Mechanisms of Erythropoietin Signal Transduction: Involvement of Calcium Channels (43756)

BARBARA A. MILLER[1] AND JOSEPH Y. CHEUNG

Departments of Pediatrics and Medicine, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033

Abstract. **Erythropoietin (Epo) induces a dose-dependent increase in intracellular free calcium concentration ($[Ca_i]$) in single human BFU-E derived erythroblasts, which is specific for stage of differentiation and mediated through a voltage-independent ion channel permeable to calcium. This minireview examines the regulation of calcium channels by Epo during normal erythroid differentiation, as a model to delineate the immediate signaling events which follow interaction of Epo with its receptor.**

[P.S.E.B.M. 1994, Vol 206]

Erythropoietin (Epo) is a glycoprotein growth factor which induces proliferation and differentiation of erythroid progenitor and precursor cells. On normal human erythroid cells, Epo receptor expression peaks at the CFU-E/proerythroblast stage, with approximately 1,100 receptors per cell, followed by gradual decline during maturation to undetectable levels at the reticulocyte stage (1). The human Epo receptor (Epo-R) has recently been cloned (2). No kinase or other enzyme motif has been identified in the cytoplasmic domain of the Epo receptor. However, as an early step in the signaling cascade, Epo has been shown to induce tyrosine phosphorylation of its own receptor (3, 4) as well as other proteins (4–9) including Janus kinase 2 (5); the c-*fps/fes* proto-oncogene product (6); the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (7); and two proteins involved in activation of *ras,* the 56-kDa SH2-containing protein Shc (8) and 120-kDa GTPase-activating protein (GAP) (9). Activation of a cascade of serine and threonine kinases, including Raf-1, likely follows tyrosine phosphorylation (10, 11).

Our research and the rest of this review will focus on the changes in intracellular free calcium concentration ($[Ca_i]$) following Epo stimulation. Using fluorescence microscopy coupled to digital video imaging, our laboratory was the first to show that Epo induces a dose-dependent increase in $[Ca_i]$ in single normal human BFU-E derived erythroblasts (12). Erythroblasts were removed from culture at different days to obtain normal erythroid precursors at defined stages of maturation (13). Treatment of cells from Day 7 of culture with Epo when colonies are small and blasts poorly hemoglobinized with a large proliferative capacity (CFU-E), resulted in no significant increase in $[Ca_i]$. On Day 10 erythroblasts which are partially hemoglobinized and have decreased proliferative capacity, Epo stimulated a large increase in $[Ca_i]$ from 49 ± 11 nmol/l at baseline to 279 ± 47 nmol/l, which persisted for at least 18 min (13). *The differential $[Ca_i]$ response observed between early and late erythroid precursors to growth factor stimulation suggests that $[Ca_i]$ may be an important signal in cell differentiation.* The lack of $[Ca_i]$ response in early erythroid precursors is unlikely to be due to reduced Epo-R density since early BFU-E progeny contain more Epo-R per cell when compared with late, more mature erythroblasts (1). The role of increased $[Ca_i]$ in erythroid differentiation is supported by the observations that the calcium ionophore A23187 induced the commitment to differentiation of murine erythroleukemia (MEL) cells (14) and that Ca^{2+} influx was required for hexamethylene bisacetamide-induced differentiation of MEl cells (15).

Ca^{2+} mobilizing hormones regulate cell function

[1] To whom requests for reprints should be addressed at Department of Pediatrics, The Milton S. Hershey Medical Center, P.O. Box 850, Hershey, PA 17033.

0037-9727/94/2063–0263$10.50/0

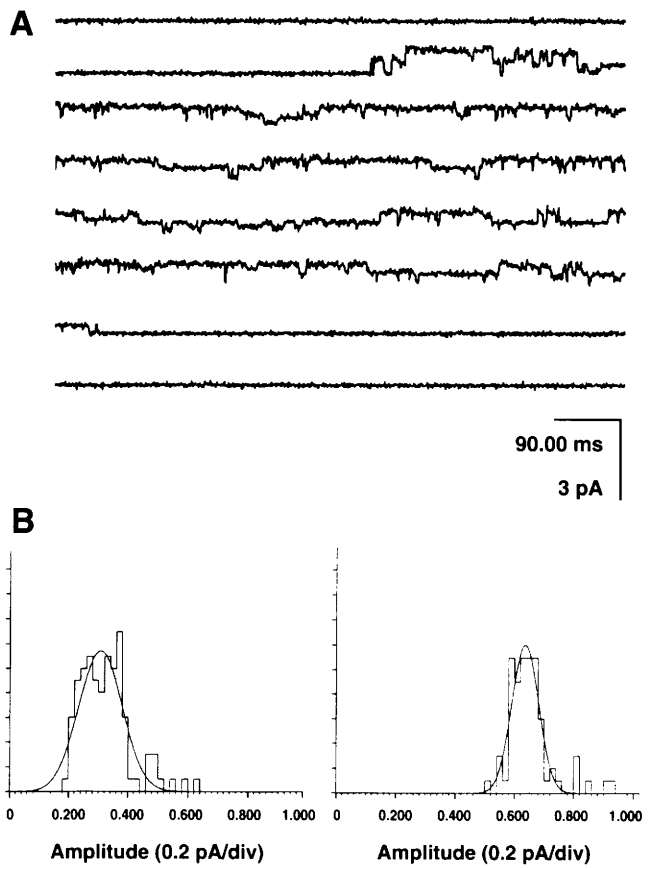

Figure 1. Calcium channels in human erythroblasts. (A) Whole-cell attached patch channel recordings of a human Day 10 BFU-E derived erythroblast incubated in media containing (in m*M*): N-methyl-D-glucamine 140, CsCl 4, $MgCl_2$ 1, EGTA 1, HEPES 20, glucose 10, pH 7.4. Sylgard-coated fire-polished pipettes (diameter 1–2 μm) were filled with millipored filling solution containing (in m*M*): N-methyl-D-glucamine 140, CsCl 4, $MgCl_2$ 0.5, $CaCl_2$ 6, tetraethylammonium 10, HEPES 20, pH 7.4. Charybdotoxin (100 n*M*) was used in pipette solution to block Ca^{2+}-activated potassium channels. Holding potential was at −20 mV. Upward deflections (inward current) indicate channel openings. There are two channels in this membrane patch. (B) Amplitude histograms showing two identical channels, each passing 0.3 pA at −20 mV. Addition of Epo (2 units/ml) to this erythroblast resulted in increase in open probability from 0.059 to 0.273 with no changes in the amplitudes.

by increasing $[Ca_i]$ through two basic mechanisms, release from internal stores via receptor-G protein-mediated stimulation of phospholipase C, or extracellular Ca^{2+} influx. We have demonstrated that the increase in $[Ca_i]$ observed in normal human erythroblasts is the result of extracellular Ca^{2+} influx via an Epo-modulated voltage independent ion channel permeable to calcium (16). This is supported by several observations: (i) chelation of extracellular free calcium with EGTA abolished the rise in $[Ca_i]$ in response to Epo; (ii) pretreatment of cells with the calcium channel blocker nifedipine or nickel chloride blocked the increase in $[Ca_i]$; (iii) levels of inositol-1,4,5-trisphosphate and inositol-1,3,4,5-tetrakisphosphate were not elevated in Day 10 cells treated with Epo; and (iv) addition of Epo did not cause significant changes in the resting membrane potential (16). Gillo *et al.* (15) also identified voltage-independent calcium channels on MEL cells. In preliminary experiments utilizing whole cell attached patch configuration, we have identified Epo-regulated calcium channels on Day 10 BFU-E-derived erythroblasts (Fig. 1). In response to Epo, there was an increase in open channel probability, but no change in conductance was evident.

Hormonal modulation of plasma membrane calcium channels which are ligand-regulated but not voltage-dependent is not well understood. In contrast, the activity of voltage-dependent calcium channels can be influenced by hormone-mediated phosphorylation by protein kinase A (17), protein kinase C (18), or tyrosine kinase (19, 20). Gastric mucosal calcium channels showed an increase in tyrosine phosphorylation of the 55- and 170-kDa channel subunits and a 78% and 48% increase in calcium uptake in response to platelet-derived growth factor or epidermal growth factor binding (19, 20). We have explored the role of protein phosphorylation in regulation of calcium channel activity by Epo (Miller *et al.*, submitted[2]). The Epo-induced increase in $[Ca_i]$ was blocked by staurosporine, a broad inhibitor of serine/threonine and tyrosine kinases. However, the Epo-induced calcium influx was unaffected by preincubation of cells with specific inhibitors of protein kinase C (Calphostin C) or the c-AMP- or C-GMP-dependent kinases (KT 5720, HA 1004). $[Ca_i]$ did not increase following stimulation with phorbol 12-myristate 13-acetate (PMA) or dibutryl c-AMP. These results suggest that neither protein kinase C nor protein kinase A mediate the Epo-induced $[Ca_i]$ increase. In contrast, preincubation with genistein, a tyrosine kinase inhibitor, blocked the Epo-induced increase in $[Ca_i]$ (Table I). The results demonstrate that tyrosine phosphorylation has a functional role in regulation of plasma membrane calcium channels by Epo. We have not yet determined whether the relevant protein(s) tyrosine phosphorylated is the Epo receptor, the calcium channel itself, or protein involved in receptor/channel coupling.

Evidence suggests that GTP-binding proteins are involved in the signaling mechanisms of Epo. Three classes of proteins (GTPases) have been described: (i) the heterotrimeric G proteins which couple ligand-bound receptors to effector proteins and are composed of α (the GTPase), β and γ subunits; (ii) the small GTP-binding proteins of the Ras family; and (iii)

[2] Miller BA, Bell L, Lynch C, Cheung JY. Erythropoietin activation of plasma membrane calcium channels: A role for tyrosine phosphorylation. (Submitted, 1993).

Table I. Effect of Tyrosine Kinase Inhibition on $[Ca_i]$ Increase[a]

Stimulation	$[Ca_i]$ (n*M*) Basal	Peak	*n*
Epo	55 ± 5	207 ± 34[b]	35
Epo + staurosporine	41 ± 3	58 ± 9	17
Epo + genistein	43 ± 4	65 ± 6	24
Mastoparan	52 ± 3	214 ± 36[b]	37
Mastoparan + genistein	54 ± 10	62 ± 8	9
Epo + PT	21 ± 6	42 ± 10	20

[a] Day 10 Fura-2-loaded BFU-E-derived erythroblasts were preincubated at 37°C for 20 min with IMDM, 100 n*M* staurosporine, 20 μ*M* genistein or 1 μg/ml pertussis toxin (PT) and then stimulated with 2 units/ml recombinant erythropoietin (Amgen), 10 μ*M* mastoparan, or IMDM in the presence of inhibitor. Mean $[Ca_i]$ ± SEM at baseline and the peak during 20 min of stimulation measured by digital video imaging are shown. *n* is the number of individual cells studied.

[b] Significant increase above control ($P < 0.05$).

GTPases used in ribosomal synthesis. We have demonstrated the important role of heterotrimeric GTP-binding proteins in the signaling pathway of Epo (21). Pertussis toxin was able to: (i) ADP ribosylate Giα subunits on the surface of human (21) and fetal rat liver erythroid percursors (22); (ii) block the Epo-stimulated rise in $[Ca_i]$ in Day 10 erythroblasts (Table I, 21); and (iii) block the Epo-dependent proliferation of an Epo-responsive cell line 32 D C123 (23) as well as Epo stimulated large colony formation in rat liver (22). The pertussis toxin-sensitive G protein α subunits in human erythroblasts were Giα1 or Giα3 and Giα2 but not Goα (21). Furthermore, mastoparan, a peptide from wasp venom which activates G-proteins including Gi, was able to induce a dose-dependent rise in $[Ca_i]$ on Day 10 erythroblasts (Table I) similar to that observed with Epo (Miller *et al.*, submitted[2]). These data provide strong evidence for the involvement of heterotrimeric G proteins in the signaling mechanism of Epo and in the Epo modulation of calcium channels. The mastoparan-induced $[Ca_i]$ increase was also dependent on tyrosine phosphorylation, since it was blocked by preincubation with genistein (Table I). This suggests that G-protein activation is proximal to tyrosine kinase activation involved in calcium channel regulation by erythropoietin. Whether these G-proteins interact directly with the plasma calcium channel or in association with the Epo receptor is not clear.

The activation of a small GTP-binding protein, Ras, in erythropoietin signal transduction has been demonstrated. Using a human erythroleukemia cell line, Torti *et al.* presented evidence that GTP-bound p21Ras is increased 5-fold in HEL cells following Epo stimulation in association with Epo-induced tyrosine phosphorylation of GAP (9). The increase in GTP-

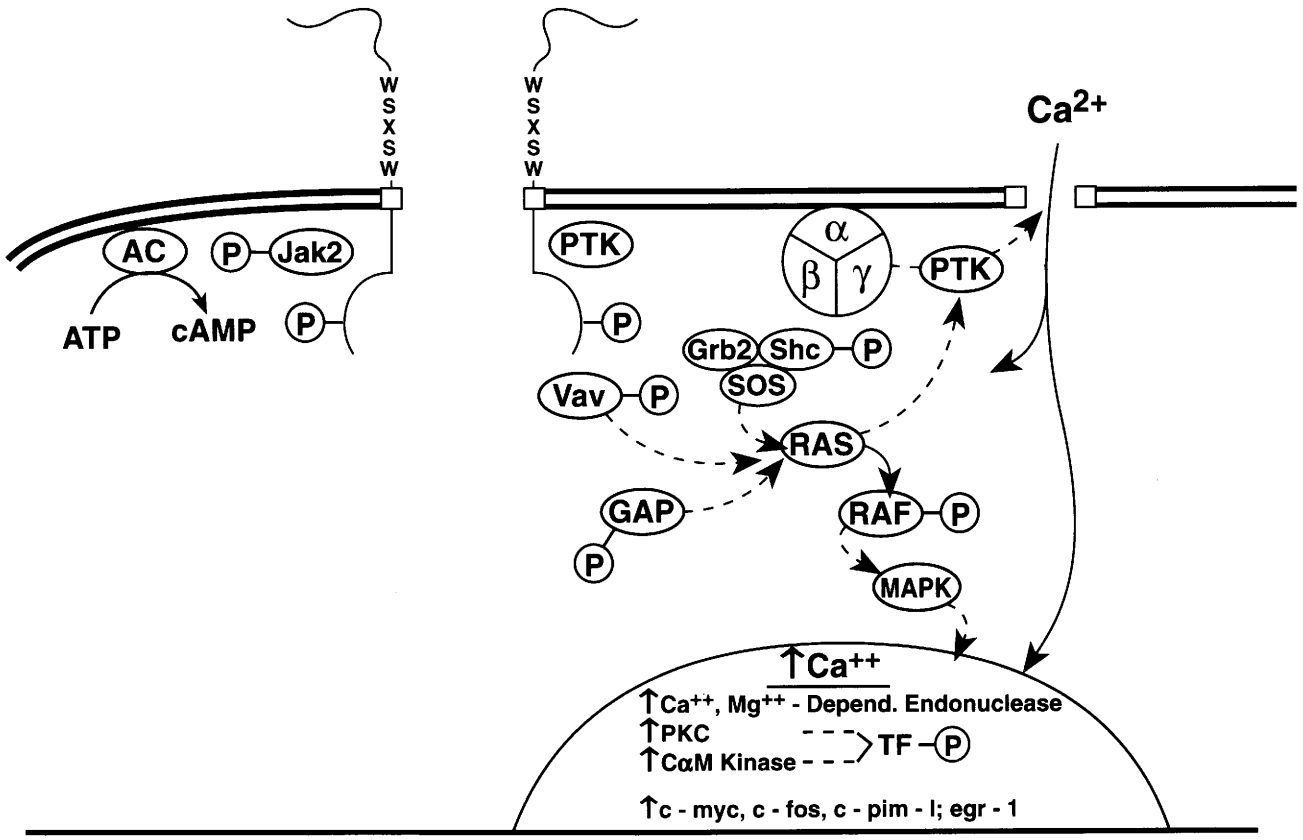

Figure 2. Model of signaling pathways of the erythropoietin receptor. Solid line, established pathway; broken lines, hypothetical pathways.

bound Ras following Epo stimulation may also result in part from Epo induced tyrosine phosphorylation of Shc and its association with Grb2 and/or a tyrosine phosphorylated 145-kDa protein (8). A third potential mechanism to increase GTP-bound Ras is activation of Vav, a hematopoietic-specific SH2- and SH3-containing molecule with Ras GDP/GTP nucleotide exchange activity (24). Tyrosine phosphorylation of Vav in response to Epo has been reported (8). The signaling cascade downstream of Ras-GTP may include phosphorylation of Raf-1 (11), followed by activation of mitogen-activated protein kinase (MAPK), with induction of immediate early genes as has been reported with GM-CSF and IL-3 (25). Another potential downstream target mechanism for activated Ras is regulation of ion channels. Ras has been shown to regulate insulin-like growth factor II-triggered calcium influx by restoring receptor G-protein coupling (26). Ras and GAP have also been shown to interfere with coupling of muscarinic receptors to the heterotrimeric G protein, G_k (27), providing evidence that Ras may control signal transducing components at the plasma membrane. Thus, inhibition of calcium channel opening by genistein could result from interference with activation of proteins involved in coupling including Ras.

The importance of increased intracellular free calcium in erythroid differentiation is not yet defined. Using optical sectioning microscopy, we demonstrated a 2- to 3-fold elevation of calcium concentration in the nucleus compared with the cytoplasm in Epo-stimulated erythroid precursors (28). Activation of Ca^{++}, Mg^{++}-dependent endonucleases (29), enhanced expression of protooncogenes (30) or phosphorylation of transcription factors following activation by calcium of protein kinase C (31) or Ca^{++}-calmodulin-dependent protein kinases (32) are possible mechanisms through which ionic signals transduced to the nucleus may play an important role in gene regulation.

A model for the known signaling components of the Epo receptor including recent observations on regulation of calcium channels is shown in Figure 2. Many of the identified components are shared by receptor superfamily members. How activation of receptors results in lineage-specific proliferation and differentiation remains to be identified.

1. Brody VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T. Erythropoietin receptor characteristics on primary human erythroid cells. Blood **77**:2583–2590, 1991.
2. Jones SS, D'Andrea AD, Haines LL, Wong GG. Human erythropoietin receptor: Cloning, expression, and biologic characterization. Blood **76**:31–35, 1990.
3. Yoshimura A, Lodish HF. *In vitro* phosphorylation of the erythropoietin receptor and associated proteins, pp 130. Mol Cell Biol **12**:706–715, 1992.
4. Dusanter-Fourt I, Casadevall N, Lacombe C, Muller O, Billat C, Fischer S, Mayeux P. Erythropoietin induces the tyrosine phosphorylation of its own receptor in human erythropoietin-responsive cells. J Biol Chem **267**:10670–10675, 1992.
5. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell **74**:227–236, 1993.
6. Hanazow Y, Chiba S, Susaki K, Mant H, Yazaki Y, Hirai H. Erythropoietin induces tyrosine phosphorylation and kinase activity of the c-*fps/fes* proto-oncogene product in human erythropoietin-responsive cells. Blood **81**:3193–3196, 1993.
7. Damen JE, Mui A, Puil L, Pawson T, Krystal G. Phosphatidylinositol 3-kinase associates, via its Src homology 2 domains, with the activated erythropoietin receptor. Blood **81**:3204–3210, 1993.
8. Damen JE, Ling L, Cutler R, Krystal G. Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-kd tyrosine phosphorylated protein. Blood **82**:2296–2303, 1993.
9. Torti M, Martin KB, Altschuller D, Yamamoto K, Lapetina EG. Erythropoietin induces p21 ras activation and p120GAP tyrosine phosphorylation in human erythroleukemia cells. J Biol Chem **267**:8293–8298, 1992.
10. Spivak JL, Fisher J, Isaacs MA, Hankins WD. Protein kinases and phosphatases are involved in erythropoietin-mediated signal transduction. Exp Hematol **20**:500–504, 1992.
11. Carroll MP, Spivak JL, McMahon M, Weich N, Rapp OR, May WS. Erythropoietin induces raf-1 activation and raf-1 is required for erythropoietin-mediated proliferation. J Biol Chem **266**:14964–14969, 1991.
12. Miller BA, Scaduto R, Tillotson D, Botti J, Cheung JY. Erythropoietin stimulates a rise in intracellular free calcium concentration in single early human erythroid precursors. J Clin Invest **82**:309–315, 1988.
13. Miller BA, Cheung JY, Tillotson DL, Hope SM, Scaduto R. Erythropoietin stimulates a rise in intracellular-free calcium concentration in single BFU-E derived erythroblasts at specific stages of differentiation. Blood **73**:1188–1194, 1989.
14. Hensold JO, Dubyak G, Housman DE. Calcium ionophore, A23187, induces commitment to differentiation but inhibits the subsequent expression of erythroid genes in murine erythroleukemia. Blood **77**:1362–1370, 1991.
15. Gillo B, Ma Y-S, Marks AR. Calcium influx in induced differentiation of murine erythroleukemia cells. Blood **81**:783–792, 1993.
16. Cheung JY, Elensky MB, Brauneis U, Scaduto RC Jr., Bell L, Tillotson DL, Miller BA. Ion channels in human erythroblasts: Modulation by erythropoietin. J Clin Invest **90**:1850–1856, 1992.
17. Nunoki K, Florio V, Catterall WA. Activation of purified calcium channels by stoichiometric protein phosphorylation. Proc Natl Acad Sci USA **86**:6816–6820, 1989.
18. O'Callahan CM, Ptasienski J, Hosey MM. Phosphorylation of the 165-kDa dihydropyridine/phenylalkylamine receptor from skeletal muscle by protein kinase C. J Biol Chem **263**:17342–17349, 1988.
19. Slomiany BL, Lui J, Slomiany A. Platelet-derived growth factor activation of gastric mucosal calcium channels. Biochem Biophys Res Commun **186**:1496–1502, 1992.
20. Slomiany BL, Liu J, Yao P, Slomiany A. Gm, -ganglioside regulation of EGF-induced gastric mucosal channel activation. Gen Pharmacol **23**:799–803, 1992.
21. Miller BA, Foster K, Robishaw JD, Whitfield CF, Bell L, Cheung JY. Role of pertussis toxin-sensitive guanosine triphosphate-binding proteins in the response of erythroblasts to erythropoietin. Blood **77**:486–492, 1991.
22. Billat C, Delemer B, Correge C, Haye B. Effect of pertussis

toxin on the erythropoietin-stimulated proliferation and differentiation of erythroid-responsive cells. Biol Neonate **601**:371–376, 1991.

23. Hilton CJ, Tan AS, Berridge MV. Evidence for direct involvement of G-proteins in erythropoietin signal transduction. Blood **74**(Suppl 1):193a, 1989.
24. Gulbins E, Coggeshall KM, Baier G, Katzav S, Burn P, Altman A. Tyrosine kinase-stimulated guanine nucleotide exchange activity of vav in T cell activation. Science **260**:822–825, 1993.
25. Miyajima A, Mui AL-F, Ogorochi T, Sakamaki K. Receptors for granulocyte-macrophage colony-stimulating factor, IL-3, and IL-5. Blood **82**:1960–1974, 1993.
26. Okamoto T, Asano T, Harada S, Ogata E, Nishimoto I. Regulation of transmembrane signal transduction of insulin-like growth factor II by competence type growth factor or viral ras p21. J Biol Chem **266**:1085–1091, 1991.
27. Yatani A, Okabe K, Polakis P, Halenbeck R, McCormick F, Brown AM. ras p21 and GAP inhibit coupling of muscarinic receptors to atrial K^+ channels. Cell **61**:769–776, 1990.
28. Yelamarty RV, Miller BA, Scaduto RC, Yu FTS, Tillotson DL, Cheung JY. Three-dimensional intracellular calcium gradients in single human BFU-E derived erythroblast induced by erythropoietin. J Clin Invest **85**:1799–1809, 1990.
29. McMahon G, Alsina JL, Levy SB. Induction of a Ca^{2+} Mg^{2+} dependent endonuclease activity during the early stages of murine erythroleukemic cell differentiation. Proc Natl Acad Sci USA **81**:7461–7465, 1984.
30. Morgan JI, Curran T. Role of ion flux in the control of c-*fos* expression. Nature **322**:552–555, 1986.
31. Greenberg ME, Greene LA, Ziff EB. Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. Science **234**:80–83, 1986.
32. Sheng M, Thompson MA, Greenberg ME. CREB:A Ca^{2+} regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science **252**:1427–1430, 1991.