Epidermal Growth Factor Receptor in Synaptic Fractions of the Rat Central Nervous System*

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The EGF receptor (EGF-R) is a single chain transmembrane glycoprotein in which the transduction mechanism involves the intrinsic protein tyrosine kinase activity residing in its intracellular domain that phosphorylates the receptor itself and several intracellular substrate proteins (9). A high content of tyrosine kinases and phosphotyrosine proteins has recently been reported in adult brain (10–12) and in synaptic vesicles (15). Thus, protein tyrosine phosphorylation, usually associated with regulation of cell growth and differentiation (9), may also be relevant to synaptic function as recently suggested (16, 17).

In this sense, it is interesting that EGF immunoreactive material is enriched in synaptosomal fractions (4) and there is also convincing evidence that nervous tissue expresses transforming growth factor α (TGF-α) (18, 19), the other known EGF-R ligand (9). The possibility exists that EGF and TGF-α have a role in synaptic function provided that the EGF-R could be identified in this region.

Whether the EGF-R is expressed or not expressed in adult neurons still remains unclear since most of the studies failed to demonstrate significant ligand binding or EGF-R kinase activity, leading to the belief that only glial cells would express the receptor in the brain (20). Two currently cited studies (21, 22) that describe EGF-R-like immunohistochemical staining in the brain are controversial regarding the appearance of the receptor during development and the staining of glial cells. Unfortunately, none of these studies has characterized the reactive immune product. Studies made in crude membrane fractions or brain homogenates from adult rats (23, 24) do not allow clear conclusions either, since glial rather than neuronal cells would be the most important contributions to the results.

On the other hand, investigations carried out in different in vitro experimental models have detected EGF-R preponderantly in glial cells (25, 26) in spite of reports showing EGF effects in both glial (27) and neuronal (28–30) cells in culture. Primary cultures of cells dissociated from the central nervous system are restricted to fetal or neonatal tissue in which neurons have not reached their terminal differentiation. Moreover, since these cultures usually take several days before reaching a workable state, this would entail substantial divergence from in vivo conditions. Therefore, EGF-R expression...
on immature neurons still seems uncertain and requires further exploration.

In this paper, we give definitive evidence for the presence of the EGF-R in newborn and adult rat neuronal tissue by using immunohistochemistry in brain tissue sections and several identificatory assays for this receptor in fresh preparations of synaptosomes and by showing its transductional activity by EGF-induced tyrosine autophosphorylation. Thus, subcellular fractions derived from the synaptic region express a functional EGF-R and would allow the examination of possible EGF effects in a model system more closely representing in vivo neuronal physiology.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials were purchased from Sigma unless otherwise specified in the text. All reagents were analytical grade.

**Purification of EGF Receptor and Antibody Preparation**—Human recombinant EGF (kindly provided by Drs. Paolo Valenzuela and Carlos George-Nascimento, Chiron Co., Emeryville, CA) was coupled (1 mg/ml packed bed volume) to Affi-Prep-10 (Bio-Rad) according to dealer specifications and used to isolate the liver EGF-R by affinity chromatography as described (24), with the following modications: the washing volume of the EGF-Affi-Prep-10 column was doubled and the wheat germ lectin column step was omitted. The identity of the isolated 170-kDa band with the EGF-R was assessed by immunoblot using the anti-EGF-R antibodies RK2 and anti-C produced in a rabbit immunized against synthetic peptides corresponding to different segments of the intracytoplasmic domain of the EGF-R (31, 32). Both antibodies were kindly given to us by Drs. J. Schlessinger and B. Margolis (New York University Medical Center, New York). Employing immunization schedules and procedures described (33, 34), the 170-kDa band was excised from a preparative gel and used as immunogen in white female New Zealand rabbits. Antisera were characterized by microtiter and immunoblot (35). Our polyclonal antibody was further probed by immunoprecipitation of cell extracts (36) prepared from 3P-labeled isolated rat hepatocytes (37) incubated in the presence and absence of EGF. The polyclonal antibody was purified by adsorption to the isolated liver EGF-R immobilized on nitrocellulose as described (38).

**Indirect Immunocytochemistry**—The encephalon was fixed by vascular perfusion of Bouin’s fluid for 30 min at room temperature and anesthetized with intraperitoneal pentobarbital and sacrificed by cervical perfusion of Bouin’s fluid for 30 min at room temperature. In this paper, we give definitive evidence for the presence of the EGF-R in newborn and adult rat neuronal tissue by using immunohistochemistry in brain tissue sections and several identificatory assays for this receptor in fresh preparations of synaptosomes and by showing its transductional activity by EGF-induced tyrosine autophosphorylation. Thus, subcellular fractions derived from the synaptic region express a functional EGF-R and would allow the examination of possible EGF effects in a model system more closely representing in vivo neuronal physiology.

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**Indirect Immunocytochemistry**—The encephalon was fixed by vascular perfusion of Bouin’s fluid for 30 min at room temperature and then immersed in the fixative for 2 h. The tissue was embedded in Paraplast. Incubations were made for 40 min with rabbit polyclonal anti-EGF-R at 1/200 dilution or immune serum adsorbed against the alkaline phosphatase substrate and 0.03% hydrogen peroxide was added. Protein was determined by Bio-Rad protein assay using bovine serum albumin as standard.

**Phosphorylation Assays**—[γ-32P]ATP was synthesized using 9 Ci/μmol [32P]orthophosphoric acid (Du Pont-New England Nuclear) as described (46). Estimated final specific activity was close to 3000 Ci/mmol. Fresh fractions of heavy synaptosomes (1 mg/0.1 ml) were opened by rapidly freezing at −80 °C and thawing at 37 °C in buffer A supplemented with 1.5 mM MnCl2, 1 mM MgCl2, 1 mM sodium orthovanadate, 0.14 trypsin inhibitory units/ml aprotinin, 2 μg/ml pepstatin, leupeptin, and antipain. These synaptosomes were preincubated for 10 min at 0 °C in the absence or presence of 1 μg of EGF, and the phosphorylation reaction was started by adding 20 μCi/1−2 μl [γ-32P]ATP in unlabeled ATP at a final concentration of 15 μM for 10 min at 0 °C. The reaction was stopped by adding either 1 ml of cold buffer B (50 mM NaCl, 150 mM NaF, 1 mM CaCl2, 2 mM EGTA; 0.5% Triton X-100; 5% glycerol; 10 mM sodium pyrophosphate; 1 mM MnCl2; 0.1 M NaF; 10 μM ammonium molybdate; 1 mM sodium orthovanadate; 20 mM phenylmethylsulfonyl fluoride; 0.14 trypsin inhibitory units/ml aprotinin; 2 μg/ml pepstatin, leupeptin, and antipain) or 100 μl of electrophoresis sample buffer, the supernatant was collected and analyzed by showing its transductional activity by EGF-induced tyrosine autophosphorylation. The phosphorylation reaction was made following the above mentioned procedure and also without using [32P]-labeled ATP, in which case intact synaptosomes were incubated 10 min at 37 °C in a 0.4-ml final volume of buffer A either in the absence or presence of 1 μg of EGF.

The assessment of EGF-induced changes in phosphorylation of synaptosomal proteins was made by densitometric analysis of bands appearing in autoradiograms.

**Immunoprecipitations**—Tyrosine-phosphorylated EGF-R was immunoprecipitated using the described anti-EGF-R antibodies, anti-C and RK2, raised against synthetic EGF-R peptides, or a polyclonal anti-phosphotyrosine antibody (31, 32), generously given to us by Dr. J. Schlessinger and B. Margolis. We also used the monoclonal antibody anti-phosphotyrosine antibody 268.66 (ATCC catalog no. HB8960). Immunoprecipitations were made following established procedures (31, 32, 47) except that we extended to 3 h the incubation time of the supernatant with the antibody-resin complex; for the monoclonal antibody was made with Sepharose protein G (Pharmacia, Uppsala, Sweden). Precipitates were analyzed by SDS-PAGE electrophoresis, immunoblot, and autoradiography exposing Kodak X-Omat AR films with intensifier screens at −80 °C.

**Electron Microscopy**—The different fractions produced by the Percoll gradients were pelleted and fixed in 2% glutaraldehyde, 0.2 M...
sodium cacodylate, pH 7.4, postfixed in osmium tetroxide, and processed for electron microscopy following conventional procedures.

**RESULTS**

*Polyclonal Antibodies against Rat EGF Receptor—*The EGF-R preparation obtained from rat liver by affinity chromatography gives two prominent bands of 170 (EGF-R) and 45 kDa (unidentified) when analyzed in SDS-PAGE (Fig. 1, lane 1). A band of 150 kDa described as a degradation product of the EGF-R (24, 32, 33) was also observed with variable intensity in different preparations. None of these peptides were retained in control chromatography on albumin-Affi-Prep-10 (not shown). In addition to the chromatographic behavior and apparent molecular mass, the identity of the 170-kDa protein with the EGF-R was further demonstrated by immunoblot (not shown) using the well characterized RK2 and anti-C antibodies against cytosolic segments of the receptor (31). The 170-kDa peptide was sliced out from SDS-polyacrylamide gels and used as immunogen (35). The antibody obtained immunoprecipitates a 170-kDa protein in which the phosphorylation in $^{32}$P-labeled hepatocytes is rapidly increased by the effect of EGF (Fig. 1, lanes 2 and 3), a hallmark of the EGF-R.

**Identification of EGF-R in Adult Rat Encephalon—*Several methods were used to search for EGF-R in rat brain. Fig. 2 illustrates results obtained by affinity chromatography experiments similar to those performed on liver extracts. The 170-kDa protein (asterisk) was not as prominent in brain- as in liver-chromatographed extracts and was accompanied by several other bands, though it was still significantly purified (Fig. 2A, lanes 1 and 2). EGF-R contents in brain homogenate would be at least 15-fold lower than in liver as grossly estimated by densitometric analysis assuming similar recovery from both tissues. A column of BSA-Affi-Prep used as control does not enrich any particular band (Fig. 2A, lane 3). Only the 170-kDa protein of the brain eluate was stained with concanavalin A-peroxidase, indicating its glycoprotein nature (Fig. 2B, lane 5). This protein also reacted in immunoblot with out affinity-purified polyclonal anti-EGF-R antibody (Fig. 2C, lane 6).

Immunocytochemistry showed positive immunoperoxidase-anti-peroxidase reaction in neurons of all brain areas examined (Fig. 3, c-e). A similar result was obtained in neonatal rats (Fig. 3b); we did not observe staining of glial cells. Both facts are in contrast with previous similar studies in the rat brain (22) but agree with the observations made by Werner et al. (21) using monoclonal antibody in human brain. Control of specificity is shown by the lack of positive reaction when liver-isolated EGF-R was co-incubated with the antibody (Fig. 3, compare a and b) Preimmune serum gave no reaction either (not shown).

**Binding of $^{125}$I-EGF to Isopycnically Isolated Synaptosomes—*Previous binding studies in the brain have been restricted to crude membrane fractions (23) that mostly represent material derived from glial cells. We used here the Percoll gradient method (40, 48) to separate functional synaptosomes preserving structural integrity from myelin and membrane fragments. Fig. 4 shows representative ultrastuctural images of the myelin (a), light synaptosomes (b), heavy synaptosomes (c), and mitochondrial (d) fractions routinely obtained in the Percoll gradient of adult brain. In addition, a synaptosomal fraction obtained from newborn rat brain equivalent to the adult heavy synaptosomes and similar to that described (49) is shown in Fig. 4e.

The fraction of heavy synaptosomes gave very reproducible binding parameters even when background levels of 40% were usually obtained. In myelin and mitochondrial fractions, only very low levels of specific $^{125}$I-EGF binding could be detected over higher background. All binding assays were thus made in freshly prepared heavy synaptosomal fractions. $^{125}$I-EGF binding as a function of increasing concentrations of synaptosomal protein was linear within the range of 12.5-400 pg of protein (Fig. 5A). Equilibrium was reached in about 45 min at 22 °C and 60 min at 4 °C (Fig. 5B). Since the binding level at 4 °C was very low, making it difficult to reliably assess kinetic parameters, all following experiments were performed at 22 °C.

Scatchard analysis of saturation binding data (Fig. 6) fit a two-binding site model. High affinity sites ($K_a = 1.42 \times 10^{-10}$ ± 0.58 M) correspond to 17% of total binding sites, whereas the great majority of total binding was contributed by low affinity sites ($K_a = 2.55 \times 10^{-11}$ ± 0.35 M) as currently described in other cellular systems (50, 51). Similar $B_{max}$ of 80.1 ± 3.48 and 76.6 fmol/mg protein was observed both in this kind of experiment and in displacement experiments with unlabeled EGF (Fig. 7), respectively. In contrast, the $^{125}$I-EGF binding capacity found in synaptosomal fractions prepared from whole rat brain (Fig. 3b) did not observe staining of glial cells. Both facts are in contrast with previous similar studies in the rat brain (22) but agree with the observations made by Werner et al. (21) using monoclonal antibody in human brain. Control of specificity is shown by the lack of positive reaction when liver-isolated EGF-R was co-incubated with the antibody (Fig. 3, compare a and b) Preimmune serum gave no reaction either (not shown).

**FIG. 2. Affinity chromatography of EGF-R in brain homogenates.** Brain homogenates were loaded either on EGF-Affi-Prep or on BSA-Affi-Prep columns and eluted as described under "Experimental Procedures." Total homogenate (4 ml) and trichloroacetic acid precipitate (500 μl) from each eluate were analyzed by SDS-PAGE by: A, Coomassie Blue staining of total homogenate (lane 1), eluate from EGF-Affi-Prep (lane 2), and eluate from BSA-Affi-Prep (lane 3); B, concanavalin A-peroxidase blot staining of total homogenate (lane 4) and 75 μl of eluate from EGF-Affi-Prep column (lane 5); C, immunoblot staining of 75 μl of brain eluate from EGF-Affi-Prep column (lane 6) and isolated rat liver EGF-R (lane 7).
Fig. 3. Immunohistochemistry of EGF-R in neonatal and adult rat brain. Positive staining for EGF-R was seen in neurons of all examined areas of neonatal and adult rat brains after incubating tissue sections with the polyclonal antibody directed against rat liver EGF-R followed by peroxidase-anti-peroxidase reaction. The figure presents tissue sections of only some areas from rat neonatal brain cortex (b) and from adult rat brain cortex (c), hippocampus (d), and cerebellar cortex (e). No staining of glial cells was detected. In a, a co-incubation with purified rat liver EGF-R was included, showing complete abolishment of the reaction. The bar represents 100 μm.

Fig. 4. Electron micrographs of rat brain subcellular Percoll step gradient fractions. A P2 fraction from brain homogenates was subjected to isopycnic sedimentation over discontinuous Percoll gradients centrifuged at 15,000 × g for 20 min as described (40). Material from each isolated fraction was sedimented, and the pellets were processed for electron microscopy. Sections of myelin (a), light synaptosomes (b), heavy synaptosomes (c), and mitochondrial (d) fractions derived from adult rats and synaptosomes from neonatal rat brain (e) were analyzed at 15,000 × g. The bar represents 1 μm.

newborn rat brain was consistently higher by at least a 2-fold factor (Fig. 8).

A clear demonstration that EGF-R would be responsible for the observed 125I-EGF binding was obtained by affinity cross-linking experiments (Fig. 9). A single band of electrophoretic migration corresponding to the complex of EGF (6 kDa) and the 170-kDa EGF-R protein was seen (Fig. 9, lane 1). Displacement of 56% was achieved by 10 ng/ml cold EGF (Fig. 9, lane 2), and the cross-linked radioative band was undetected in the presence of 400 ng/ml cold EGF (Fig. 9, lane 3). This is consistent with the results already presented in the binding displacement experiments (Fig. 7).

EGF-induced Tyrosine Phosphorylation of Synaptosomal Proteins Including the EGF-R—To test whether the EGF-R intrinsic tyrosine kinase activity was functional in the synaptic region, we made protein phosphorylation assays using heavy synaptosomal fractions prepared from total adult rat brain. Freeze-thawed permeabilized synaptosomes were preincubated with or without EGF and then loaded with [γ-32P] ATP at 0 °C for 10 min. Of several proteins seen phosphorylated in variable degrees, only those in the range of 170 and 126–150 kDa appeared to increase their 32P contents after EGF treatment (Fig. 10A, lanes 1 and 2). Immunoprecipitation using a specific polyclonal anti-phosphotyrosine antibody showed that these two groups of proteins were phosphorylated in tyrosine (Fig. 10A, lanes 3 and 4). The band at 170 kDa probably corresponds to the EGF-R as shown below, and proteins in the 126–150-kDa region could include phospholipase C-γ, a reported substrate of the EGF-R (47, 52, 53). Besides, another group of proteins with apparent molecular masses of 124, 113, 98, and 70 kDa, in which tyrosine phosphorylation was fainter but increased by EGF treatment, was also detected in these immunoprecipitates. That the EGF-R was indeed phosphorylated by the effect of EGF was shown by immunoprecipitating it with anti-C antibody (Fig. 10B, lanes 5 and 6). Furthermore, it was phosphorylated in tyrosine
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Fig. 6. Binding of different $^{125}\text{I-EGF}$ concentrations to rat brain synaptosomes under equilibrium conditions. Heavy Percoll synaptosomes from rat brain cortex were incubated with increasing concentrations (0.2-20 ng/ml) of $^{125}\text{I-EGF}$ at 22 °C for 60 min. Each point represents specific binding, average ± S.E. of triplicates. At the highest concentrations, nonspecific binding was about 40% of total binding. The inset shows a Scatchard analysis performed by the LIGAND program fit to a two-binding site model.

Fig. 7. Competition binding assays with unlabeled EGF. Heavy synaptosomes were incubated at 22 °C for 1 h in 0.75 nM $^{125}\text{I-EGF}$ either without (100% binding) or with increasing concentrations of unlabeled EGF (0.01-100 ng/assay). 56% displacement was achieved with 12.5 ng/ml EGF. fm, femtomoles.

As is demonstrated by incubating intact synaptosomes with EGF for 10 min at 37 °C followed by immunoprecipitating them with the monoclonal anti-phosphotyrosine antibody 2G8.D6 and immunoblotting with anti-C antibody after SDS-PAGE (Fig. 10B, lanes 7 and 8). It is clearly seen that the amount of EGF-R recovered in the immunoprecipitate of EGF-treated synaptosomes greatly exceeds that found in the absence of the ligand. Thus, the overall evidence showed that the EGF-R tyrosine kinase is activated by EGF in the synaptosomal fractions as expected for a functional receptor.

DISCUSSION

In this paper, we give definitive evidence for the expression of the EGF-R protein in neural tissue, showing for the first time its presence and functional activity in synaptic regions of adult rat brain. This was also demonstrated in neonatal synaptic fractions. The evidence presented encompasses affinity chromatography enrichment, immunohistochemistry, binding, and tyrosine phosphorylation assays complemented with immunobiochemical characterization.

Previous studies have focused mainly on embryonic and neonatal nerve tissue and provided inconclusive or ambiguous evidence regarding the expression of EGF-R in incompletely differentiated neurons. A prevalent notion has been that only glial cells would express significant levels of this receptor in the brain (20) since neither the 170-kDa EGF-R protein nor specific binding sites have been rigorously demonstrated in nerve cells. In fact, recent comparative studies made in dissociated cells from fetal or neonatal brain, after several days in culture, have shown 5-fold higher binding of $^{125}\text{I-EGF}$ in glial rather than in neuronal cells (26). Results of cross-linking and phosphorylation experiments were positive only
EGF-induced tyrosine phosphorylation in synaptosomal proteins including the EGF-R. Synaptosomes were either permeabilized by freeze-thawing and then preincubated with EGF (1 μg) for 10 min at 0 °C followed by 20 μCi of [γ-32P]ATP (lanes 1–6) or incubated intact with EGF during 10 min at 37 °C (lanes 7 and 8). Autoradiograms of total phosphorylated proteins (A, lanes 1 and 2) or proteins immunoprecipitated by polyclonal anti-phosphotyrosine antibody (anti-PTyr) (A, lanes 3 and 4) or by anti-C antibody against EGF-R (B, lanes 5 and 6) and resolved in 7.5% SDS-PAGE are shown. Proteins in which tyrosine phosphorylation increases by EGF treatment are indicated by their apparent molecular masses. It can also be seen that EGF pretreatment of synaptosomes during 10 min at 37 °C increases in 32P content of the immunoprecipitated EGF-R (compare lanes 5 and 6). When intact synaptosomes were incubated in the absence (B, lane 7) or presence (B, lane 8) of EGF, a clear demonstration of EGF-R tyrosine phosphorylation was achieved by first immunoprecipitating with anti-phosphotyrosine monoclonal antibody (2G8.D6) and then immunoblotting with anti-EGF-R (anti-C antibody). The results of a similar experiment made in A431 cells are shown for comparison (B, lanes 9 and 10).

in the glial-enriched culture (26). Binding parameters in cell membranes reported by these authors do not agree with ours or with those currently described in other kinds of cultured cells (50, 51). Other authors also suggest that EGF-R would be preferentially present in astrocytes, as found by autoradiography made on 3-day cultures of brain cells from rats aged 4–5 days that less than 1% of neuronal cells express detectable levels of the receptor (25). In a more recent study, EGF-induced tyrosine phosphorylation of proteins has been shown in highly enriched cultures of embryonic neurons and astrocytes. However, the authors mention that tyrosine phosphorylation was inconsistently detected by immunofluorescence in neuronal cells but clearly showed EGF-increased immunoreactivity in astrocytes (30). On the other hand, EGF increases the survival in culture and processes outgrowth of cerebellar (29) and subneocortical telencephalic neurons of neonatal rat brain (28), implying that these cells express functional EGF-R at least under culture conditions. Our present results showed immunohistochemical reaction in newborn rat brain neurons with a polyclonal antibody prepared against the rat liver EGF-R and specific EGF binding sites in the synaptic fractions from the animals of the same age. Furthermore, the binding capacity of these synaptosomes exceeds by 2-fold that found in similar preparations from adult animals.

Primary cultures of cells dissociated from fetal or neonatal brain usually take several days before becoming enriched in neuronal or glial cells. Culture conditions can dramatically change the expression of various proteins including the EGF-R (54, 55). EGF-R expression could also change in culture according to the proliferative activity of the cells (56) and thus could be affected differently in the more actively proliferating glial cells. All this suggests that although many characteristics of glial and neuronal cells would be maintained during their primary culture, the level of EGF-R expression may be so altered as to not represent the in vivo situation.

The available information supporting EGF-R expression in completely differentiated, nonproliferating neurons of adult organisms in situ is even scantier. EGF has been shown to affect long term potentiation in hippocampal slices (57). Results of immunohistochemistry performed in human (21, 58) and rat (22) brain do not agree with respect to glial cell staining and to the time in which neurons become immunoreactive. Werner et al. (21), using a monoclonal antibody, found EGF-R-like immunoreactivity widely distributed in neurons of all regions of the human brain but not in astrocytes or oligodendrocytes. The immunoreactivity was detectable at least since week 28 of gestation through adulthood. In contrast, Gómez-Pinilla et al. (22) found positive immunoreactivity in rat brain only from the 11th postnatal day, being at that age restricted to the cerebellar Purkinje’s cells. They also reported positive staining of glial cells. Since these authors do not provide any characterization either of the antibody used or of their EGF-R purification from A431 cells, the reason for this discrepancy remains unclear. Also, the identity of the protein being detected in both studies mentioned was not assessed by other means. With our polyclonal antibody prepared against affinity-purified rat liver EGF-R, we observed positive immunoreactivity in neonatal (1 day) and adult rat brain, present only in neurons and not confined to specific areas of the brain. The staining was abolished by co-incubating with the purified liver receptor. Thus, our immunohistochemical observations would appear to be more consistent with the results of Werner et al. (21).

The binding and tyrosine phosphorylation assays that we made in freshly prepared synaptosomal fractions gave further proof of EGF-R occurrence in adult neurons, also showing evidence of its functional state in the synaptic region. Binding
of $^{125}$I-EGF in fresh brain material has been previously shown only in crude rat brain membranes (23) and in rat brain homogenates (24). Total membrane fractions showed just one kind of binding site ($K_a = 7 \times 10^{-9}$ M), contrasting with our results, and a $B_{	ext{max}}$ of 5.3 fmoI/mg protein (23) that is 12-fold below our observed $B_{	ext{max}}$. Since glial cells account for more than half of the total weight of the brain and outnumber neurons by as much as 10:1 or even 50:1 (59), a crude membrane preparation is expected to be relatively poor in neuron-derived material, having instead a high content of glial membranes, especially myelin.

To circumvent this problem, we used synaptosomal fractions prepared from the brains of adult as well as newborn rats following the Percoll method of isopycnic centrifugation in iso-osmotic media. This established method gives a relatively homogeneous fraction of synaptosomes that maintain several synaptic functions and preserve ultrastructural integrity (40, 48). This fraction is mostly depleted of identifiable myelin and other membranous material (40, 48), as we corroborated in our own fractions by electron microscopy analysis. This preparation allowed us to implement a reliable and highly reproducible $^{125}$I-EGF binding assay.

We found specific and saturable $^{125}$I-EGF binding sites with two different affinities. High affinity ($K_a = 1.42 \times 10^{-10} \pm 0.58$ M) binding sites accounted for 14 fmoI of $^{125}$I-EGF bound/mg of protein, whereas the highest proportion (80 fmoI/mg of protein) of binding sites were of low affinity ($K_a = 2.55 \times 10^{-9} \pm 0.35$ M). Saturation with $^{125}$I-EGF and cold displacement experiments gave similar results. Furthermore, cross-linking experiments made under conditions defined by cold displacement showed that the EGF-R 170-kDa protein was responsible for this binding. The affinity constants as well as the relative amount of high to low affinity binding sites that we observed in synaptosomes are congruent with those reported in A431 cells (50) and in other cultured cells transfected with the cDNA encoding the human EGF-R (51).

Tyrosine phosphorylation is crucial to the transduction mechanism used by the EGF-R as it possesses a tyrosine kinase domain in its intracellular segment that becomes activated after EGF binding (9). Tyrosine kinase activity seems abundant in adult brain (11-13). During comparisons between several tissues, Cohen et al. (24) detected in brain homogenates a very faintly phosphorylated 170-kDa protein that adsorbs to EGF-Affi-Gel. However, the demonstration of EGF-induced tyrosine phosphorylation has been elusive in neuronal tissue. We already mentioned that evidence for EGF-inducible tyrosine phosphorylation is controversial in cultured immature neurons, but is clear, however, in astrocytes (26, 30). In synaptosomes prepared from adult animals, tyrosine kinase activity has been found to be intense both in membrane and soluble (synaptosoma) fractions; however, attempts to detect EGF-dependent increases in protein phosphorylation have been heretofore unfruitful (13, 14). Using completely different conditions for synaptosomal isolation and phosphorylation assays, we presented here clear evidence that EGF treatment increases tyrosine phosphorylation in several synaptosomal proteins (pp170, pp126-150, pp124, pp113, pp98, and pp70) that were immunoprecipitated with anti-phosphotyrosine antibodies. The EGF-R was among these proteins, as demonstrated by subsequently accomplished immunoblot. Direct immunoprecipitations of the EGF-R from $^{32}$P-labeled synaptosomes also showed higher phosphorylation of the receptor after preincubating with EGF.

Thus, the presence of functional EGF-R in synaptosomes prepared from adult animals, its wide distribution in all groups of neurons, and evidence suggesting that tyrosine phosphorylation may play a role in synaptic functions (15-17) all point to some previously unsuspected function of this receptor influencing brain physiology, certainly not related to proliferation and differentiation. This is also suggested by evidence showing EGF immunoreactive material (2-4) and mRNA for the other known EGF-R ligand, TGF-α (18, 19), in specific areas of the brain. The effects of EGF on long term potentiation in hippocampal slices (57) and food intake behavior in rats (60) may be a reflection of such a function.

Even when the EGF-R appears widely distributed in every region of the brain, a distinct regulation of its function may occur in different zones depending on the regionalized distribution of other kinds of receptors. The EGF-R is a substrate of protein kinase C (9) and its high affinity form can be converted in cultured cells to the low affinity form by stimulating this kinase with phorbol esters (61) or with the small peptide hormone bombesin (62). Platelet-derived growth factor has a similar effect through a different mechanism (63, 64). Platelet-derived growth factor receptor is concentrated in the amygdala and midbrain and is poorly represented in cerebral cortex and medulla oblongata (65), whereas bombesin receptors are found in cerebral cortex, hippocampus, and other brain regions but are absent in cerebellum (66). On the other hand, protein kinase C participates in processes of hippocampal long term potentiation and memory (67, 68) and would also be involved in the mechanism of some psychotropic drugs (69, 70). We are focusing future studies on the exploration of EGF-R functions in the brain based on these suggestive relationships.

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REFERENCES