Biochemical and Biophysical Research Communications **279,** 293–297 (2000)
doi:10.1006/bbrc.2000.3926, available online at http://www.idealibrary.com on IDEAL®

Erythropoietin Inhibits Calcium-Induced Neurotransmitter Release from Clonal Neuronal Cells

Masakatsu Kawakami,* Satoshi Iwasaki,*,[1] Kazuki Sato,† and Masami Takahashi*,†,[2]
**Department of Life Sciences (Biology), Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan; and †Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194-8511, Japan*

Received November 7, 2000

Erythropoietin (EPO) and EPO receptor (EPO-R) are expressed in the brain but their neuronal functions are not yet clarified. The effects of EPO on neurosecretion were studied using clonal rat pheochromocytoma PC12 cells. EPO suppressed Ca^{2+}-induced dopamine release from PC12 cells in a concentration- and time-dependent manner. Inhibition was also produced by an EPO mimetic peptide 1 (EMP1), a small synthetic peptide agonist of EPO-R, but not by its inactive analogue in which Cys residues were substituted with Ser. Inhibition was abolished by genistein but not by genistin. EPO and EMP1 induced autophosphorylation of Janus kinase 2 (JAK 2), a tyrosine kinase that associates with EPO-R, and dephosphorylation of GAP-43 in a tyrosine kinase-dependent fashion. These results suggest that EPO suppresses neurotransmitter release through activation of EPO-R linked to JAK2. © 2000 Academic Press

Key Words: **erythropoietin; neurotransmitter release; JAK2; GAP-43; PC12 cells.**

The plasticity of synaptic transmission is the cellular basis of learning and memory, and elucidation of the mechanisms involved is important to gain an understanding of these higher brain functions. Various endogenous factors are implicated in the regulation of synaptic transmission (1). Cytokines and growth factors promote differentiation, maturation, and survival of various neuronal populations during development. In addition to these effects, there is an increasing interest in the involvement of cytokines and growth factors in synaptic plasticity in mature brain (2). For example, brain-derived neurotrophic factor is essential for the survival and differentiation of neurons in central nervous system; however, it also plays an important role in synaptic plasticity in adult brain, probably through the regulation of neurotransmitter release from presynaptic nerve terminals (3).

The hematopoietic growth factor erythropoietin (EPO) is the primary regulator of mammalian erythropoiesis and is produced by the kidney and liver in an oxygen-dependent manner (4, 5). The EPO receptor (EPO-R) is a member of the type 1 superfamily of single-transmembrane cytokine receptors. EPO binding to EPO-R induces receptor oligomerization and subsequent activation by autophosphorylation of Janus kinase 2 (JAK2), a protein tyrosine kinase associated with EPO-R. JAK2 phosphorylates the signal transducer and activator of transcription (STAT), a cytoplasmic transcription factor, leading to translocation of STAT into the nucleus and regulation of transcription (6). Recently, EPO and EPO-R were found to be expressed in the brain, and hypoxia was shown to strongly stimulate EPO production (7, 8). Although activation of EPO-R has been suggested to play a neuroprotective role (8, 9), little is known about the action of EPO in the nervous system.

In the present study, we investigated the effect of EPO on neurotransmitter release using clonal rat pheochromocytoma PC12 cells and found that EPO-R activation attenuates Ca^{2+}-induced dopamine (DA) release through activation of JAK2.

MATERIALS AND METHODS

Materials. EPO mimetic peptide 1 (EMP1) was synthesized by the solid-phase methodology of Fmoc chemistry. After trifluoroacetic acid cleavage, crude peptide was purified by preparative HPLC with an ODS column by applying isocratic elution with 30% acetonitrile in 0.1% aqueous trifluoroacetic acid. The structure and purity of a synthetic peptide was confirmed by analytical HPLC, amino acid analysis, and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids, Fmoc-NH-

Abbreviations used: DA, dopamine; EMP, erythropoietin mimetic peptide; EPO, erythropoietin; EPO-R, EPO receptor; JAK, Janus kinase; PAGE, polyacrylamide gel electrophoresis; STAT, signal transducer and activator of transcription.

[1] Present address: Satoshi Iwasaki, Mitsubishi Chemical Co. Yokohama Research Center; Kazuki Sato, Faculty of Human Environmental Science, Fukuoka Women's University.

[2] To whom correspondence should be addressed at Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194-8511, Japan. Fax: (+81) 42-724-6314. E-mail: masami@libra.ls.m-kagaku.co.jp.

0006-291X/00 $35.00

resin, and other reagents used on the peptide synthesizer were obtained from Applied Biosystems Japan (Chiba, Japan). The sources of the following materials are: inactive analogue of EMP1 (iEMP1), Sawady Technology (Tokyo); polyvinylidene difluoride (PVDF) membranes (Immobilon), Millipore Corporation (Bedford, MA); Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and horse serum, GIBCO BRL, Life Technologies, Inc. (Rockville, MD); Human recombinant EPO, Sigma (St. Louis, MO); genistein, Calbiochem (La Jolla, CA); genistin, Nakarai Tesque (Kyoto).

Antibody. Anti-phosphorylated GAP-43 antibody was raised to a peptide with the sequence (C)TKIQAS(Pi)FRGHI, equivalent to GAP-43 residues 36–46, which includes a phospho-serine residue at position 41, according to a published method (10). The antibody was purified from the antiserum by affinity chromatography on phosphopeptide antigen-conjugated Sepharose as described (10). The purified antibody gave a single band at the migration position of GAP-43 in Western blots of rat brain homogenates, and the band disappeared when the antibody was preadsorbed with the phosphopeptide but not with the unphosphorylated peptide (data not shown). Anti EPO-R and anti-phospho-JAK2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Biosource International (Camarillo, CA), respectively.

Cell culture. The PC12 cells (clone 11, a gift from Dr. Yasuhisa Fukui, University of Tokyo) were maintained in DMEM containing 5% FBS and 5% heat-inactivated horse serum. Two days before experiments, 10^6 PC12 cells were plated on polyethyleneimine-coated 35 mm plastic culture dishes.

DA release assay. Before the experiments, PC12 cells were washed three times with a low-K^+ solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose and 15 mM Hepes–NaOH, and pH 7.4) and incubated in the low-K^+ solution for 10 min. After pretreating the cells in various conditions as indicated in the figure legends, they were incubated for 2 min with the low-K^+ solution and then incubated for 2 min with 1 μM ionomycin in the low-K^+ solution. To assay DA, the solution was immediately transferred to a microtube containing 150 μl of 1 M perchloric acid (PCA) at the end of each incubation period. At the end of experiments, cells were sonicated on ice with 500 μl chilled 0.2 M PCA and 0.1 mM EDTA. The samples were centrifuged at 15,000 rpm for 5 min at 4°C and the supernatant was stored at −80°C until use.

DA assay by HPLC. Released and cellular DA were assayed by HPLC using a reverse-phase column (TSK gel ODS-80TM, 4.6 mm × 10 cm, Tosoh, Tokyo) and an ECD as described (11). The mobile phase was composed of 85 mM NaH_2PO_4 buffer (pH 3.7) containing 15% methanol, 20 μM EDTA, and 2.5 mM sodium 1-octanesulfonate. The column was maintained at 34°C and a flow rate of 1 ml/min. The applied potential at the working electrode was at +700 mV against Ag/AgCl and the detector range was 2 nA full scale. The amount of DA released is expressed as a percentage of the total cellular DA content.

Immunoblotting. Proteins were denatured at 100°C for 5 min in sodium SDS sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl and pH 6.8) in the presence of 10% 2-mercaptoethanol. SDS–polyacrylamide gel electrophoresis (PAGE) was performed on linear 2–15% acrylamide gradient gels (Daiichi Pure Chemicals, Tokyo). After separation by SDS-PAGE, the proteins were transferred to PVDF membrane following standard procedures with a semi-dry transblotting apparatus. Membranes were blocked in 10% nonfat milk in Tris-buffered saline and incubated with antibodies overnight at 4°C. After washing in Tris-buffered saline containing 0.05% Tween 20, membranes were incubated for 1 h at room temperature with peroxidase-labeled anti-rabbit IgG antibody in Tris-buffered saline containing 10% milk. After washing, the immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala) and a luminescent image analyzer with an electronically cooled CCD camera system (LAS-1000, Fuji Photo Film Co., Tokyo).

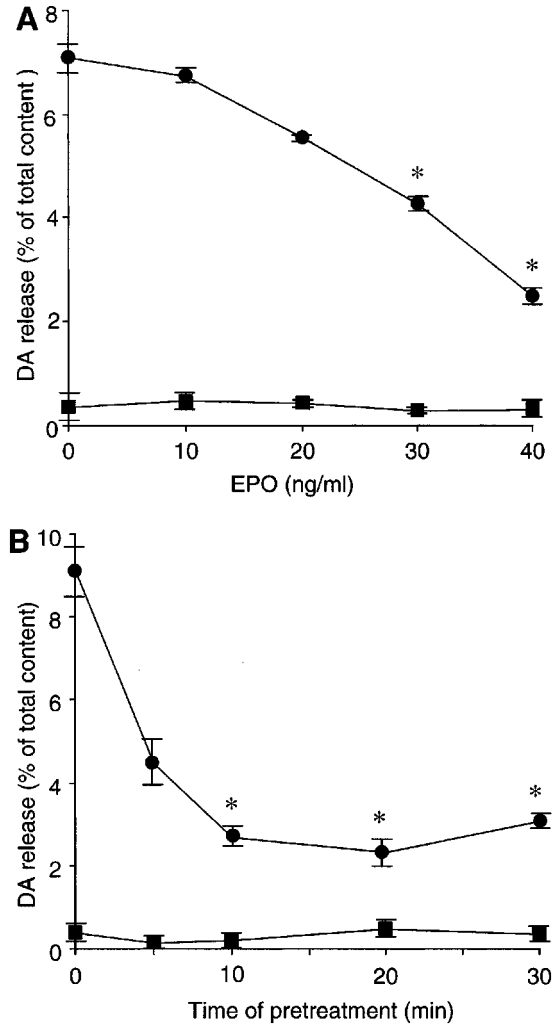

FIG. 1. Effects of EPO on basal and ionomycin-induced DA release from PC 12 cells. (A) Cells were preincubated in low-K^+ solution containing various concentrations of EPO for 10 min. The cells were further incubated in low-K^+ solution with or without EPO for 2 min, and the release of DA during this period represented basal release (■). Then the cells were incubated for 2 min in low-K^+ solution containing 1 μM ionomycin with or without EPO for 2 min; release during this period represented Ca^{2+}-induced DA release (●). (B) Cells were incubated with 30 ng/ml EPO for various periods as indicated. Basal release (■) and Ca^{2+}-induced DA release (●) were measured as above. The amount of DA was quantified using HPLC as described under Materials and Methods. The amount of DA released is expressed as a percentage of the total cellular DA content. The values represent means ± SE ($n = 3$). *, significantly different ($P < 0.01$) from the control level.

RESULTS

EPO-R-Mediated Suppression of Ca^{2+}-Induced DA Release from PC 12 Cells

PC12 cells release a substantial amount of DA in response to 1 μM ionomycin treatment in the presence of extracellular Ca^{2+}. As shown in Fig. 1, EPO suppressed Ca^{2+}-induced DA release in a concentration-

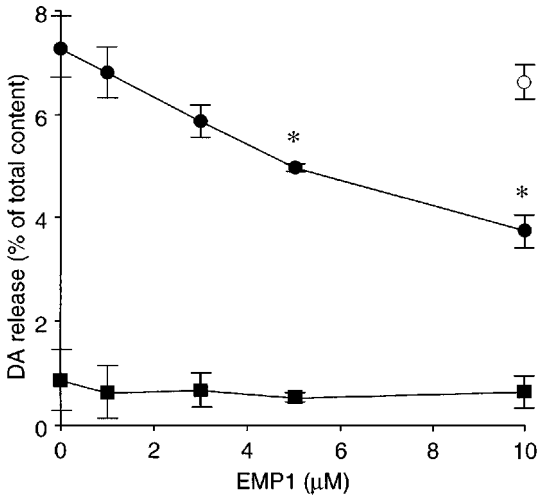

FIG. 2. Effects of EMP1 on basal (■) and ionomycin-induced DA release (●) from PC 12 cells. Cells were preincubated in low-K^+ solution containing various concentrations of EMP1 for 10 min. The cells were further incubated in low-K^+ solution with or without EMP for 2 min, and the release of DA during this period represented basal release. Then the cells were incubated for 2 min in low-K^+ solution containing 1 μM ionomycin with or without EMP for 2 min; the release during this period represented Ca^{2+}-induced DA release. (○) Ionomycin-induced DA release in the presence of 10 μM iEMP1. The amount of DA was quantified using HPLC as described under Materials and Methods and the amount of DA released is expressed as a percentage of the total cellular DA content. The values represent the means ± SE ($n = 3$). *, significantly different ($P < 0.05$) from control level.

(Fig. 1A) and time-dependent manner (Fig. 1B). The inhibition appeared at concentrations above 10 ng/ml (0.28 nM) and significant suppression was observed at 30 and 40 ng/ml (Fig. 1A). The inhibition of DA release by EPO appeared very quickly and attained a maximal level within 10 min after treatment with 30 ng/ml EPO (Fig. 1B). No significant change in the basal DA release in the low-K^+ solution was observed.

The inhibition of Ca^{2+}-induced DA release was also induced by another EPO-R agonist, EMP1. EMP1 is a synthetic 20-mer peptide having no sequence similarity with EPO (12). EMP1 binds to an extracellular region of EPO-R distinct from the EPO binding site and induces receptor dimerization and activation (13). EMP1 has two Cys residues and substitution of these Cys residues with Ser causes a loss of activity (12). As shown in Fig. 2, EMP1 also suppressed Ca^{2+}-induced DA release from PC12 cells in a concentration-dependent manner. Suppression by EMP was specific since no significant inhibition of DA-release was observed with iEMP1, an inactive analogue of EMP1 in which both of the Cys residues were mutated to Ser (12). These results indicate that activation of EPO-R induces inhibition of Ca^{2+}-induced DA release from PC 12 cells.

Activation of JAK2 by EPO and EMP1

JAK2 is a tyrosine kinase associated with EPO receptor and the activation of JAK2 is essential for the signal transduction of EPO receptor. As shown in Fig. 3A, genistein, a tyrosine kinase inhibitor, but not with genistin, an inactive analogue of genistein, abolished EPO-mediated inhibition of DA release. Activation of JAK2 was further investigated by immunoblotting of cell homogenates using an antibody specific for tyrosine phosphorylated JAK2. As shown in Fig. 3B, the intensity of the immunoreactive band increased mark-

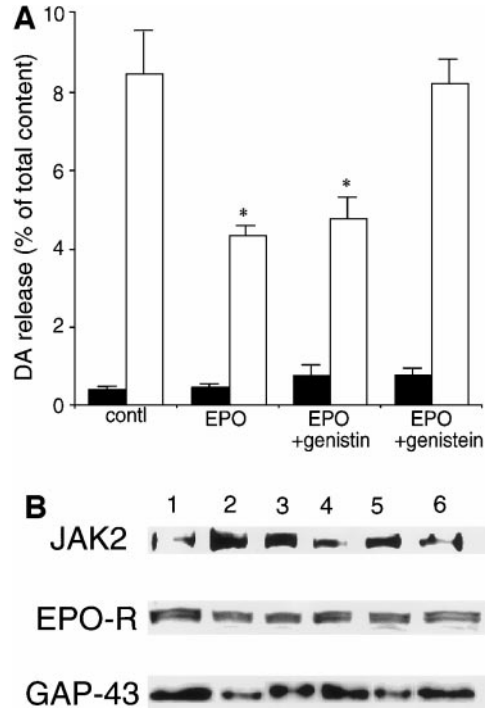

FIG. 3. Involvement of JAK2 in EPO receptor mediated inhibition of DA release from PC12 cells. (A) Cells were preincubated in low-K^+ solution for 10 min in the presence or absence of 50 ng/ml EPO with or without 10 μM genistein or genistin as indicated in the figure. After washing, the cells were further incubated in the same solution for 2 min, and the release of DA during this period represented basal release (closed bar). Then the cells were incubated for 2 min in low-K^+ solution containing 1 μM ionomycin with or without EPO and the inhibitor for 2 min, the release during this period represented Ca^{2+}-induced DA release (open bar). The amount of DA was quantified using HPLC as described under Materials and Methods and the amount of DA released is expressed as a percentage of the total cellular DA content. The values represent the means ± SE ($n = 3$). *, significantly different ($P < 0.05$) from control level. (B) EPO/EMP1 induced phosphorylation of JAK2 and dephosphorylation of GAP-43 in PC12 cells. PC12 cells were incubated under various conditions as indicated below, for 10 min at 37°C. Proteins in cell homogenates (20 μg per lane) were separated by SDS–PAGE and analyzed by immunoblotting using antiphosphorylated JAK2, anti-EPO-R, or antiphosphorylated GAP-43 antibodies. Lane 1, control; lane 2, +EPO (50 ng/ml); lane 3, +EPO and genistin (10 μM); lane 4, +EPO and genistein (10 μM); lane 5, +EMP1 (10 μM); lane 6, +iEMP1 (10 μM). The immunoreactive bands were visualized by the ECL detection system and a luminescent image analyzer LAS-1000.

edly after treatment with EPO and EMP1 but not with iEMP1. Pretreating the cells with genistein but not with genistin blocked the tyrosine phosphorylation of JAK2. Amounts of EPO-R estimated by immunoblotting did not change significantly in these conditions. These results suggest that activation of JAK2 is involved in EPO-R-mediated suppression of DA release from PC12 cells.

EPO Induces Dephosphorylation of GAP-43

GAP-43 is an abundant protein in the presynaptic compartment of the adult synapse and has been suggested to play an important role in the regulation of neurotransmitter release (14). GAP-43 is a good substrate for protein kinase C (PKC) and Ser^{41} was identified as the primary phosphorylation site (15, 16). We examined the effect of EPO on the phosphorylation of GAP-43 by immunoblotting of cellular homogenates using an antibody specific for GAP-43 phosphorylated at Ser^{41}. As shown in Fig. 3B, a strong immunoreactive GAP-43 band was identified in untreated cells, indicating that a substantial amount of GAP-43 was phosphorylated in normal conditions. The intensity of the band was markedly reduced in EPO-treated cells, and this decrement was suppressed by genestein but not by genistin. The phosphorylation of GAP-43 was also decreased by treatment with EMP1, but not by iEMP1. These results indicate that the phosphorylation of GAP-43 could be regulated by EPO-R, and that a tyrosine kinase was involved.

DISCUSSION

We showed in the present study that EPO and EMP1 inhibit Ca^{2+}-induced DA release from PC 12 cells and induce dephosphorylation of GAP-43 in a tyrosine kinase-dependent manner.

The expression of EPO-R in PC12 cells has been demonstrated by immunoblotting, Northern blotting, and more directly by cDNA cloning of EPO-R from a PC12 cell cDNA library (8, 17). The amino acid sequence of the EPO-R expressed in PC12 cells is identical to that from rat erythroid cells (17). Ligand binding experiments using ^{125}I-labeled EPO have revealed a single class of EPO binding sites with a K_d = 16 nM (17). In the present study, the inhibitory effect of EPO on Ca^{2+}-induced DA release was observed in a nM concentration range. Inhibition was also induced by EMP1, another EPO-R agonist having a structure and a binding site on EPO-R completely different from those of EPO (12, 13). EPO and EMP1 induced tyrosine phosphorylation of JAK2, which is known to associate with EPO-R, and EPO-mediated inhibition of DA release was abolished by a tyrosine kinase inhibitor in activity-dependent manner. All of these results clearly indicate that the inhibitory effect of EPO and EMP1 on Ca^{2+}-induced DA release is mediated through the activation of EPO-R in PC12 cells.

Previously, EPO was reported to activate dihydropyridine-sensitive L-type Ca^{2+} channels (17, 18), and to induce DA release from PC12 cells (18). We did not detect significant EPO-induced DA release in the present study. There are several possibilities to account for this apparent discrepancy. In the previous study, DA release was studied in differentiated PC12 cells cultured in the presence of nerve growth factor (NGF) (18). In contrast, we used NGF-untreated PC12 cells throughout the present study. It is well established that NGF-treatment induces dramatic changes in signal transduction mechanisms (19, 20). Thus, it is possible that variability in the degree of cell differentiation accounts for the different effect of EPO on the DA release. However, this possibility seems to be unlikely since we also observed EPO-mediated suppression of Ca^{2+}-dependent DA release from NGF-treated PC12 cells. PC12 cells become heterogeneous after a large number of cell divisions due to spontaneously generated mutations and many laboratories including ours have conducted subcloning of PC12 cells, using their intrinsic heterogeneity to isolate lines with a particular phenotype (see 21). Thus another possible explanation of the discrepancy is heterogeneity of PC12 cells among different laboratories. We also found that EPO inhibits Ca^{2+}-dependent glutamate release from cultured rat cerebellar and hippocampal neurons,[3] indicating that EPO-induced suppression of neurotransmitter release is not restricted to PC12 cells and may be one of the important regulatory mechanisms of presynaptic function in the central nervous system.

EPO and EMP1 inhibited DA release induced by ionomycin. Since voltage-dependent Ca^{2+} channels are not involved in DA release in these conditions, inhibition must occur subsequent to Ca^{2+} entry. The onset of inhibition by EPO/EMP occured very rapidly after application, suggesting that EPO/EMP regulates the release machinery by a post-translational modulation. In the present study, we found that phosphorylation of GAP-43 at Ser^{41} was decreased after EPO/EMP1-treatment. Both tyrosine phosphorylation of JAK2 and dephosphorylation of GAP-43 induced by EPO/EMP1 were suppressed by the tyrosine kinase inhibitor, genestein, but not by its inactive analogue, genistin, indicating that a tyrosine kinase, probably JAK2, plays an essential role in the regulation of GAP-43 phosphorylation. PKC phosphorylates GAP-43 at Ser^{41} *in vivo* and *in situ* (15, 16) and PKC is involved, at least in part, in the regulation of neurotransmitter release in variety of neuronal preparations including PC12 cells (10, 22, 23). Thus, it is quite possible that GAP-43 has an important role in the EPO-R-mediated regulation of DA release from PC12 cells. The expression of EPO in brain is up-regulated by hypoxia/ischemia (7, 8) and hypoxia/ischemia induces dephos-

[3] M. Kawakami *et al.*, in preparation.

phorylation of GAP-43 in rat brain (24). It will be interesting to elucidate the physiological consequences of the dephosphorylation of GAP-43 in EPO-mediated neuroprotection in ischemia.

So far, erythroid precursor cells have been considered to be the main target for EPO (4, 5). However, recent reports suggest that, beside its role in erythropoiesis, EPO exerts a function in the brain. Analysis of primary cerebral cells isolated from newborn mice revealed that astrocytes, but not microglial cells, expressed EPO in response to ischemic conditions, suggesting that EPO may protect neurons from ischemia-induced cell death (7–9). In addition, the present study suggests that EPO may also be involved in synaptic plasticity via the inhibition of neurotransmitter release. Various kinases including PKC, cAMP-dependent protein kinase, mitogen-activated protein kinase, and TrkA receptor protein tyrosine kinase, stimulate neurotransmitter release from several neuronal preparations (25, 26), however, reports on protein kinases involved in negative regulation are extremely scarce to date. It is crucial to elucidate the mechanism of EPO-R-mediated inhibition of neurotransmitter release to understand the functional role of the EPO/EPO-R system in the plasticity of synaptic transmission.

ACKNOWLEDGMENTS

This work has been supported in part by CREST of JST (Japan Science and Technology). We thank Dr. M. J. Seagar for critical reading of the manuscript and Ms. Atsuko Ohtake for technical assistance in peptide synthesis.

REFERENCES

1. Sanes, J. R., and Lichtman, J. W. (1999) Can molecules explain long-term potentiation? *Nat. Neurosci.* **2,** 597–604.
2. Jankowsky, J. L., and Patterson, P. H. (1999) Cytokine and growth factor involvement in long-term potentiation. *Mol. Cell. Neurosci.* **14,** 273–286.
3. McAllister, A. K., Katz, L. C., and Lo, D. C. (1999) Neurotrophins and synaptic plasticity. *Annu. Rev. Neurosci.* **22,** 295–318.
4. Krantz, S. B. (1991) Erythropoietin. *Blood* **77,** 419–434.
5. Jelkmann, W. (1992) Erythropoietin: Structure, control of production, and function. *Physiol. Rev.* **72,** 449–489.
6. Wojchowski, D. M., Gregory, R. C., Miller, C. P., Pandit, A. K., and Pircher, T. J. (1999) Signal transduction in the erythropoietin receptor system. *Exp. Cell Res.* **253,** 143–156.
7. Marti, H. H., Wenger, R. H., Rivas, L. A., Straumann, U., Digicaylioglu, M., Henn, V., Yonekawa, Y., Bauer, C., and Gassmann, M. (1996) Erythropoietin gene expression in human, monkey and murine brain. *Eur. J. Neurosci.* **8,** 666–676.
8. Morishita, E., Masuda, S., Nagao, M., Yasuda, Y., and Sasaki, R. (1997) Erythropoietin receptor is expressed in rat hippocampal and cerebral neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience* **76,** 105–116.
9. Sakanaka, M., Wen, T. C., Matsuda, S., Masuda, S., Morishita, E., Nagao, M., and Sasaki, R. (1998) In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl. Acad. Sci. USA* **95,** 4635–4640.
10. Iwasaki, S., Kataoka, M., Sekiguchi, M., Shimazaki, Y., Sato, K., and Takahashi, M. (2000) Two distinct mechanisms underlie the stimulation of neurotransmitter release by phorbol esters in clonal rat pheochromocytoma PC12 cells. *J. Biochem.* **128,** 407–414.
11. Nishiki, T., Shoji-Kasai, Y., Sekiguchi, M., Iwasaki, S., Kumakura, K., and Takahashi, M. (1997) Comparison of exocytotic mechanisms between acetylcholine- and catecholamine-containing vesicles in rat pheochromocytoma cells. *Biochem. Biophys. Res. Commun.* **239,** 57–62.
12. Wrighton, N. C., Farrell, F. X., Chang, R., Kashyap, A. K., Barbone, F. P., Mulcahy, L. S., Johnson, D. L., Barret, R. W., Jolliffe, L. K., and Dower, W. J. (1996) Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* **273,** 458–463.
13. Livnah, O., Stura, E. A., Johnson, D. L., Middleton, S. A., Mulcahy, L. S., Wrighton, N. S., Dower, W. J., Jolliffe, L. K., and Wilson, I. A. (1996) Functional mimicry of a protein hormone by a peptide agonist: The EPO receptor complex at 2.8 Å. *Science* **273,** 464–471.
14. Skene, J. H. P. (1989) Axonal growth-associated proteins. *Annu. Rev. Neurosci.* **12,** 127–156.
15. Coggins, P. J., and Zwiers, H. (1989) Evidence for a single protein kinase C-mediated phosphorylation site in rat brain protein B-50. *J. Neurochem.* **53,** 1895–1901.
16. Meiri, K. F., Bickerstaff, L. E., and Schwob, J. E. (1991) Monoclonal antibodies show that kinase C phosphorylation of GAP-43 during axonogenesis is both spatially and temporally restricted in vivo. *J. Cell Biol.* **112,** 991–1005.
17. Masuda, S., Nagao, M., Takahata, K., Konishi, Y., Ferenc, Gallyas, Jr., Tabira, T., and Sasaki, R. (1993) Functional erythropoietin receptor of the cells with neural characteristics. *J. Biol. Chem.* **268,** 11208–11216.
18. Koshimura, K., Murakami, Y., Sohmiya, M., Tanaka, J., and Kato, Y. (1999) Effects of erythropoietin on neuronal activity. *J. Neurochem.* **72,** 2565–2572.
19. Shafer, T. J., and Atchison, W. D. (1991) Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: A model for neurotoxicological studies. *Neurotoxicology* **12,** 473–492.
20. Kaplan, D. R., and Miller, F. D. (1997) Signal transduction by the neurotrophin receptors. *Curr. Opin. Cell Biol.* **9,** 213–221.
21. Shoji-Kasai, Y., Morishima, M., Kuwahara, R., Kondo, K., Itakura, M., and Takahashi, M. (2000) Establishment of variant PC12 subclones deficient in stimulation-secretion coupling. *Biochim. Biophys. Acta,* in press.
22. Pozzan, T., Gatti, G., Dozio, N., Vicentini, L. M., and Meldolesi, J. (1984) Ca^{2+}-dependent and -independent release of neurotransmitters from PC12 cells: A role for protein kinase C activation? *J. Cell Biol.* **99,** 628–638.
23. Shimazaki, Y., Nishiki, T., Omori, A., Sekiguchi, M., Kamata, Y., Kozaki, S., and Takahashi, M. (1996) Phosphorylation of 25-kDa synaptosome-associated protein. *J. Biol. Chem.* **271,** 14548–14553.
24. Huang, K. P., Huang, F. L., and Chen, H. C. (1999) Hypoxia/ischemia induces dephosphorylation of rat brain neuromodulin/GAP-43 in vivo. *J. Neurochem.* **72,** 1294–1306.
25. Greengard, P., Valtorta, F., Czernik, A. J., and Benfenati, F. (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259,** 780–785.
26. Gottschalk, W. A., Jiang, H., Tartaglia, N., Feng, L., Figurov, A., and Lu, B. (1999) Signaling mechanisms mediating BDNF modulation of synaptic plasticity in the hippocampus. *Learn. Mem.* **6,** 243–256.