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Protein Tyrosine Kinase Activity and Its Endogenous Substrates in Rat Brain: A Subcellular and Regional Survey

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Abstract

The rat CNS contains high levels of tyrosine-specific protein kinases that specifically phosphorylate the tyrosine-containing synthetic peptide poly(Glu⁸⁰, Tyr²⁰). The phosphorylation of this peptide is rapid and occurs with normal Michaelis–Menten kinetics. Using this peptide to assay for enzyme activity, we have measured the protein tyrosine kinase activity in homogenates from various regions of rat CNS. A marked regional distribution pattern was observed, with high activity present in cerebellum, hippocampus, olfactory bulb, and pyriform cortex, and low activity in the pons/medulla and spinal cord. The distribution of protein tyrosine kinase activity was examined in various subcellular fractions of rat forebrain. The majority of the activity was associated with the particulate fractions, with enrichment in the crude microsomal (P₃) and crude synaptic vesicle (LP₂) fractions. Moreover, the subcellular distribution of pp60^{csrc}, a well-characterized protein tyrosine kinase, was examined by immunoblot analysis using an affinity-purified antibody specific for pp60^{csrc}. The subcellular distribution of pp60^{csrc} paralleled the overall protein tyrosine kinase activity. In addition, using an antibody specific for phosphotyrosine, endogenous substrates for protein tyrosine kinases were demonstrated on immunoblots of homogenates from the various regions and the subcellular fractions. The immunoblots revealed numerous phosphotyrosine-containing proteins that were present in many of the CNS regions examined and were associated with specific subcellular fractions. The differences in tyrosine-specific protein kinase activity, and in phosphotyrosine-containing proteins, observed in various regional areas and subcellular fractions may reflect specific functional roles for protein tyrosine kinase activity in mammalian brain.

Keywords

Rat CNS; Poly(Glu⁸⁰, Tyr²⁰); Phosphotyrosine; Anti-phosphotyrosine antibody; pp60^{csrc}

Protein phosphorylation has been widely recognized as one of the major regulatory mechanisms in the control of cellular metabolism. Recent studies have provided evidence that protein phosphorylation plays a prominent role in the regulation of neuronal function, including synaptic transmission (Browning et al., 1985; Nairn et al., 1985). Many neurotransmitters and other first messengers regulate the levels of intracellular second messengers such as cyclic AMP, cyclic GMP, calcium, and diacylglycerol, and many of the actions of these second messengers are mediated by the activation of specific classes of serine- and threonine-specific protein kinases (Nestler and Greengard, 1984). Tyrosine-specific protein kinases are a newly discovered class of protein kinases that were originally

shown to be involved in the regulation of cell transformation and cell proliferation (for review, see Hunter and Cooper, 1985).

Tyrosine-specific protein kinases were first identified as the protein products of the oncogenes of transforming retroviruses (Hunter and Cooper, 1985). Growth factor receptor kinases have been identified as a related class of tyrosine kinases. These include the epidermal growth factor receptor, the insulin receptor, the platelet-derived growth factor receptor, and the insulin-like growth factor receptor (Cohen et al., 1980; Kasuga et al., 1983; Heldin et al., 1983; Jacobs et al., 1983). It appears that the tyrosine-specific protein kinase activity intrinsic to these receptors and to the viral oncogene products mediates many of the effects of these proteins on cellular growth (Hunter and Cooper, 1985).

Many of the viral oncogenes have been shown to have normal cellular homologues (for review see Hunter and Cooper, 1985). Recent studies have demonstrated that the gene products of the cellular homologues of the *src* and *yes* viral oncogenes are very abundant in nervous tissue, suggesting that protein tyrosine kinases may be involved in the regulation of neuronal function (Barnekow et al., 1982; Cotton and Brugge, 1983; Sorge et al., 1984; Fults et al., 1985; Sudol and Hanafusa, 1986). During the development of the brain, the level of pp60^{src} begins to rise after differentiation and remains high in the adult when cell division is very low (Cotton and Brugge, 1983; Sorge et al., 1984; Fults et al., 1985). In addition, recent studies have reported the partial purification and characterization of several protein tyrosine kinases from adult brain (Neer and Lok, 1985; Braun et al., 1986). The presence of high levels of protein tyrosine kinase activity in adult brain, a nonproliferating tissue, suggests that protein tyrosine kinases may play a specific role in the regulation of neuronal function. The nature of this role is as yet unclear.

As part of a study of the role of tyrosine-specific protein kinases in neuronal function, we have begun to characterize these kinases in rat brain. In the present investigation, we have studied the tyrosine-specific protein kinase activity in rat CNS using a tyrosine-containing synthetic peptide as exogenous substrate. Synthetic polymers rich in tyrosine and glutamic acid, such as poly(Glu⁸⁰, Tyr²⁰), have been shown to be effective substrates for various tyrosine-specific protein kinases (Braun et al., 1984). Using this exogenous substrate, we have determined the regional and subcellular distributions of protein tyrosine kinase activity within the rat CNS. We have also characterized the regional and subcellular distribution of the endogenous substrates for the protein tyrosine kinases in rat CNS using phosphotyrosine-specific antibodies.

MATERIALS AND METHODS

Male Sprague-Dawley rats (180–250 g) were obtained from Charles River. Ouabain, phosphotyrosine, phosphoserine, phosphothreonine, Nonidet P-40 (NP-40), poly(Glu⁸⁰, Tyr²⁰), benzamidine, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma. Sodium vanadate and trichloroacetic acid (TCA) were obtained from Fisher. Leupeptin and antipain were obtained from Chemicon (El Segundo, CA, U.S.A.). Trasylol was obtained from Mobay Chemical. DEAE-Sephacel and Sepharose 4B were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Nitrocellulose sheets (0.2 μ m pore size) were obtained from Schleicher & Schuell (Keene, NH, U.S.A.). [γ -³²P]ATP (2,900 Ci/mmol) and ¹²⁵I-labeled Protein A (90 μ Ci/ μ g) were obtained from New England Nuclear.

Phosphorylation assays

Phosphorylation reactions were carried out under the following standard conditions: 20 mM Tris-HCl, pH 7.4/20 mM MgCl₂/2 mM MnCl₂/1 mM EGTA/0.5 mM EDTA/1 mM ouabain/1 mM sodium vanadate/0.1 mM dithiothreitol/10–50 μ g protein/100–200 μ M [γ -³²P]ATP

(1,000 cpm/pmol) in a final volume of 100 μ l. The reaction was initiated by the addition of the ATP and carried out for 10 min at 30°C. For samples to be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the reaction was stopped by the addition of 50 μ l of 6% SDS/375 mM Tris-HCl, pH 6.8/30% glycerol (wt/vol)/15% β -mercaptoethanol (BME)/0.05% bromphenol blue. For filter paper assays the reactions were carried out in the presence of 0.1% Triton X-100 in the absence or presence of 500 μ g/ml poly(Glu⁸⁰, Tyr²⁰), and 10 μ l of 10 mM ATP/0.25 mM EDTA was used to quench the reaction (Braun et al., 1984). A 55- μ l aliquot of the reaction mixture was then spotted onto Whatman 3MM filter paper (2.3 cm diameter). The filters were washed five times in 10% TCA (wt/vol)/10 mM sodium pyrophosphate, once in 95% ethanol, and rinsed with diethyl ether. The filters were air dried, placed in Liquiscint, and counted by liquid scintillation spectrometry. Specific tyrosine phosphorylation was taken as the difference in ³²P incorporation in the absence and presence of 500 μ g/ml poly(Glu⁸⁰, Tyr²⁰) in the reaction mixture. A unit of specific activity was defined as 1 pmol of ³²P incorporation/min/mg of protein.

Phosphoamino acid analysis

To identify the amino acids phosphorylated, reaction mixtures were stopped by the addition of excess nonradioactive ATP and then precipitated with 10% TCA. The precipitates were hydrolyzed in 6 M HCl under vacuum for 1.5 h at 105°C. The acid-hydrolyzed peptides were lyophilized, resuspended in a small volume of H₂O (1,000 cpm/2 μ l), and spotted 4 cm from the end of 20 $\times$ 20 cm cellulose thin-layer plates. Phosphotyrosine, phosphoserine, and phosphothreonine standards with a trace of phenol red were also spotted at the origin. Electrophoresis was carried out in formic acid/acetic acid/H₂O, 10:1:89 (by vol), pH 1.9, at 500 V until the phenol red had moved 6 cm (Nairn and Greengard, 1987); then the plate was subjected to electrophoresis at 500 V in the same direction in acetic acid/pyridine/H₂O, 10:1:89 (by vol), pH 3.5, until the phenol red had moved another 6 cm. The cellulose plate was dried, developed in 1% ninhydrin in acetone to detect the internal phosphoamino acid standards, and subjected to autoradiography to identify the ³²P-labeled phosphoamino acids.

Subcellular fractionation

Subcellular fractionation was carried out essentially according to Huttner et al. (1983) and Cotman et al. (1974). Ten male Sprague–Dawley rats (180–200 g) were stunned and decapitated. The brains were quickly removed and the forebrains (neocortex, hippocampus, and striatum) were dissected away from the diencephalon and underlying structures. The forebrains were homogenized in 10 volumes of ice-cold 0.32 M sucrose with 10 strokes in a glass-Teflon homogenizer rotating at 1,200 rpm. All centrifugation was done at 4°C. The homogenate was centrifuged for 10 min at 800 g, and the pellet (P₁, the crude nuclear fraction) was saved, while the supernatant (S₁) was centrifuged at 9,200 g for 15 min yielding P₂ (the crude synaptosomal fraction) and S₂. S₂' and P₂' were obtained by resuspending the P₂ pellet in 120 ml of sucrose and recentrifuging for 15 min at 10,200 g. The supernatants S₂ and S₂' were pooled and centrifuged for 1 h at 165,000 g to yield a microsomal fraction (P₃) and a cytosol fraction (S₃). The P₂ pellet was resuspended in 0.32 M sucrose, lysed by hypoosmotic shock in nine volumes of H₂O, quickly adjusted to 1 mM HEPES/KOH, pH 7.4, and continuously stirred on ice for 30 min. The lysate (L) was then centrifuged for 20 min at 25,000 g to yield the lysate pellet (LP₁) and the lysate supernatant (LS₁) fraction. The LS₁ fraction was centrifuged for 2 h at 165,000 g to yield LS₂ and LP₂ (the crude synaptic vesicle fraction). The LP₁ fraction was resuspended in 0.25 M sucrose, layered onto a discontinuous sucrose gradient containing 10 ml each of 0.8 M/1.0 M/1.2 M sucrose, and centrifuged for 2 h at 65,000 g in a Beckman SW-28 rotor. The gradient yielded a floating myelin fraction, a light membrane mixture at the 0.8 M/1.0 M sucrose interface, a

synaptosomal plasma membrane fraction at the 1.0 *M*/1.2 *M* sucrose interface, and a mitochondrial fraction as a pellet. The various subcellular fractions were assayed for protein tyrosine kinase activity under standard conditions.

Dissection of brain regions

Male Sprague–Dawley rats (180–200 g) were stunned and decapitated. The brains and spinal cords were rapidly placed in ice-cold 0.32 *M* sucrose, pH 7.4. The various brain regions were microdissected according to Walaas et al. (1983a,b), and homogenized in a buffer consisting of 20 *mM* Tris-HCl, pH 7.4/10 *mM* EGTA/5 *mM* EDTA/10 *mM* BME/20 $\mu\text{g/ml}$ leupeptin/20 $\mu\text{g/ml}$ antipain/20 U/ml Trasylol/10% glycerol.

Antibody preparation and immunoblot analysis

Antiserum against phosphotyrosine was prepared as described by Ohtsuka et al. (1984). In brief, phosphotyrosine (20 mg) was coupled to keyhole limpet protein (20 mg) or to bovine serum albumin (20 mg) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and dialyzed overnight against phosphate-buffered saline. Rabbits were injected intradermally as described by Ohtsuka et al. (1984). The resulting antiserum was affinity purified on a Sepharose 4B column coupled to phosphotyrosine (Ohtsuka et al., 1984). The antibody against pp60^{csrc} was prepared against a synthetic peptide corresponding to the autophosphorylation site of pp60^{csrc} and affinity purified as described by Casnellie et al. (1984).

Aliquots of the homogenates from each brain region (50 μg) and from each subcellular fraction (50 μg) were phosphorylated under standard conditions using nonradioactive ATP. The proteins were separated by electrophoresis on 8% SDS-polyacrylamide gels by the method of Laemmli (1970). The proteins were then transferred to nitrocellulose sheets at 200 mA overnight (12–18 h) using the buffer system of Towbin et al. (1979). Immunoblotting with the anti-phosphotyrosine antibody was performed with ¹²⁵I-labeled Protein A as described by Jahn et al. (1984), except that a 1:100 dilution of the affinity-purified anti-phosphotyrosine antibody was used. The blots were subjected to autoradiography on Kodak X-AR film to identify the ¹²⁵I-labeled phosphoproteins. The immunoblots using the affinity purified anti-pp60^{csrc} antibody were performed using alkaline phosphatase-coupled goat anti-rabbit immunoglobulin G as described by Pinter et al. (1983).

Protein determinations

Protein concentrations were measured by the procedure of Peterson (1977) with bovine serum albumin as standard.

RESULTS

Protein tyrosine kinase activity in rat brain homogenates

To measure the endogenous protein tyrosine kinase activity of rat CNS, homogenates were assayed for protein phosphorylation in the presence and absence of the synthetic substrate poly(Glu⁸⁰, Tyr²⁰). In the absence of added substrate, a low level of phosphorylation of endogenous substrates could be seen. Phosphoamino acid analysis demonstrated that although most of this phosphorylation occurred on serine and threonine residues a detectable level occurred on tyrosine residues. With the addition of the synthetic substrate poly(Glu⁸⁰, Tyr²⁰), the endogenous protein tyrosine kinase activity rapidly phosphorylated the exogenous substrate (see Fig. 1). Phosphoamino acid analysis indicated that the observed increase in phosphorylation occurred on tyrosine residues (see Fig. 1). To quantitate the

tyrosine kinase activity directly, we used a filter paper assay to measure the specific incorporation of ^{32}P into poly(Glu 80 ,Tyr 20). The phosphorylation of the synthetic substrate was enhanced by the addition of 0.1% Triton X-100 and was linear with respect to time and the amount of homogenate added to the assay mixture.

Kinetics of phosphorylation of poly(Glu 80 ,Tyr 20)

The rate of phosphorylation of the poly(Glu 80 , Tyr 20) substrate showed normal Michaelis–Menten kinetics with respect to the concentration of ATP and of poly(Glu 80 ,Tyr 20). Linear Lineweaver–Burk plots for the phosphorylation of poly(Glu 80 ,Tyr 20) are shown as a function of ATP in Fig. 2A and as a function of poly(Glu 80 ,Tyr 20) in Fig. 2B. In the presence of a saturating amount of poly(Glu 80 ,Tyr 20), the apparent K_m value for ATP was 19 μM . The synthetic peptide proved to be a very effective substrate for the endogenous protein tyrosine kinases with an apparent K_m of 14 $\mu\text{g/ml}$ and a V_{max} of 666 pmol/min/mg protein.

Regional distribution of protein tyrosine kinase activity

A comparison of tyrosine-specific protein kinase activity in homogenates of 16 microdissected brain regions and the spinal cord revealed a marked regional distribution, with the specific activities varying in magnitude over a sixfold range (140–800 pmol/min/mg) (Fig. 3). In general, the tyrosine kinase activity was highest (>600 pmol/min/mg) in cerebellum, hippocampus, pyriform cortex, and olfactory bulb, and lowest (<200 pmol/min/mg) in the pons/medulla and spinal cord.

Subcellular distribution of protein tyrosine kinase activity

Initial fractionation of rat forebrain homogenate into particulate and soluble fractions by centrifugation at 100,000 g for 45 min at 4°C indicated that the majority (>95%) of the tyrosine kinase activity remained in the pellet. To characterize further the subcellular localization of the tyrosine-specific protein kinase activity, a detailed subcellular fractionation of rat forebrain tissue was carried out by differential centrifugation followed by discontinuous sucrose density gradient centrifugation (Fig. 4). Measurement of specific activity showed low amounts of protein tyrosine kinase activity in the cytosol fractions (S_3 and LS_2) and in the myelin and mitochondrial fractions. In contrast, the crude microsomal fraction (P_3) and the crude synaptic vesicle fraction (LP_2) showed the highest levels of protein tyrosine kinase activity.

Subcellular distribution of pp60^{csrc}

To examine the subcellular distribution of pp60^{csrc}, a well-characterized protein tyrosine kinase, we have used an affinity-purified antibody prepared against a synthetic peptide corresponding to the autophosphorylation site of pp60^{csrc}. This antibody specifically inhibits 40% of the tyrosine kinase activity in rat neocortex and specifically immunoprecipitates a phosphotyrosine-containing 60-kilodalton (kD) protein from neocortex homogenates phosphorylated in vitro (data not shown). As shown in Fig. 5, this antibody recognizes a 60-kD protein on immunoblots of homogenates of rat neocortex. The 56-kD protein is a proteolytic breakdown product of pp60^{csrc}. The subcellular distribution of pp60^{csrc} parallels the overall protein tyrosine kinase activity with very low levels of pp60^{csrc} in the cytosol fractions (S_3 and LS_2) and in the mitochondrial fraction. In contrast, the highest levels were found in the crude microsomal (P_3) and crude synaptic vesicle (LP_2) fractions.

Substrates of the tyrosine-specific protein kinases

To begin to examine the major substrates of the tyrosine-specific protein kinases, the homogenates from the various regions and the subcellular fractions were incubated under

phosphorylating conditions with unlabeled ATP and analyzed by immunoblotting techniques using antibodies specific for phosphotyrosine. Although the tyrosine phosphorylation of exogenous substrates was enhanced by nonionic detergents the tyrosine phosphorylation of endogenous membrane substrates has been found to be selectively inhibited by detergents (Huganir et al., 1984; Pang et al., 1988). We have therefore examined the in vitro phosphorylation of endogenous substrates in the absence of detergent. Control blots using preimmune serum showed no immunoreactivity and preincubation of the affinity-purified anti-phosphotyrosine antibodies with 1 mM phosphotyrosine eliminated labeling of the phosphoproteins. Preincubation of the antibodies with 1 mM phosphoserine or phosphothreonine had no effect on the labeling pattern observed. In addition, when homogenates were phosphorylated using [γ - 32 P]ATP, the anti-phosphotyrosine antibodies specifically immunoprecipitated the major phosphotyrosine-containing phosphoproteins (data not shown).

Regional distribution of substrates for the protein tyrosine kinase activity

Many phosphotyrosine-containing proteins were detected in all brain regions examined (Fig. 6). Prominent among these phosphoproteins were proteins of molecular masses 26, 38, 56, 70, 85, 96, 114, 135, 164, and 196 kD. Most of these phosphoproteins were present in all of the brain regions examined and in general they appeared to parallel the tyrosine kinase activity. The cerebellum appeared to be particularly rich in the 87-kD phosphoprotein. The hypothalamus and thalamus showed unusually low immunoreactivity in the experiment illustrated; other experiments consistently showed a higher level of labeling.

Subcellular distribution of substrates for protein tyrosine kinase activity

The anti-phosphotyrosine antibodies recognized many tyrosine-phosphorylated proteins, which varied in their subcellular distribution (Fig. 7). Crude homogenates of rat forebrain contained 10 major substrates with molecular masses of 26, 38, 56, 70, 85, 96, 114, 135, 164, and 196 kD. Most of these phosphotyrosine-containing phosphoproteins were enriched in the particulate fractions. Phosphoproteins with molecular masses of 26, 38, 85, and 96 kD were enriched in the crude synaptic vesicle fraction (LP₂) but also appeared in other particulate fractions such as the light membrane fraction and the synaptic plasma membrane (SPM) fraction. A phosphoprotein with a molecular mass of 70 kD was highly enriched in the SPM fraction, whereas phosphotyrosine-containing proteins with molecular masses of 114, 164, and 196 kD were enriched in the SPM and light membrane fractions. A distinct protein with a molecular mass of 68 kD appeared to be enriched in the crude nuclear (P₁) fraction. The major soluble phosphotyrosine-containing substrate had a molecular mass of 58 kD.

DISCUSSION

The results presented here demonstrate that rat brain contains high levels of tyrosine-specific protein kinase activity, which can be characterized using the synthetic peptide substrate poly(Glu⁸⁰,Tyr²⁰). Under the optimized conditions of the assay, the phosphorylation reaction proceeds rapidly and with normal Michaelis–Menten kinetics.

Protein tyrosine kinase activity was detected in all areas of the CNS examined; however, there were marked differences in the levels of activity in the various regions. High protein tyrosine kinase activity was found in the cerebellum, hippocampus, olfactory bulb, and pyriform cortex. The regional distribution of pp60^{csrc} has been studied, and in general is similar to the distribution of protein tyrosine kinase activity reported here (Walaas et al., 1988). The highest levels of pp60^{csrc} were seen in the hippocampus, pyriform cortex, and

neocortex, followed by subcortical forebrain regions, cerebellum, diencephalic (thalamus, hypothalamus), and brainstem regions and spinal cord (Walaas et al., 1988).

The uneven distribution of protein tyrosine kinase activity observed in the present study differs from the relatively even distribution seen for cyclic AMP-dependent protein kinase activity (Walaas et al., 1983a,b). In addition, it is different from the distribution of calcium/calmodulin-dependent protein kinase activity, e.g., protein tyrosine kinase activity is high in the cerebellum where calcium/calmodulin-dependent protein kinase activity is low (Walaas et al., 1983a,b). The regional distribution of protein tyrosine kinase activity is most similar to the distribution of protein kinase C in rat brain (Walaas et al., 1983a,b).

Using the phosphotyrosine-specific antibodies, we found many phosphotyrosine-containing proteins with similar molecular masses in all regions of the CNS. These results suggest that some common class of cellular elements may serve as endogenous substrates.

The protein tyrosine kinase activity in rat brain, like that of many other protein tyrosine kinases, is associated with particulate fractions (Hunter and Cooper, 1985). Indeed, the high-speed pellet fractions, such as the P₃ and LP₂ fractions, showed the highest specific activity. In contrast, the cytosolic fractions such as the S₃ and LS₂ fractions contained little enzyme activity. On subfractionation of the lysed synaptosome fraction, most of the activity resided in the light membrane and SPM fractions. The presence of high levels of protein tyrosine kinase activity in the LP₂ fraction which is highly enriched in synaptic vesicles and in the SPM fraction suggests that tyrosine phosphorylation may play a role in synaptic physiology.

The subcellular distribution of pp60^{src}, one of the well-characterized protein tyrosine kinases, parallels that of the overall protein tyrosine kinase activity. Very low levels of pp60^{src} were found in cytosolic fractions (S₃ and LS₂) and high levels were found in the crude microsomal (P₃) and crude synaptic vesicle (LP₂) fractions. These results provide evidence that pp60^{src} may play a role in the modulation of neurotransmitter release.

In agreement with the subcellular distribution of kinase activity and pp60^{src}, the majority of phosphotyrosine-containing proteins were present in the particulate fractions. Some of these phosphoproteins showed a very distinct distribution among the various fractions. The 26-, 38-, 85-, and 96-kD phosphoproteins were highly enriched in the LP₂ fractions. Recently, it has been reported that 30-, 38-, 94-, and 105-kD phosphotyrosine-containing proteins are highly enriched in purified synaptic vesicles (Pang et al., 1988). It is likely that these proteins are identical to the 26-, 38-, 85-, and 96-kD proteins observed in the present study. Moreover, the 38-kD protein was identified as synaptophysin, or p38, a major integral membrane protein in synaptic vesicles (Pang et al., 1988). In addition, we have found that phosphotyrosine-containing proteins with molecular masses of 70 and 114 kD were enriched in synaptic plasma membranes. These results suggest that phosphorylation of these substrate proteins on tyrosine residues may play a role in synaptic function.

Except for the growth factor receptors, which have been reported to be present at low concentrations in brain tissue, the physiological regulators of the brain tyrosine kinases are at present unknown. It is unlikely that, in adult neuronal tissue, the majority of the tyrosine kinase activity is involved in cell proliferation. However, the tyrosine kinases could play a role in the development/maturation of neuronal processes (Skene, 1983). The membrane association of these kinases, and their presence in the synaptosomal compartment, are compatible with this idea. Indeed, developmental studies by Maness and co-workers (Sorge et al., 1984; Fults et al., 1985) showed that immunoreactivity to pp60^{src} appears in neuronal processes at the time of differentiation.

Another possible role for the tyrosine kinases may be in signal transduction. The presence of high levels of the protein tyrosine kinases and their endogenous substrates in the LP₂ and SPM fractions is consistent with tyrosine phosphorylation being involved in the regulation of synaptic functions such as neurotransmitter release and the modulation of neurotransmitter receptors or ion channels. Recently it was reported that chromaffin granule membranes contain high levels of pp60^{csrc} and suggested that pp60^{csrc} may play a role in secretion (Parsons and Creutz, 1986). Moreover, Pang et al. (1988) have shown that high levels of endogenous tyrosine phosphorylation occur in purified synaptic vesicles and that synaptophysin (p38) is a major substrate for the endogenous kinase activity. In addition, the nicotinic acetylcholine receptor in postsynaptic membranes from *Torpedo* electroplax is phosphorylated by several protein kinases (Huganir, 1987), among them an endogenous protein tyrosine kinase (Huganir et al., 1984). Phosphorylation of the nicotinic acetylcholine receptor by the cyclic AMP-dependent protein kinase in vitro and in vivo has been shown to regulate the rate of desensitization of the receptor to acetylcholine (Huganir et al., 1986; Middleton et al., 1986; Albuquerque et al., 1986; Miles et al., 1987). Since the tyrosine phosphorylation sites of the receptor appear to be in the vicinity of the phosphorylation sites for cyclic AMP-dependent protein kinase (Huganir et al., 1984), it is attractive to hypothesize that tyrosine phosphorylation of the receptor will also regulate its rate of desensitization. Tyrosine phosphorylation, like serine and threonine phosphorylation of proteins, may in general play a major role in the mediation and modulation of signal transduction in the brain.

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Abbreviations used

BME	β -mercaptoethanol
kD	kilodalton
NP-40	Nonidet P-40
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPM	synaptic plasma membrane
TCA	trichloroacetic acid

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FIG. 1. Demonstration of tyrosine-specific protein kinase activity in rat forebrain. The phosphorylation reaction was carried out under standard conditions for 30 min with 50 μ g of protein in the absence (–) or presence (+) of 0.5 mg/ml poly(Glu⁸⁰, Tyr²⁰). **Left:** SDS-PAGE analysis. **Right:** Phosphoamino acid analysis. The spots near the origin of the TLC plates are unhydrolyzed phosphopeptides.

FIG. 2.

Lineweaver-Burk plots of the phosphorylation of poly(Glu⁸⁰,Tyr²⁰) by the endogenous protein tyrosine kinase activity of rat forebrain. Initial rates were measured under standard conditions for 5 min, using the filter paper assay. ATP concentration (**A**) and Poly(Glu⁸⁰,Tyr²⁰) concentration (**B**) were varied as indicated.

FIG. 3.

Regional distribution of protein tyrosine kinase activity in rat CNS. The material dissected from the various regions was homogenized and the protein tyrosine kinase activity was measured under standard conditions, using the filter paper assay. These data are the averages from three different dissections. The results represent picomoles of phosphate incorporated into the synthetic substrate, presented as means $\pm$ SEM. Olf. bulb, olfactory bulb; Olf. tube., olfactory tubercle; Caud-put., caudatoputamen; N. acc., nucleus accumbens; Glob. pall., globus pallidus; Hypothal., hypothalamus; Thal., thalamus; Septum, septum; Hippo., hippocampus; Pyr. ctx., pyriform cortex; Neoctx., neocortex; V. Mes., ventral mesencephalon; M. Mes., middle mesencephalon; D. Mes., dorsal mesencephalon; Cblm., cerebellum; Pons/med., pons/medulla; Sp. cord, spinal cord.

FIG. 4.

Subcellular distribution of protein tyrosine kinase activity in rat forebrain. Protein tyrosine kinase activity was assayed under standard conditions, using the filter paper assay. These data are the averages of three separate subcellular fractionations, assayed in duplicate. Results represent picomoles of phosphate incorporated into the synthetic substrate, presented as means $\pm$ SEM. The various fractions are as defined in Materials and Methods.

FIG. 5. Subcellular distribution of pp60^{csrc} in rat forebrain. Each subcellular fraction (50 μ g of protein) was subjected to SDS-PAGE on 8% gels, and transferred onto nitrocellulose. The nitrocellulose filters were probed with the anti-pp60^{csrc} antibodies and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG. The various fractions are as defined in Materials and Methods.

FIG. 6.

Regional distribution of phosphotyrosine-containing proteins in rat CNS. An aliquot (50 μ g of protein) of the homogenate from each region was phosphorylated under standard conditions using nonradioactive ATP, subjected to SDS-PAGE on 8% gels, and transferred onto nitrocellulose. The nitrocellulose filters were probed with the anti-phosphotyrosine antibodies, developed with 125 I-Protein A, and subjected to autoradiography. For abbreviations used see legend to Fig. 3.

FIG. 7.

Subcellular distribution of phosphotyrosine-containing proteins in rat forebrain. Each subcellular fraction (50 μ g of protein) was phosphorylated under standard conditions using nonradioactive ATP, subjected to SDS-PAGE on 8% gels, and transferred onto nitrocellulose. The nitrocellulose filters were probed with the anti-phosphotyrosine antibodies, developed with 125 I-Protein A, and subjected to autoradiography.