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Epidermal Growth Factor-Mediated Inhibition of Neurotransmitter Glutamate Release from Rat Forebrain Synaptosomes

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SUMMARY

We investigated the possibility that receptor tyrosine kinases are involved in modulating neurotransmitter release from isolated nerve terminals. We examined the effects of epidermal growth factor on the release of neurotransmitter glutamate evoked from rat forebrain synaptosomes by KCl and 4-aminopyridine. We detected a significant inhibition of the Ca^{2+}-dependent component of release. This effect appears to be mediated by a reduction in the depolarization-evoked increase in cytosolic free calcium levels, in the absence of significant effects on the plasma membrane potential. On depolarization, a Ca^{2+}-dependent increase was observed in the phosphotyrosine content of bands at molecular masses of ~107 and ~40 kDa. The addition of epidermal growth factor before depolarization induced a significant phosphorylation of the growth factor receptor in the absence of detectable changes in the phosphotyrosine pattern of total synaptosomal proteins, suggesting that phosphorylation of a minor protein is responsible for the epidermal growth factor-mediated inhibition of glutamate release.

Phosphorylation of proteins on tyrosine residues has in general been thought to be associated with cell growth, differentiation, and transformation. However, there is increasing evidence suggesting that protein tyrosine phosphorylation is a signaling pathway also used in mature neurons. Nerve terminals of adult mammalian neurons possess all components necessary for this signaling pathway, i.e., tyrosine-specific kinases (1–3), phosphotyrosine phosphatases (4), and substrate proteins (2, 5, 6). However, the roles of tyrosine phosphorylation at the nerve terminal are poorly understood.

The detection of EGF and its receptor in the adult central nervous system (7) has prompted the idea that this growth factor might be involved in the regulation of important neuronal functions, such as synaptic activity. Physiological studies have suggested a role for EGF in the positive modulation of synaptic transmission (8, 9). However, the effects described thus far appear to be mediated primarily by a postsynaptic action of EGF. It is unknown whether EGF or other growth factors might act presynaptically, i.e., by modulating neurotransmitter release. In the present study, we used a preparation of isolated nerve terminals (synaptosomes) from adult rat forebrain to investigate the effect of EGF on neurotransmitter glutamate release and examined in parallel the phosphotyrosine patterns generated in response to depolarization and the growth factor. The results indicate that the acute application of EGF is able to negatively influence the depolarization-dependent increases in intraterminal Ca^{2+} levels, thus inhibiting the evoked release of neurotransmitter.

Experimental Procedures

Materials. Percoll and protein G-Sepharose beads were obtained from Pharmacia (Uppsala, Sweden); Fura-2 acetoxymethyl ester and $DiSC_2(5)$, Molecular Probes (Eugene, OR); anti-phosphotyrosine antibodies, UBI (Lake Placid, NY); ^{125}I-labeled goat anti-mouse IgGs, Amersham (Buckinghamshire, England); EGF, phenylmethylsulfonyl fluoride, pepstatin A, and glutamate dehydrogenase, Sigma Chemical Co. (St. Louis, MO); and Sprague-Dawley rats, Charles

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ABBREVIATIONS: 4-AP, 4-aminopyridine; BSA, bovine serum albumin; EGF, epidermal growth factor; HBM, HEPES-buffered medium; IGF-1, insulin-like growth factor 1; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; $[Ca^{2+}]_c$, cytoplasmic free calcium concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

River (Calco, Italy). Anti-EGF receptor antibodies (10) and NIH-3T3 cells transfected with the human EGF-receptor cDNA (11) were a gift of Dr. L. Beguinot (DIBIT, Milano, Italy).

Preparation of synaptosomes. Synaptosomes were prepared from forebrains of male Sprague-Dawley rats (body weight, 125–150 g) and purified on Percoll gradients as described by Dunkley *et al.* (12), with the following modifications: (i) the 0.32 M sucrose medium used for homogenization included 4 mM HEPES-Na, pH 7.4, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml pepstatin A, and (ii) the four-step Percoll gradient was replaced by a three-step gradient consisting of 23%, 10%, and 3% Percoll. A synaptosome fraction was collected from the 23%/10% interface and washed in HBM consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM HEPES-Na, pH 7.4. After being washed, the synaptosomes were stored as pellets on ice and resuspended in HBM immediately before use (within 3–4 hr after their preparation).

Glutamate release assay. Glutamate release was determined by the continuous fluorimetric assay previously described by Nicholls *et al.* (13). Briefly, synaptosomes (at a protein concentration of 0.33 mg/ml) were preincubated at 37° for 5 min in HBM containing 0.1 mM $CaCl_2$ in a thermostated, stirred cuvette. We then added 1 mM $NADP^+$, 65 units glutamate dehydrogenase, and either 1.2 mM $CaCl_2$ or 0.3 mM EGTA, and the fluorescence emission of NADPH followed in a Perkin-Elmer LS-50 spectrofluorometer. Excitation and emission wavelengths were 340 and 460 nm, respectively, with 2.5- and 7.5-nm slit width. Data points were collected every 2 sec. A glutamate standard was added to a separate trace and used to quantify released glutamate after importing the data into a Lotus 1-2-3 spreadsheet.

Determination of cytoplasmic free Ca^{2+} levels. The cytoplasmic free Ca^{2+} concentration was measured with the fluorescent indicator Fura-2 (14). Synaptosomes (1 mg protein/ml) were loaded with Fura-2 by a 35-min preincubation at 37° in HBM containing 1 mg/ml BSA, 0.1 mM $CaCl_2$, and 5 μM Fura-2 acetoxymethyl ester. After Fura-2 loading, synaptosomes were centrifuged in an Eppendorf microcentrifuge (10,000 × g_{max}) for 20 sec. The resulting pellet was resuspended at a protein concentration of 0.33 mg/ml in HBM containing 0.1 mM $CaCl_2$ and transferred to a stirred, thermostated cuvette in a Perkin-Elmer LS-50 spectrofluorometer. Fluorescence emission at a 505-nm wavelength was determined in response to alternate excitation at 340 and 380 nm, and the $[Ca^{2+}]_c$ was calculated (14) using the Intracellular Biochemistry program of the Perkin-Elmer FLDM package.

Determination of synaptosomal plasma membrane potential. Either the cationic carbocyanine dye $DiSC_2(5)$ or bisoxonol (15) were used to fluorimetrically monitor synaptosomal membrane potential changes. Synaptosomes (0.66 mg protein/ml) were resuspended in HBM containing 0.1 mM $CaCl_2$ and incubated for 5 min in a stirred, thermostated cuvette at 37° before the addition of either $DiSC_2(5)$ (2 μM) or bisoxonol (1 μM). The dye was allowed to equilibrate for 2 min, and the membrane potential determination was carried out within the subsequent 10 min. Excitation and emission wavelengths were 649 and 680 nm and 545 and 570 nm, for the $DiSC_2(5)$ and bisoxonol experiments, respectively.

EGF receptor immunoprecipitation. Synaptosomes or NIH-3T3 cells were solubilized in lysis buffer consisting of 1% Triton X-100, 10% glycerol, 1% aprotinin, and 20 mM HEPES-Na, pH 7.4, and then incubated with EGF-receptor antibodies for 2 hr at 4°. Protein G-Sepharose beads (30 μl) were added, and the incubation was continued for 45 min. The immune complexes were isolated by centrifugation and washed 5 times with 20 mM HEPES-Na, pH 7.4, 150 mM NaCl, 10% glycerol, and 0.1% Triton-X-100. The washed pellets were then boiled in Laemmli's stop solution (3% SDS, 5% v/v β-mercaptoethanol, 1 mM EDTA, 8% w/v sucrose, 62 mM Tris·HCl, pH 6.7) (16) for 5 min, separated by electrophoresis, and immunoblotted as described below.

Other procedures. Protein was determined according to the method of Bradford (17) with the Bio-Rad protein assay reagent and BSA as a standard. For the electrophoretic separation of proteins, samples were solubilized in Laemmli's stop solution, boiled for 2 or 5 min, and separated on SDS/7.5% polyacrylamide gels according to the method of Laemmli (16). After electrophoresis, the proteins were transferred to nitrocellulose membrane sheets (0.2-μm pore), as described (18). The protein blots were processed for phosphotyrosine immunoreactivity as follows: (i) incubation for 2 hr in TBS (100 mM Tris-Cl, pH 7.5, 150 mM NaCl) with 5% (w/v) BSA added; (ii) overnight incubation in TBS/5% BSA, including a 1:1000 dilution of anti-phosphotyrosine antibody; (iii) washing with five changes of TBS/5% BSA/0.5% (v/v) Tween 20 over a total period of 20 min; (iv) incubation for 2 hr in TBS/5% BSA, including ^{125}I-labeled sheep anti-mouse antibody (0.25 μCi/ml); and (v) extensive washing with several changes of TBS/5% BSA/0.5% Tween 20, followed by two changes of TBS alone. Labeling was revealed by autoradiography of the air-dried blots with Kodak X-OMAT films.

Results

The effect of EGF and of two other receptor tyrosine kinase ligands, insulin and IGF-1, on neurotransmitter glutamate release was investigated in rat forebrain synaptosomes. The Ca^{2+}-dependent component of the glutamate release evoked by the K^+ channel blocker 4-AP (3 mM) was significantly inhibited by the previous addition of 100 nM EGF (Fig. 1, A and B) but not by similar concentrations of either insulin or IGF-1 (data not shown). Table 1 illustrates the extent of inhibition mediated by EGF. Sixty seconds after the 4-AP addition, the Ca^{2+}-dependent release of glutamate was re-

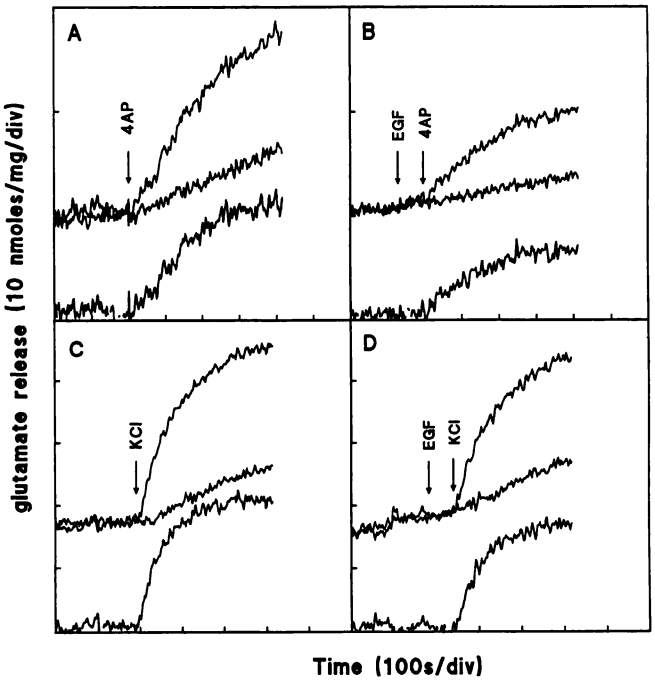

Fig. 1. EGF inhibits the 4-AP- or KCl-induced, Ca^{2+}-dependent release of glutamate. *Top traces*, glutamate release in the presence of 1.3 mM $CaCl_2$; *middle traces*, release in the presence of 0.3 mM EGTA; *bottom traces*, difference between the two (*top* minus *middle*), which gives the Ca^{2+}-dependent component of release. Depolarization was evoked by 3 mM 4-AP (A and B) or 30 mM KCl (C and D). B and D, 100 nM EGF was added 1 min before the depolarizing agent (*arrows*). Synaptosomes (0.33 mg/ml) were incubated in HBM, and glutamate release was measured as described in Experimental Procedures. Traces from representative experiments are shown.

TABLE 1

Quantification of the effects of EGF on Ca^{2+}-dependent glutamate release and rise in $[Ca^{2+}]_c$

EGF (100 nM) was added 1 min before evoking release with 3 mM 4-AP. Glutamate release from synaptosomes and $[Ca^{2+}]_c$ were measured as described in Experimental Procedures. Results are mean ± standard deviation from five independent experiments.

	Time after 4-AP addition			
	30	60	100	300
	sec			
Control release (nmol/mg)	1.94 ± 0.70	3.80 ± 0.92	5.72 ± 0.66	9.64 ± 1.34
Release + 100 nM EGF (nmol/mg)	1.90 ± 0.34	3.0 ± 0.60	4.00 ± 0.82[b]	6.58 ± 1.38[b]
$[Ca^{2+}]_c$ control (nM)	206 ± 2.65	206 ± 2.25	204 ± 1.76	208 ± 3.55
$[Ca^{2+}]_c$ + 100 nM EGF (nM)	192 ± 2.16[a]	189 ± 2.46[a]	191 ± 3.72	192 ± 0.69[a]

[a] $p < 0.04$, Student's t test.
[b] $p < 0.01$, Student's t test.

duced to 79% of the control release; 5 min after the 4-AP addition, release was reduced to 68% of the control release. A similar degree of inhibition of the Ca^{2+}-dependent component of glutamate release by EGF was observed when release was stimulated by depolarization with 30 mM KCl (Fig. 1, C and D). Insulin and IGF-1 were ineffective (data not shown).

To investigate the mechanism responsible for the EGF-mediated inhibition of release, synaptosomal $[Ca^{2+}]_c$ levels were determined with the Fura-2 assay. Fig. 2 shows that EGF *per se* did not induce changes in the resting values of $[Ca^{2+}]_c$. However, the increases in $[Ca^{2+}]_c$ induced by depolarization were partially inhibited by 100 nM EGF. Although the rise in $[Ca^{2+}]_c$ induced by KCl was higher than that induced by 4-AP, a similar fractional inhibition was observed with the two stimulation protocols (Table 1 and data not shown).

In principle, the inhibition in the elevation of $[Ca^{2+}]_c$ might be ascribed either to a direct modulation by EGF of Ca^{2+} entry through voltage-dependent Ca^{2+} channels or to secondary effects resulting from, for example, modulation of K^+ channels with the consequent alteration in plasma membrane potential. To discern between these two possibilities, the plasma membrane potential was measured with two different fluorescent dyes, $DiSC_2(5)$ and bisoxonol. With either method, no significant effect of EGF on plasma membrane potential was observed either under resting conditions or on 4-AP or KCl stimulation (data not shown), thus indicating that the effect of EGF on $[Ca^{2+}]_c$ is most likely due to a direct modulation of the activity of voltage-dependent Ca^{2+} channels .

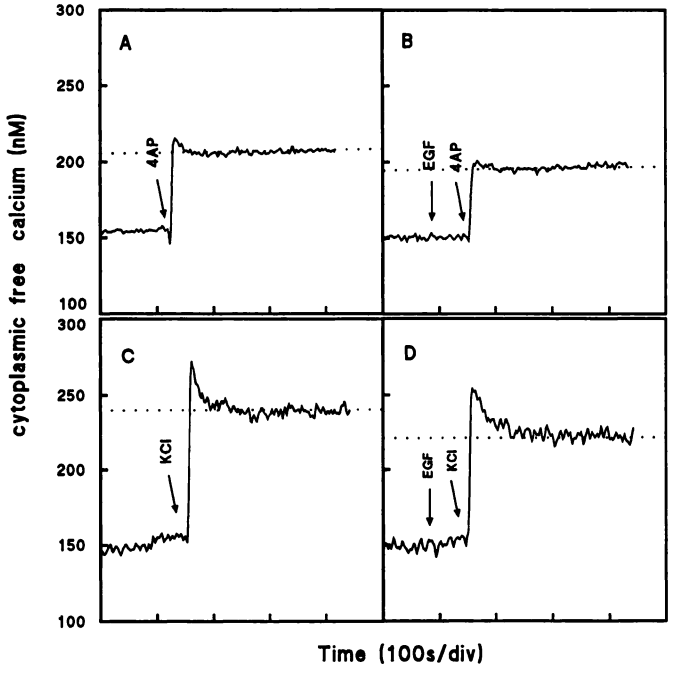

Fig. 2. The depolarization-evoked increase in $[Ca^{2+}]_c$ is reduced by EGF. Depolarization in the presence of external 1.3 mM $CaCl_2$ was evoked by 3 mM 4-AP (A and B) or 30 mM KCl (C and D). B and D, 100 nM EGF was added 1 min before the depolarizing agent (*arrows*). Cytosolic free Ca^{2+} levels in synaptosomes were determined with the Fura-2 assay as described in Experimental Procedures. Traces from representative experiments are shown.

We also determined the pattern of tyrosine phosphorylated proteins from synaptosomes. In polarized synaptosomes, major bands labeled by anti-phosphotyrosine antibodies were detected at approximate molecular masses of 170, 120, 95, 57, 47, and 42 kDa (Fig. 3A). Depolarization with 3 mM 4-AP in the presence of 1.3 mM $CaCl_2$ resulted in the appearance of two additional major bands at approximate molecular masses of 107 and 40 kDa (Fig. 3B). The depolarization-dependent phosphorylation of these bands required the presence of extracellular calcium (data not shown). Occasionally, we also observed the phosphorylation of a band at ~145 kDa and a slight-to-moderate increase in the intensity of unresolved bands in the molecular mass range of 90–95 kDa. The phosphotyrosine patterns induced by KCl-depolarization were indistinguishable from those induced by 4-AP (data not shown).

Treatment with 100 nM EGF before depolarization with 3 mM 4-AP apparently did not alter the phosphotyrosine pattern of total synaptosomal proteins (Fig. 3C). However, the level of tyrosine phosphorylation of the EGF receptor, which could be detected in our rat forebrain synaptosomes (Fig. 4A), was markedly increased after a 10-min incubation with 100 nM EGF (Fig. 4B) and was decreased below basal levels after a 10-min preincubation with 10 μM tyrphostin 23, a receptor tyrosine kinase inhibitor (19) (Fig. 4C).

Discussion

We describe EGF-mediated inhibition of Ca^{2+}-dependent glutamate release from rat forebrain synaptosomes. The degree of inhibition is comparable when either 4-AP or KCl is used as a depolarizing agent. It has been found that the parallel use of 4-AP and KCl in release studies helps to distinguish between the involvement of voltage-dependent Ca^{2+} channel modulation versus K^+ channel modulation in the regulation of neurotransmitter glutamate release (20).

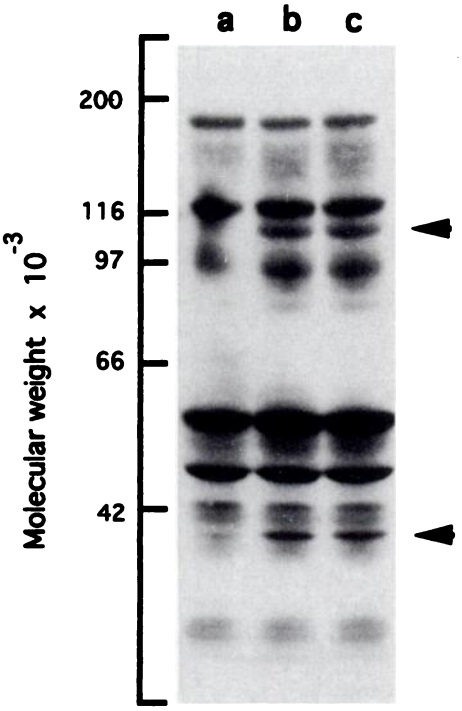

Fig. 3. Effects of depolarization and of EGF treatment on the protein tyrosine phosphorylation pattern in synaptosomes. Synaptosomes (1 mg/ml) were incubated for 2 min in HBM containing 1.3 mM $CaCl_2$ at 37° in the absence (A) or presence (B) of 3 mM 4-AP. C, 100 nM EGF was added 1 min before the addition of 3 mM 4-AP. Incubations were stopped by adding stop solution and boiling for 5 min. Samples were separated by SDS-polyacrylamide gel electrophoresis (7.5%), and the proteins were transferred to a nitrocellulose membrane before probing with anti-phosphotyrosine antibody followed by ^{125}I-labeled sheep anti-mouse antibody, as described in Experimental Procedures.

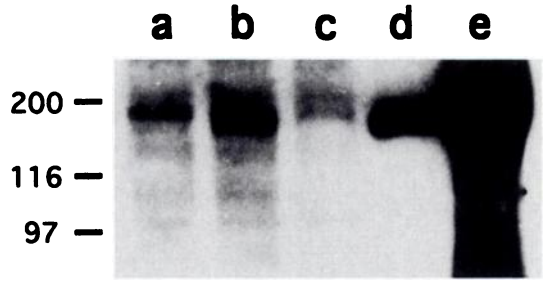

Fig. 4. The EGF receptor is present in rat forebrain synaptosomes and is tyrosine phosphorylated. Synaptosomes (1 mg/ml) were incubated for 10 min in HBM containing 1.3 mM $CaCl_2$ at 37° under control conditions (A), in the presence of 100 nM EGF (B), or in the presence of 10 μM tyrphostin 23 (C). D and E, NIH-3T3 cells (1 mg/ml) expressing the human EGF receptor (~$4 \cdot 10^5$ receptors/cell) were used as a positive control. Before immunoprecipitation, cells were incubated in Dulbecco's minimum essential medium in the presence (D) or absence (E) of 50 nM EGF. The immunoprecipation procedure was carried out as described in Experimental Procedures. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (7.5%), transferred to a nitrocellulose membrane, and probed with anti-phosphotyrosine antibody, as described in Experimental Procedures.

Although their manner of depolarizing the synaptosomal plasma membrane is different, both agents invoke the same voltage-sensitive, non-inactivating Ca^{2+} channel, which is coupled to the exocytotic release of glutamate (21). 4-AP is a K^+ channel inhibitor that causes a Ca^{2+}-dependent, tetrodotoxin-sensitive release of glutamate from synaptosomes by inducing spontaneous action potentials (22). The addition of KCl also causes a Ca^{2+}-dependent release of glutamate due to the ability of the polarized presynaptic membrane to conduct K^+ ions. However, in our experimental set-up, because of the inability to rapidly change the solution, KCl causes a "clamped" depolarization. This clamped depolarization will inactivate ion channels that require a repolarization of the plasma membrane to reset, making the channels unavailable for modulation.

The inhibition of glutamate release by EGF is accompanied by a parallel decrease in the depolarization-induced rise in $[Ca^{2+}]_c$, which could reflect either a direct inhibition of the exocytosis-coupled Ca^{2+} channel or an inhibition of the activation of this Ca^{2+} channel, secondary to hyperpolarization of the synaptosomal plasma membrane. The latter hypothesis appears unlikely for the following reasons: (i) the inhibitory effect of EGF is also observed when KCl is used as a depolarizing agent, a condition in which transient channels that require repolarization of the plasma membrane to reset are inactivated. Many of the K^+ channels of the nerve terminal are of this transient nature; (ii) the plasma membrane potential is unaffected by the addition of EGF before depolarizing with either KCl or 4-AP.

In other (non-neuronal) systems, EGF has been reported to cause alterations in the resting levels of plasma membrane potential, i.e., hyperpolarization due to the activation of Ca^{2+}-dependent K^+ channels (23). This effect is most likely due to the increase in $[Ca^{2+}]_c$ that results from the generation of inositol-1,4,5-trisphosphate and mobilization of Ca^{2+} from the internal stores, a well known event in the signal transduction pathway elicited by the interaction of EGF with its tyrosine kinase receptor (24). Surprisingly, in our system we were unable to detect changes in either the resting $[Ca^{2+}]_c$ or the plasma membrane potential as a consequence of exposure to EGF. It is possible that the inability of EGF to evoke increases in the resting $[Ca^{2+}]_c$ is due to the lack of efficient Ca^{2+} stores in the presynaptic compartment. The role, if any, of presynaptic Ca^{2+} stores in synaptic physiology is still debated. There is no precedence for the modulation of voltage-dependent Ca^{2+} channels by tyrosine phosphorylation. However, recent reports implicate tyrosine phosphorylation in the direct modulation of the activity of several ion channels, such as the delayed rectifier K^+ channel (25), a charybdotoxin-sensitive Ca^{2+}-activated K^+ channel in fibroblasts (26), and the protein kinase A-regulated cation channel of Aplysia bag cell neurons (27).

The EGF receptor has been detected through immunoprecipitation in rat cerebrocortical synaptosomes (28). We now show that it is present in our preparation and that its level of tyrosine phosphorylation increases on exposure to the ligand, indicating that the receptor is functionally active. Thus, the absence of obvious effects of EGF on the synaptosomal tyrosine phosphorylation pattern is compatible with the hypothesis that a minor protein is being phosphorylated, e.g., an ion channel. We also observe a depolarization- and Ca^{2+}-dependent (but EGF-independent) increase in the tyrosine phosphorylation of bands at apparent molecular masses of 107 and 40 kDa and minor increases in bands at 145 and 90–95 kDa. There are various reports in the literature regarding basal and depolarization-induced tyrosine phosphoproteins in various preparations from developing and adult mammalian brain. Tyrosine phosphoproteins have been de-

tected in rat hippocampal slices (29, 30), synaptosomal membranes (1, 3), synaptosomes (6), synaptic vesicles (5), and cultured neurons (30, 31). In neuronal preparations, the increase in the phosphotyrosine content of a 40-kDa protein on various treatments has been reported by several groups. These treatments include electroconvulsive shock, stimulation of glutamate receptors, phorbol esters, neurotransmitters, and growth factors. In several instances, the identity of this band with mitogen-activated protein kinase has been suggested (e.g., see Ref. 32).

EGF has been reported to exert a modulatory effect on phenomena of synaptic plasticity, such as short- and long-term potentiation (8, 9). Consistently, tyrosine kinase inhibitors depress the ability to develop long-term potentiation (33). However, these effects appear to be exerted at postsynaptic sites of action. An effect of EGF on the release process had never been reported. EGF has been shown to improve recovery in cortical neurons that have been subjected to anoxic stress, and this protective role of EGF is more dramatic when anoxia is applied to mature neurons (34). Thus, the EGF-mediated inhibition of Ca^{2+}-dependent glutamate release could be of physiological importance in hypoxic/ischemic conditions, where glutamate release from nerve terminals has been implicated as a major factor underlying neuronal damage (35).

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