Role of Pertussis Toxin-Sensitive Guanosine Triphosphate-Binding Proteins in the Response of Erythroblasts to Erythropoietin

By Barbara A. Miller, Karen Foster, Janet D. Robishaw, Carol F. Whitfield, Laurie Bell, and Joseph Y. Cheung

Human progenitor-derived erythroblasts have been recently shown to respond to erythropoietin (Epo) with an increase in intracellular free calcium concentration ([Ca]i). To explore the role of guanosine triphosphate (GTP)-binding proteins in mediating the rise in [Ca]i, single day 10 erythroid burst forming unit (BFU-E)-derived erythroblasts loaded with Fura-2 were pretreated with pertussis toxin (PT), stimulated with Epo, and [Ca]i measured over 18 minutes with fluorescence microscopy coupled to digital video imaging. The [Ca]i increase in day 10 erythroblasts stimulated with Epo was blocked by pretreatment with PT in a dose-dependent manner but not by heat-inactivated PT. These observations provided strong evidence that a PT-sensitive GTP-binding protein is involved. To further characterize the GTP-binding protein, day 10 erythroblast membrane preparations were solubilized, electrophoresed, and immunoblotted with antibodies specific for the known PT-sensitive G-protein sub-units: the three subtypes of Gα1, Gα2, and Gα3 and Gαi, Gαo, or Gαo.

GROWTH FACTORS that influence the proliferation and differentiation of erythroid cells include erythropoietin (Epo),1,2 human granulocyte-macrophage colony-stimulating factor (GM-CSF),3 and human interleukin-3 (IL-3).4 While the precise stages of erythroid differentiation at which these factors exert their effects is uncertain, Epo has clearly been shown to be required for the proliferation/differentiation of more mature erythroid progenitors and precursors.5,6 The biochemical mechanism through which Epo exerts this effect has been of great interest. Using fluorescence microscopy coupled to digital video imaging, single normal human erythroblasts loaded with the Ca2+-sensitive probe Fura-2 have been shown to respond to Epo with an increase in intracellular free calcium concentration ([Ca]i). This [Ca]i increase is Epo dose-dependent and is related to the stage of erythroid differentiation.5 These observations on single normal cells at defined stages of differentiation are consistent with other reports using murine erythroleukemia (MEL) cells,7 Friend virus (FV)-infected cells,8 or normal human bone marrow9 that Epo stimulation causes alterations in cellular Ca2+ homeostasis.

Signal transduction mechanisms have been well described for certain hormone/receptor/effector pathways, such as adenylyl cyclase inhibition and activation.10,11 Receptors for certain hormones activate membrane-bound transducing proteins that bind guanosine triphosphate (GTP) (G-protein).10,12 G-proteins can modulate the activity of multiple specific effectors including adenylyl cyclase,13 ion channels in the plasma membrane,14 and enzymes that regulate phosphatidylinositol metabolism.15,16 Activation of some effectors results in an increase in intracellular free calcium.16-18 Pertussis toxin (PT) can inactivate the α subunit of certain G-proteins by adenosine diphosphate (ADP)-ribosylation, which decreases or eliminates receptor-mediated responsiveness.15 Treatment with PT can block the potentiation of calcium channel currents in response to GTP-γ-S in dorsal root ganglion cells16 and the decrease in phosphatidylinositol bisphosphate and increase in [Ca]i, which follows stimulation of neutrophils with chemotactic peptides.17 PT-catalysed ADP-ribosylation of G proteins in certain cell types also results in modulation of growth factor-induced cellular responses including change in [Ca]i.

To determine whether the [Ca]i increase that follows Epo stimulation is mediated via a G-protein, progenitor-derived erythroblasts at a defined stage of differentiation were pretreated with PT before Epo stimulation. The [Ca]i increase in single erythroblasts that followed Epo stimulation was inhibited by preincubation with PT in a dose-dependent manner. This result was not observed after pretreatment with heat-inactivated PT. PT ADP-ribosylated membrane proteins of approximately 40 Kd from progenitor-derived erythroblasts. Immunoblotting of erythroblast membranes with antibodies that recognize Gα1 showed the presence of Gα1 but not Gα2-proteins in the erythroblast membrane, suggesting that the family of G proteins is the PT-sensitive substrate.
GTP-BINDING PROTEINS IN EPO SIGNALLING

MATERIALS AND METHODS

Preparation of erythroid burst-forming unit (BFU-E)-derived erythroid precursors. Adult blood was obtained in acid citrate dextrose (ACD) according to a protocol approved by The Milton S. Hershey Medical Center Committee on Clinical Investigation. Adult blood BFU-E were partially purified by 2-aminoethyolithio-urokon bromide hydrobromide (AET)-treated sheep red blood cell (RBC) rosetting, adherence to plastic, and panning, as described previously. Partially purified mononuclear cells from adult blood were cultured in 0.9% methylcellulose media with 2 U/mL recombinant Epo (rEpo) (>10,000 U/mg; Amgen, Thousand Oaks, CA), and rGM-CSF (gift of Dr Steven Clark, Genetics Institute, Cambridge, MA; 25 ng/mL final concentration) at plateau concentrations. To study erythroid precursors at specific stages of differentiation, cells from maturing BFU-E-derived colonies were plucked from culture on day 10. Day 10 cells are partially hemoglobinized and respond to Epo with an increase in [Ca2+]i.

Measurement of [Ca2+]i in early erythroid precursor cells. BFU-E-derived cells were removed from culture on day 10 and labeled with antihuman β2 microglobulin. Cells were then bound to antimouse Ig-coated glass coverslips by incubating at 37°C for 1 hour. During this 1-hour period at 37°C, cells were exposed to Isove's Modified Dulbecco's Media (IMDM) (control cells) or to 0.05, 0.10, 0.25, 1, or 5 µg/mL active or heat-inactivated PT (List Biological Laboratories, Campbell, CA). Media was removed and the cells were then incubated in phosphate-buffered saline (PBS) at 37°C for 20 minutes and containing the same concentrations of PT as well as 1 µmol/L Fura-2 acetoxymethyl ester (Molecular Probes, Inc, Eugene, OR). Thus, the total PT pretreatment period was 80 minutes. Total time lapse from removal of cells from culture to completion of Fura-2 loading was 3 to 5 hours. Cell viability as judged by trypan blue exclusion was >98%. [Ca2+]i and its changes in response to Epo (2 U/mL), IMDM, or dibutyryl cyclic adenosine monophosphate (cAMP) (5 µmol/L; Sigma, St Louis, MO) in Fura-2-loaded cells incubated in PBS with physiologic concentrations of calcium were measured with the digital video imaging system described previously. Cells were chosen based on visible Fura-2 fluorescence and measured with the digital video imaging system described previously.

Identification of PT-sensitive G-proteins with antisera. Membrane pellets were prepared as described above for ADP-ribosylation and resuspended in 20 µL of 20 mmol/L HEPES, 2 mmol/L MgCl2, and 1 mmol/L EDTA. The pellets were frozen at −70°C until immunoblot was performed. Seventeen different membrane preparations from progenitor-derived erythroblasts were pooled, electroblotted onto a 11% acrylamide gel, and blotted as previously described.

Adenylyl cyclase activity in progenitor-derived erythroblasts. To determine the influence of Epo on adenylyl cyclase activity, day 10 BFU-E-derived erythroblasts were removed from culture and incubated in IMDM at 37°C for 60 minutes. Epo (4 U/mL) was added where specified during the 60-minute incubation. In some experiments, cells were preincubated with PT (1 µg/mL) for 60 minutes at 37°C and then incubated with Epo for 20 minutes. Membranes were prepared from cells and adenylyl cyclase activity was determined by measuring the formation of cAMP from unlabeled ATP in the presence of theophylline as described by Bonanou-Tzedaki et al. Briefly, the reaction was initiated by addition of 50 µL of the membrane fraction to 150 µL of assay mixture followed by incubation at 37°C for 20 minutes. The adenylyl cyclase assay mixture contained (final concentration): 2 mmol/L ATP, 3 mmol/L MgCl2, 10 mmol/L NaCl, 10 mmol/L KCl, and 6 mmol/L theophylline as a phosphodiesterase inhibitor in 50 mmol/L Tris-HCl buffer pH 7.4. The reaction was terminated by heating the tubes at 99°C in a heat block for 5 minutes followed by centrifugation at 3,000g for 10 minutes at 4°C. Portions of the supernatant were removed and cAMP measured with cAMP assay kit (Amersham, Arlington Heights, IL). Results are expressed as pmol/mg per 1 × 107 cells. Protein content was not determined because of insufficient sample size but assuming 200 µg protein/2 × 107 cells, picomole content is similar to that previously reported.

Influence of PT on adenylyl cyclase activity was assayed by treatment of intact cells followed by measurement of adenylyl cyclase activity in membranes prepared from stimulated cells. This
method was chosen because it had been reported that membranes isolated from cells previously treated with Epo exhibited adenylate cyclase activity greater than or equal to that obtained from isolated membrane preparations stimulated with Epo.28

RESULTS

Effect of PT on the increase in [Ca], in BFU-E-derived erythroblasts stimulated by Epo. To explore the possibility that a GTP-binding protein may be involved in Epo action, day 10 BFU-E-derived cells preincubated with 0 to 5.0 μg/mL PT were loaded with Fura-2 and the time course of [Ca], changes after Epo addition was followed. Table 1 shows the baseline [Ca], peak [Ca],, and the peak increase in [Ca], above baseline of cells treated with Epo or IMDM and followed for 18 minutes. Pretreatment with PT inhibited the increase in [Ca], observed with Epo stimulation in a dose-dependent manner. Treatment with IMDM resulted in no significant change in [Ca].

To demonstrate that the inhibition in [Ca], increase was not due to nonspecific cytotoxicity of PT, cells were preincubated with 5 μg/mL PT or 5 μg/mL heat-inactivated PT and subsequently stimulated with Epo. PT was heat-inactivated by incubating at 95°C for 20 minutes. Figure 1 shows the time course of the change in [Ca] for BFU-E-derived erythroblasts pretreated with IMDM, PT, or heat-inactivated PT and then stimulated with Epo. The increase in [Ca], observed with Epo treatment followed a similar time course in cells pretreated with media or heat-inactivated PT. The change in [Ca], in cells pretreated with heat-inactivated PT and stimulated with Epo was significantly different from that observed following pretreatment with PT (P < .01), which was similar to cells not exposed to Epo.

ADP-ribosylation of erythroblast membrane proteins by PT. To demonstrate the presence of PT-sensitive substrates in human erythroblast membranes, the ability of activated PT to ADP-ribosylate erythroblast membrane preparations was studied. PT consistently ADP-ribosylated membrane proteins of approximately 40 Kd. These results are shown in Fig 2A and were consistently seen in nine different membrane preparations. When either PT or membrane preparations were eliminated from the ADP-ribosylation reaction mixture, no bands were observed.

Identification of G-proteins in erythroblast membrane with antisera. To confirm that PT-sensitive substrates were G-proteins and to identify which G-proteins were ADP-ribosylated by PT, erythroblast membrane proteins were immunoblotted with antipeptide antibodies specific for the α subunits of G, G, and G,. The immunoblots in Fig 2 show the presence of a 41-Kd G, band (Fig 2B) and a 40-Kd G, band (Fig 2C). No G, was observed in erythroblast membranes (Fig 2D). Therefore, the substrates for ADP-ribosylation by PT appear to be one or more forms of G,.

Effect of Epo stimulation on adenylate cyclase activity. To determine whether Epo inhibited adenylate cyclase after interaction with a G-type G-protein in erythroblasts, adenylate cyclase activity was measured in membranes prepared from day 10 cells treated with Epo for 0 to 60 minutes. Adenylate cyclase activity significantly increased following Epo treatment and the stimulatory effect plateaued at 20 minutes after Epo addition (Fig 3). To explore the influence of PT on Epo-stimulated adenylate cyclase.

Table 1. Effect of PT on the [Ca] Response to Epo

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Baseline</th>
<th>Peak</th>
<th>Δ[Ca]</th>
<th>No. of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo</td>
<td>20 ± 3</td>
<td>207 ± 61*</td>
<td>187 ± 60</td>
<td>36</td>
</tr>
<tr>
<td>0.05 Epo</td>
<td>7</td>
<td>32 ± 6</td>
<td>82 ± 20*</td>
<td>51 ± 22</td>
</tr>
<tr>
<td>0.10 Epo</td>
<td>13</td>
<td>31 ± 8</td>
<td>76 ± 18*</td>
<td>45 ± 16</td>
</tr>
<tr>
<td>0.25 Epo</td>
<td>8</td>
<td>37 ± 12</td>
<td>76 ± 22</td>
<td>39 ± 14</td>
</tr>
<tr>
<td>1.0 Epo</td>
<td>20</td>
<td>21 ± 6</td>
<td>42 ± 10</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>5.0 Epo</td>
<td>21</td>
<td>27 ± 4</td>
<td>35 ± 8</td>
<td>8 ± 7</td>
</tr>
<tr>
<td>IMDM</td>
<td>12</td>
<td>31 ± 12</td>
<td>45 ± 13</td>
<td>14 ± 7</td>
</tr>
</tbody>
</table>

Day 10 BFU-E-derived erythroblasts preincubated at 37°C for 80 minutes with 0 to 5 μg/mL PT and then stimulated with 2 U/mL Epo. Mean ± SEM are shown for 15 experiments.

*Statistically increased above baseline after Epo treatment by Student's t-test (P < .05).
Fig 2. ADP-ribosylation and immunoblots of membrane proteins from day 10 erythroblasts. Proteins with or without PT labeling were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred from gels to nitrocellulose. The 32P-labeled membrane blot was subjected to autoradiography. The unlabeled blots were treated with [125I]labeled goat antirabbit IgG F(ab')2 fragments after incubation with primary antiserum at a 1:500 dilution. Numbers to the left (A) mark the location of molecular weight standards. Lane 1, standard lane containing 2.5 µg of a mixture of the 41-Kd α subunits of G11, the 40-Kd α subunit of G12, and the 39-Kd α subunit of G13 from bovine brain. Lane 2 contains 25 µg membrane protein from erythroblasts. (A) 1.4 µg membrane protein ADP-ribosylated by PT; (B) G12 peptide antiserum A-56; (C) G13 peptide antiserum A-54; (D) G11 peptide antiserum A-10. Film was exposed to blots with an intensifying screen at -70°C for 7 days.

Activity, day 10 erythroblasts were preincubated with PT or media for 60 minutes and then stimulated with Epo for 20 minutes (Table 2). Stimulation with Epo resulted in a significant increase in cAMP levels (P < .025). Pretreatment with PT resulted in a further increase in cAMP levels above that observed with Epo alone but results did not reach statistical significance.

To study the role of cAMP in mediating the [Ca2+] increase in Epo-stimulated erythroblasts, day 10 BFU-E-derived erythroblasts were loaded with Fura-2 and then visualized with digital video imaging. [Ca2+] measurements were taken at baseline and during 20 minutes of stimulation with the cell-permeant analogue dibutyl cAMP. Results are shown in Table 3. [Ca2+] increased significantly above control after Epo stimulation (P < .001) but there was no significant change following incubation of cells with dibutryl cAMP.

DISCUSSION

Two general mechanisms of signal transduction for hormones and growth factors are well described: coupling of receptors to effectors through G-protein11-13 or coupling through activation of tyrosine kinase activity intrinsic to the receptor.19-29 G-proteins are GTP-binding heterotrimers that function as intermediaries in transmembrane signaling by linking receptors to a variety of specific effectors including phosphoinositidase (resulting in inositol lipid breakdown),20 adenylate cyclase,31 and ion channels.31 Agonist/receptor interaction for many of these signal transduction mechanisms results in an increase in [Ca2+].16-14,16,30-32 G-proteins are substrates for ADP-ribosylation by PT and/or

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Table 2. Influence of PT on Adenylate Cyclase Activity in Epo-Treated Cells

<table>
<thead>
<tr>
<th></th>
<th>Preincubated With</th>
<th>Stimulated With</th>
<th>cAMP (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMDM</td>
<td>IM DM</td>
<td>0.47 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>IMDM</td>
<td>Epo</td>
<td>1.05 ± 0.18*</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>Epo</td>
<td>1.43 ± 0.39*</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>IM DM</td>
<td>0.55 ± 0.17</td>
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</table>

Day 10 erythroblasts were pretreated with or without PT (1 µg/mL) at 37°C for 60 minutes and then stimulated with or without Epo (4 U/mL) for 20 minutes. Membranes were prepared and adenylate cyclase activity assayed in the presence of 6 mmol/L theophylline. Mean ± SEM pmol cAMP produced by membranes from 1 x 10⁶ cells shown for seven experiments.

Differences among means were tested with one-way analysis of variance. A priori comparisons of means of control versus Epo-treated groups were then performed, using F-tests as tests of significance. There were no differences between IMDM/IMDM versus PT/IMDM groups.

*P < .025 compared with IMDM/IMDM.
2-day Epo-dependent proliferation of an Epo-responsive cell line 32D Cl 23. These results using different approaches also support the concept that G-proteins are involved in Epo signal transduction.

To further identify PT-sensitive substrates in day 10 erythroblasts, membrane preparations were ADP-ribosylated by PT. At least four different polypeptides have been reported to be ADP-ribosylated by PT. By denaturing gel electrophoresis, these polypeptides have apparent molecular masses of 39 to 41 Kd. PT-sensitive substrates of approximately 40 Kd were reproducibly observed, consistent with the molecular weight of previously described Gα subunits. Resolution of the ADP-ribosylation band was insufficient to distinguish the precise number of bands present. If sufficiently low amounts of membrane were loaded it may have been possible to resolve 41-Kd and 40-Kd bands on gels by silver stain, but the resolution would be partially lost due to 32P-scatter. Therefore, immunoblots were performed with antibodies specific for the different PT substrates to define more precisely the PT-sensitive α protein subunits present. Immunoblot analysis showed that the substrates for ADP-ribosylation by PT present on erythroblast membranes are G11, G12, or G13. The Gα subunit, which has a molecular mass of 39 Kd, was not detected.

The nature of the effector system to which the Epo receptor is coupled has not been explored here. With respect to Epo-induced [Ca2+] increase, phosphoinositidase,30,32,34 or direct G-protein gating of calcium channels31,33 are two likely possibilities because both are associated with G-proteins and with change in [Ca2+]. Although Gα is classically associated with direct G-protein gating of ion channels,33,34 recent evidence shows that the family of Gα proteins can also modulate Ca2+ currents. For example, in the adrenocortical cell line Y1, angiotensin II stimulated voltage-dependent Ca2+ currents that were blocked by PT. Y1 cells also contain Gγ-type G-proteins but no Gβ, suggesting that Gβγ-type G-proteins can mediate stimulation of calcium currents.35 The possibility was considered that Epo interaction with a Gγ-type G-protein might inhibit adenylyl cyclase, resulting in a decrease in cAMP levels. This decrease in turn might relieve tonic inhibition (by cAMP) of phosphoinositidase hydrolysis or affect activity of ion channels, resulting in a secondary increase in [Ca2+]. This hypothesis appears unlikely because this study and others have demonstrated the ability of Epo to stimulate adenylyl cyclase and cause an increase in cAMP levels.36 The influence of Epo on cAMP levels is controversial because other investigators have not demonstrated an increase in cAMP levels in response to Epo.37,40 This controversy may partially be due to the use of different cell lines or erythroleukemia cells. However, no studies to our knowledge have demonstrated a decrease in cAMP levels in response to Epo stimulation. In addition, our current results show that direct stimulation with dibutyl cAMP resulted in no appreciable increase in [Ca2+] in day 10 erythroblasts. Our data suggest that the PT-sensitive and Epo-stimulated increase in [Ca2+] is not mediated via suppression of adenylyl cyclase activity.41 In this light, it is interesting to note that other investigators have shown that dibutyl cAMP and other cAMP-enhancing agents were not effective in inducing cell growth in Epo-dependent cell lines,38 MEL cells,42 or immature rabbit erythroblasts.43 These investigators concluded that cAMP does not play a direct role as the second messenger in Epo signal transduction. Indeed, Tsuda et al found that Epo-stimulated growth was inhibited by cAMP enhancing agents.44

### Table 3. Effect of Dibutyl cAMP on [Ca2+] Levels

<table>
<thead>
<tr>
<th>Stimulated With</th>
<th>No. of Cells</th>
<th>[Ca2+] (nmol/L)</th>
<th>Δ[Ca2+] (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMDM</td>
<td>13</td>
<td>22 ± 5</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Epo</td>
<td>13</td>
<td>22 ± 5</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>DiButryl cAMP</td>
<td>10</td>
<td>24 ± 6</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

Day 10 BFU-E-derived erythroblasts stimulated with 2 U/mL Epo, 5 mmol/L dibutyryl cAMP, or IMDM for 20 minutes. Mean ± SEM shown for four experiments. Significance of differences among the means of six groups (three controls, three treatments) was determined by one-way analysis of variance. A priori comparisons of means of any n groups (n < 6) were then performed, using F-tests as tests of significance. There were no statistically significant differences among the means of baseline [Ca2+] values.

*P < .001.
The evidence presented here suggests that in day 10 human BFU-E-derived erythroblasts, signal transduction of Epo occurs through a PT-sensitive G-type GTP-binding protein. Recently, the effects of two other hematopoietic growth factors, IL-3 and GM-CSF, on myeloid cells, have also been shown to be mediated through G-proteins.27,43 Determination of the precise identity of the G-protein(s) and effector system(s) involved in the response of different hematopoietic lineages at distinct stages of differentiation to hematopoietic growth factors will be required for a fuller biochemical understanding of the variety of proliferative and differentiation pathways observed.

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