks calcium stores (38). Although the mechanisms by which Epo regulates TRPC2 activity were not addressed in this exploratory study, in erythroid cells, we were unable to demonstrate an increase in [Ca\textsuperscript{2+}] in the first 2 min after Epo stimulation, arguing against activation of TRPC by depletion of calcium stores in these cells. Of note, two other TRP family channels have also been shown to be regulated by growth factors: (a) GRC, a mouse homologue of VRL-1, by insulin-like growth factor-1 through regulation of membrane trafficking (39) and (b) TRPC3 by brain-derived nerve growth factor through activation of the neurotrophin receptor TrkB and phospholipase C (40).

In summary, we have shown plasma membrane expression of both mTRPC2 clone 14 and Epo-R in erythroid cells. Using CHO cells doubly transfected with Epo-R and TRPC channels, we demonstrated the ability of Epo-R to regulate calcium influx through mTRPC2 but not mTRPC6. Whereas our data clearly demonstrate a role for mTRPC2, it is probably not the only TRP channel of importance in hematopoietic cells. LTRPC2 is expressed on hematopoietic cells, has an important role in calcium influx in immune cells, and is involved in tumor necrosis factor-\alpha-induced cell death (41, 42). Sequence homology analysis using the Kimura pairwise near neighbor approach has placed mTRPC2 near the TRPM family channels LTRPC1 and LTRPC2 (24). Several TRP channels have been shown to form heteromultimers in vivo (22, 43, 44). LTRPC2 is a candidate calcium channel for regulation by hematopoietic growth factors and for interaction with mTRPC2. Important challenges will be to identify the other TRP channels expressed on hematopoietic cells, including ones that have not yet been cloned, and to determine the regulation and function of TRP homo- and heteromultimeric channels in hematopoietic growth factor-regulated proliferation, differentiation, and cell survival.

REFERENCES

1. Damen, J. E., and Krystal, G. (1996) \textit{Exp. Hematol.} \textbf{24}, 1455–1459
2. Wojcik, D. M., Gregory, R. C., Miller, C. P., Pandit A. K., and Porcher, T. J. (1999) \textit{Exp. Cell Res.} \textbf{253}, 143–156
3. Cheung, J. Y., and Miller, B. A. (2001) \textit{Nephron} \textbf{87}, 215–222
4. Miller, B. A., Scaduto, R. C., Jr., Tillotson, D. L., Beil, J. J., and Cheung, Y. (1988) \textit{J. Clin. Invest.} \textbf{82}, 309–315
5. Miller, B. A., Cheung, J. Y., Tillotson, D. L., Hope, S. M., and Scaduto, R. C., Jr. (1989) \textit{Blood} \textbf{73}, 1184–1194
6. Mladenovic, J., and Kay, N. E. (1988) \textit{J. Lab. Clin. Med.} \textbf{112}, 23–27
7. Misiti, J., and Spivak, J. L. (1979) \textit{J. Clin. Invest.} \textbf{64}, 1573–1579
8. Gillo, B. M., and Marks, A. R. (1993) \textit{Blood} \textbf{81}, 785–792
9. Harteneck, C., Plant, T. D., and Schultz, G. (2000) \textit{J. Neurochem.} \textbf{77}, 1362–1370
10. Levenson, R., Housman, D., and Cantley, L. (1980) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{77}, 5948–5952
11. Sawyer, S. T., and Krantz, S. B. (1984) \textit{Biological Chem.} \textbf{259}, 2769–2774
12. Cheung, J. Y., Elenkay, M. B., Brauneis, U., Scaduto, R. C., Jr., Bell, L. L., Tillotson, D. L., and Miller, B. A. (1997) \textit{Blood} \textbf{89}, 92–100
13. Ogivie, M. Y., Xu, N., Nicolas-Metrail, V., Pudlo, S. M., Liu, C., Ruegg, U. T., and Noguchi, C. T. (2000) \textit{J. Biol. Chem.} \textbf{275}, 39754–39761
14. Masuda, S., Nagao, M., Takahata, K., Konishi, Y., Gallay, F. A., Jr., Tahira, T., and Sasaki, R. (1993) \textit{J. Biol. Chem.} \textbf{268}, 11208–11216
15. Dame, C., Juul, S. E., and Christensen, R. D. (2001) \textit{Biol. Neonate} \textbf{79}, 228–235
16. Cerami, A., Brines, M. L., Ghezzi, P., and Cerami, C. J. (2001) \textit{Semin. Oncol.} \textbf{28}, 66–70
17. Sireen, A.-L., Fratelli, M., Brines, M., Goeman, C., Casagranda, S., Lewczuk, P., Keenan, S., Greiter, C., Pasquali, C., Capobianco, A., Mennini, T., Heuner, W., and Ghezzi, P. (1999) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{96}, 4044–4049
18. Koshimura, K., Makita, Y., Seishi, Y., Tanaka, J., and Kato, Y. (1999) \textit{Biochem. Biophys. Res. Commun.} \textbf{265}, 397–402
19. Ghosh, A., and Greenberg, M. E. (1995) \textit{Science} \textbf{268}, 239–247
20. Harteneck, C., Plant, T. D., and Schultz, G. (2000) \textit{Trends Neurosci.} \textbf{23}, 159–166
21. Monell, C. (2001) \textit{Science’s STKE}, 2001:90:RE1
22. Miller, B. A., Barber, D. L., Bell, L. L., Beil, J. J., Beattie, B. K., Zhang, M.-Y., Neel, B. G., Yoakim, M., Rothblum, L. I., and Cheung, J. Y. (1999) \textit{J. Biol. Chem.} \textbf{274}, 20645–20647
23. Vannier, B., Peyton, M., Boulay, G., Brown, D., Qin, N., Jiang, M., Zhu, X., and Birnbaumer, L. (1999) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{96}, 2060–2064
24. Barber, D. L., Beattie, B. K., Mason, J. M., Nguyen, M. H.-H., Yoakim, M.,
Epo Modulates Calcium Influx through TRPC2

34382

Neel, B., D’Andrea, A. D., and Frank, D. A. (2001) Blood 97, 2230–2237
26. Krystal, G. (1983) Exp. Hematol. 11, 649–660
27. Kina, T., Itokata, K., Takaokaya, E., Wada, K., Majumdar, A. S., Weissman, I. L., and Katsura, Y. (2000) Br. J. Hematol. 109, 280–287
28. Zhu, X., Jiang, M., Peyton, M., Boulay, G., Hurst, R., Stefani, E., and Birnbaumer, L. (1996) Cell 85, 661–671
29. Zhang, M.-Y., Hurhaj, E. W., Bell, L., Sun, S.-C., and Miller, B. A. (1998) Blood 92, 1225–1234
30. Mitani, A., Takeyasu, S., Yanase, H., Nakamura, Y., and Kato, A. (1994) J. Neurochem. 62, 620–634
31. Yoshino, M., and Kamiya, H. (1995) Brain Res. 695, 179–185
32. Wu, Y., and Clusin, W. T. (1997) Am. J. Physiol. 273, H2161–H2169
33. Kurebayashi, N., Harkins, A. B., and Baylor, S. M. (1993) Biophys. J. 64, 1934–1960
34. Hofmann, T., Schaeffer, M., Schultz, G., and Gudermann, T. (2000) Biochem. J. 351, 115–122
35. Billestrup, N., Rouchelouche, P., Allevato, G., Ilono, M., and Nielsen, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2725–2729
36. Jungnickel, M. K., Marrero, H., Birnbaumer, L., Lemos, J. R., and Florman, H. M. (2001) Nat. Cell Biol. 3, 499–502
37. Stowers, L., Holy, T. E., Meister, M., Dulac, C., and Koentges, G. (2002) Science 295, 1496–1500
38. Liman, E. R., Corey, D. P., and Dulac, C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5791–5796
39. Kanzaki, M., Zhang, Y.-Q., Mashima, H., Li, L., Shibata, H., and Kojima, I. (1999) Nat. Cell Biol. 1, 165–170
40. Li, H. S., Xu, X. Z., and Montell, C. (1999) Neuron 24, 261–273
41. Sano, Y., Inamura, K., Muto, A., Mochizuki, S., Yokoi, H., Matsushime, H., and Furuta, Y. (2001) Science 293, 1327–1330
42. Har, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshida, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K., and Mori, Y. (2002) Mol. Cell 9, 163–173
43. Lintschinger, B., Balzer-Geldsetzer, M., Baskaran, T., Graier, W. F., Romanin, C., Zhou, M. X., and Grosch, K. (2000) J. Biol. Chem. 275, 27799–27805
44. Strubing, C., Krapivinsky, G., Krapivinsky, L., and Clapham, D. E. (2001) Neuron 29, 645–655

Downloaded from http://www.jbc.org/ by guest on July 19, 2018
