REGIONAL DISTRIBUTION OF CALCIUM- AND CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE-REGULATED PROTEIN PHOSPHORYLATION SYSTEMS IN MAMMALIAN BRAIN

II. Soluble Systems¹

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Abstract

The regional distribution of phosphoproteins whose phosphorylation is regulated either by cyclic AMP or by calcium in combination with calmodulin or phospholipid has been investigated in soluble preparations from rat CNS. About 40 distinct phosphoproteins were observed. These cytosolic phosphoproteins exhibited widely different patterns of regional distribution. Based upon distribution patterns, we have divided these phosphoproteins into three categories: *category A*, phosphoproteins found in all parts of the CNS in approximately equal amounts; *category B*, phosphoproteins which are widely distributed within the CNS, but which show large regional variations; and *category C*, phosphoproteins which show a highly restricted regional distribution. We have tentatively interpreted the results on cytosolic phosphoproteins in the following way: some are present in all or nearly all brain cells, others are present only in certain classes of brain cells, and still others have an even more limited distribution, being present in only a single type of brain cell.

The regional distribution of soluble protein kinase activity was also studied. Calcium/phospholipid-dependent protein kinase and calcium/calmodulin-dependent protein kinase had marked regional distributions. Cyclic AMP-dependent protein kinase was more evenly distributed throughout the CNS.

This investigation thus demonstrates striking differences in the regional distribution of cytosolic protein phosphorylation systems in mammalian brain. These regional differences may reflect highly specific functional roles for certain of these protein phosphorylation systems. Similar conclusions concerning particulate protein phosphorylation systems are described in the preceding paper (Walaas, S. I., A. C. Nairn, and P. Greengard (1983) *J. Neurosci.* 3: 291-301).

In the preceding paper (Walaas et al., 1983), we reported the occurrence of about 30 distinct particulate phosphoproteins in the rat CNS. In addition, in that paper we reported the results of studies on the regional distribution of those phosphoproteins and of particulate

calcium-dependent and cyclic AMP-dependent protein kinase activities. In the present paper we report the results of studies on soluble protein phosphorylation systems in the rat CNS.

Materials and Methods

Materials. Materials were obtained as described in the preceding paper (Walaas et al., 1983).

Sample preparation. Eighteen regions of the CNS were microdissected as described in the preceding paper (Walaas et al., 1983). Samples from four to six animals were pooled, weighed, and homogenized in 10 vol of ice-cold standard buffer (containing 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 50 units/ml of Trasylol, 2 µg/ml of Pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride) with 20 strokes in a glass-Teflon homogenizer rotating at 2,100 rpm. One- to two-milliliter aliquots were

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then separated into particulate and soluble fractions by centrifugation at $150,000 \times g$ for 30 min at 4°C . The supernatants were collected on ice, and the pellets were resuspended by vortexing for 20 to 40 sec in the same volume of standard buffer and were subjected to a second centrifugation at $150,000 \times g$ for 30 min. The supernatant and the wash were pooled and used as the soluble fraction. Parts of the samples were used for analysis of protein kinase activity and for analysis of endogenous protein kinase substrates. Another aliquot of the samples was used for analysis of protein content by a detergent-based Folin reagent assay using BSA as standard, after precipitation of protein with deoxycholate and trichloroacetic acid (Peterson, 1977).

In initial experiments we studied the patterns of protein phosphorylation in the supernatant and wash separately. The two patterns of phosphorylation observed for any given brain region were qualitatively indistinguishable. The supernatant from the first centrifugation contained 6 times more protein than did the wash. When equal amounts of protein from the two preparations were analyzed for endogenous phosphorylation in the presence of calcium or cyclic AMP, considerably more ^{32}P was incorporated into the different protein substrates in the wash. Therefore, the two preparations were pooled in further experiments, to make a total soluble fraction. This preparation was then desalted, either by gel filtration through a small Sephadex G-25 column (Helmerhorst and Stokes, 1980) or by dialysis against 1 to 2 liters of cold standard buffer for 2 to 5 hr. Approximately 60% of the total protein was recovered by the gel filtration technique. The patterns of phosphoproteins and the specific activities of the protein kinases were similar for the two types of desalted preparations.

Removal of calmodulin. In some experiments, calmodulin was removed from the soluble preparation by ammonium sulfate fractionation (Yamauchi and Fujisawa, 1979). In this procedure, ammonium sulfate was added to 1-ml aliquots to 55% (w/v) saturation. The suspension was stirred on ice for 15 min and centrifuged at $28,000 \times g$ for 20 min. The supernatant was removed, and the pellet was resuspended in standard buffer and dialyzed against the same buffer overnight. This procedure removed calmodulin but left the calcium/calmodulin-dependent protein kinase activity and most major substrate proteins in the sample.

In preliminary experiments we attempted an alternate procedure (Wrenn et al., 1980) for removal of calmodulin, in which aliquots were subjected to ion exchange chromatography on DEAE-cellulose (Whatman) columns. Samples were eluted with 0.26 M NaCl dissolved in standard buffer. Under these conditions, calmodulin remained on the ion exchange column. The eluate was then dialyzed against standard buffer overnight. An incomplete recovery of protein substrates was observed using this procedure. Therefore, the ammonium sulfate procedure was used to remove calmodulin from the soluble preparations in all such experiments reported in this paper.

Demonstration of endogenously phosphorylated proteins and protein kinase assays were carried out as described in the preceding paper (Walaas et al., 1983)

except that a 60-sec incubation time was found to be optimal for the demonstration of endogenously phosphorylated proteins.

Terminology. Endogenous phosphoproteins were assigned to categories as follows: *category A*, phosphoproteins found throughout the CNS, in concentrations which varied between different regions by a factor of less than five; *category B*, phosphoproteins found to be widely distributed in the CNS, but in concentrations which varied between different regions by a factor of more than five; and *category C*, phosphoproteins found in only one or a few regions of the CNS.

Results

Figure 1 shows the pattern of phosphorylation of endogenous proteins in the soluble fraction of the rat neocortex. Under basal conditions, ^{32}P was incorporated into only a few protein bands. Addition of cyclic AMP plus isobutylmethylxanthine (IBMX) stimulated the phosphorylation of a restricted number of proteins under the conditions used. In contrast, addition of calcium stimulated the phosphorylation of a considerable number of protein bands. As will be described, this effect of calcium appears to be through an increase in calcium/calmodulin-dependent protein kinase activity. The greatest increase in total phosphorylation was achieved by the combination of calcium plus phosphatidylserine, which led to a great number of both high and low molecular weight proteins being phosphorylated (Fig. 1). Addition of phosphatidylserine alone under the conditions employed was without effect on the phosphorylation reaction (not shown). These protein phosphorylation systems were then examined in greater detail in various CNS regions.

Cyclic AMP-stimulated protein phosphorylation

Addition of the phosphodiesterase-resistant analogue 8-bromo-cyclic AMP or of cyclic AMP plus IBMX to the caudatoputamen sample markedly increased the ^{32}P content of several phosphoproteins. The major proteins phosphorylated by this treatment are indicated by arrows in Figure 2. Small increases also were observed in several other proteins with apparent M_r ranging between 180,000 and 17,000. The regional distribution patterns of these various phosphoproteins will now be described.

Category A phosphoproteins. The 280,000-dalton protein, which probably represents the microtubule-associated protein MAP₂ (Sloboda et al., 1975; Vallee, 1980), a 130,000-dalton phosphoprotein (Figs. 2 and 3), and 55,000-, 52,000-, and 43,000-dalton phosphoproteins were found in all regions. The 280,000-dalton phosphoprotein appeared as a doublet in all regions except the olfactory bulb. In this region, only one 280,000-dalton phosphoprotein band was seen.

Category B phosphoproteins. None were observed.

Category C phosphoproteins. Several phosphoproteins were found to be localized specifically in the following subdivisions of the basal ganglia: the caudatoputamen, the nucleus accumbens, the olfactory tubercle, the globus pallidus, and the substantia nigra. Included in this category were proteins of M_r 98,000, 96,000, 81,000, 40,000, 34,000, 32,000, and 21,000. Both the category A and

Figure 1. Autoradiogram illustrating cyclic AMP-dependent and calcium-dependent phosphorylation of soluble proteins from rat cerebral cortex. Aliquots containing 30 μ g of protein were incubated under standard assay conditions in the absence or presence of either 2 μ M cyclic AMP plus 1 mM IBMX, 1.5 mM CaCl_2 , or 1.5 mM CaCl_2 plus 50 μ g/ml of phosphatidylserine. Aliquots containing 20 μ g of protein were separated by SDS-PAGE and the phosphoproteins were visualized by autoradiography. **A**, 11% polyacrylamide gel, illustrating lower molecular weight phosphoproteins; **B**, 6% polyacrylamide gel allowing visualization of higher molecular weight phosphoproteins.

Figure 2. Autoradiogram illustrating cyclic AMP-dependent phosphorylation of soluble proteins from rat caudatoputamen. Aliquots containing 30 μ g of protein were incubated under standard conditions in the absence or presence of 2 μ M cyclic AMP plus 1 mM IBMX. Aliquots containing 20 μ g of protein

category C phosphoproteins (the latter indicated by *arrows*) can be visualized in Figure 3.

The category C phosphoproteins located specifically in the basal ganglia displayed two subtypes of regional distribution. One group, namely the 98,000-, 96,000-, and 81,000-dalton proteins, was observed in significant amounts only in the caudatoputamen, the nucleus accumbens (Fig. 3), and the olfactory tubercle; that is, in the dopamine-innervated dorsal and ventral parts of the neostriatum (Heimer, 1978; Björklund and Lindvall, 1978; Fallon and Moore, 1978). The second group, namely the 40,000-, 32,000-, and 21,000-dalton proteins (and, faintly, the 34,000-dalton protein), was observed in the same regions as well as in the globus pallidus and substantia nigra (Fig. 3), regions which are densely innervated by efferent fibers from the neostriatum (Nauta and Dome-sick, 1979).

Calcium-dependent and calcium/calmodulin-dependent protein phosphorylation

Addition of calcium alone stimulated the phosphorylation of a number of proteins in soluble preparations

were separated by SDS-PAGE, and the phosphoproteins were visualized by autoradiography. **A**, 11% polyacrylamide gel, illustrating low molecular weight phosphoproteins; **B**, 6% polyacrylamide gel, allowing visualization of high molecular weight phosphoproteins. *Arrows* indicate major substrates for cyclic AMP-dependent protein kinase.

from the cerebral cortex (Fig. 4). Separate experiments showed that addition of calmodulin to the medium did not change the pattern of endogenously phosphorylated proteins (not shown), and analysis of preparations where calmodulin had been removed showed that the calcium-stimulated phosphorylation of these proteins exhibited an absolute dependence upon addition of calmodulin (Fig. 5). The calcium-induced protein phosphorylation, therefore, appears to be mediated by endogenous calmodulin.

Category A phosphoproteins. Addition of calcium alone stimulated the phosphorylation of a 280,000-dalton doublet protein, which probably represents MAP₂ (Sloboda et al., 1975; Vallee, 1980), in all regions examined (except the olfactory bulb, where both basal and calcium-stimulated patterns showed only one 280,000-dalton band).

Category B phosphoproteins. Addition of calcium alone stimulated the phosphorylation of three major phosphoproteins ($M_r = 62,000, 58,000$, and $48,000$) (Figs. 1 and 4) and of one minor phosphoprotein ($M_r = 18,000$) (not shown). The soluble 62,000-dalton phosphoprotein was highly concentrated in cortical samples (Fig. 4) and was clearly seen in all other forebrain regions and cerebellum, but was almost absent from the pons/medulla (Fig. 4) and spinal cord (not shown). The 58,000-dalton

phosphoprotein was seen most clearly in the pons/medulla and faintly in the cerebellum (Fig. 4), spinal cord (not shown), and most forebrain regions (Fig. 4). The 48,000-dalton phosphoprotein was highly concentrated in the cortex and in forebrain regions but was not seen in the cerebellum, pons/medulla (Fig. 4), or spinal cord. These patterns of distribution were qualitatively similar to those seen for the $M_r 62,000, 58,000$, and $48,000$ phosphoproteins in the particulate preparations incubated with calcium plus calmodulin (Walaas et al., 1983). The soluble 18,000-dalton phosphoprotein seen in the presence of calcium alone was observed only in the pons/medulla (Fig. 6) and the spinal cord.

Category C phosphoproteins. None were observed.

Calcium/phospholipid-dependent protein phosphorylation

When soluble preparations of cerebral cortex were incubated in the presence of calcium plus phosphatidylserine, a large number of phosphoprotein bands were observed in addition to those seen in the presence of calcium alone. The same patterns of phosphoprotein bands were observed whether untreated cytosol (Figs. 1 and 6) or calmodulin-deficient cytosol was used (Fig. 5). The phosphorylation of most proteins with M_r above 40,000 was much less intense in the pons/medulla (and

Figure 3. Autoradiogram illustrating cyclic AMP-dependent phosphorylation of soluble proteins in six brain regions. Aliquots containing 30 μ g of protein were incubated under standard assay conditions in the absence or presence of 2 μ M cyclic AMP plus 1 mM IBMX. Aliquots containing 20 μ g of protein were separated by SDS-PAGE on 8% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. Arrows indicate major substrates for cyclic AMP-dependent protein kinase which are specific for the basal ganglia. A, Comparison of cortical and neostriatal (nucleus accumbens) phosphoproteins; B, comparison of diencephalic (hypothalamus) and paleostriatal (globus pallidus) phosphoproteins; C, comparison of rhombencephalic (pons/medulla) and ventral mesencephalic (i.e., substantia nigra) phosphoproteins.

Figure 4. Autoradiogram illustrating calcium/calmodulin-dependent phosphorylation of soluble proteins in various rat brain regions. Aliquots containing 50 μg of protein were incubated under standard assay conditions in the absence or presence of 1.5 mM CaCl_2 . Aliquots containing 20 μg of protein were separated by SDS-PAGE on 7% polyacrylamide gels and the phosphoproteins were visualized by autoradiography. Arrows indicate major substrates for calcium/calmodulin-dependent protein kinase.

Figure 5. Autoradiogram illustrating calcium-dependent, calcium/phospholipid-dependent, and calcium/calmodulin-dependent phosphorylation of soluble proteins in calmodulin-free cytosol from rat cerebral cortex. Calmodulin was removed from the cytosol as described in the text. Aliquots containing 30 μg of protein were incubated under standard conditions in the absence or presence of 1.5 mM CaCl_2 , 1.5 mM CaCl_2 plus 50 $\mu\text{g}/\text{ml}$ of phosphatidylserine, or 1.5 mM CaCl_2 plus 10 $\mu\text{g}/\text{ml}$ of calmodulin (*CaM*). Aliquots containing 20 μg of protein were separated by SDS-PAGE on 9% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. Arrows indicate some of the major substrates for calcium/phospholipid-dependent and calcium/calmodulin-dependent protein kinases.

Figure 6. Autoradiogram illustrating calcium/phospholipid-dependent phosphorylation of soluble proteins from various regions of rat brain. Aliquots containing 30 μ g of protein were incubated under standard assay conditions in the presence of 1.5 mM CaCl_2 , in the absence or presence of 50 μ g/ml of phosphatidylserine. Aliquots containing 20 μ g of protein were separated by SDS-PAGE on 11% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. Arrows indicate some of the major substrates for calcium/phospholipid-dependent protein kinase.

spinal cord) than in cortex, cerebellum, and other CNS regions, whereas the phosphorylation of proteins with M_r below 40,000 appeared to be of greater intensity in the lower brain stem (and spinal cord) (e.g., see Fig. 6).

Category A phosphoproteins. Three major phosphoprotein bands (M_r = 280,000 doublet (MAP2), 87,000, and 19,000), less prominent phosphoproteins of M_r 24,000 and 15,000, and about 15 to 20 other phosphoprotein bands (Figs. 1 and 6) were observed in all regions examined.

Category B phosphoproteins. Two phosphoprotein bands (M_r = 20,000 and 18,000) were observed with more restricted distribution. They were highly concentrated in the pons/medulla (Fig. 6) and spinal cord (not shown) and were less prominent in all other regions examined (Fig. 6).

Category C phosphoproteins. None were observed.

Cyclic AMP-dependent and calcium-dependent protein kinase activities

Cyclic AMP-dependent protein kinase activity, assayed with protein I as substrate (Huttner et al., 1981; Walaas et al., 1983), was fairly evenly distributed in most brain regions (Table I). Only the neostriatal regions (the caudatoputamen, nucleus accumbens, and olfactory tubercle) contained consistently higher activity of this enzyme than the rest of the brain, in parallel with the strong stimulation of endogenous phosphorylation induced by cyclic AMP and the large number of category

TABLE I

Regional distribution of cytosolic cyclic AMP-dependent and calcium/calmodulin-dependent protein I kinase activity in rat CNS

Results represent nanomoles of ^{32}P incorporated into protein, presented as mean $\pm$ SEM. Protein I (20 μ g) purified from bovine brain was used as substrate. Basal protein kinase activity was measured under standard conditions (see the text), cyclic AMP-dependent protein kinase activity was measured in the presence of 1 μ M cyclic AMP plus 1 mM IBMX, and calcium/calmodulin-dependent protein kinase activity was measured in the presence of 1.5 mM CaCl_2 plus 10 μ g/ml of calmodulin.

Region	N ^a	Basal Activity	Cyclic AMP-dependent Activity	Calcium/Calmodulin-dependent Activity
nmol/min/mg of protein				
Neocortex	3	0.47 $\pm$ 0.15	2.3 $\pm$ 0.6	2.8 $\pm$ 0.8
Hippocampus	4	0.47 $\pm$ 0.16	1.6 $\pm$ 0.2	6.3 $\pm$ 1.5
Amygdala	3	0.77 $\pm$ 0.17	3.0 $\pm$ 0.4	7.5 $\pm$ 3.3
Olfactory bulb	2	0.67 $\pm$ 0.07	2.6 $\pm$ 0.4	2.4 $\pm$ 1.0
Olfactory tubercle	2	0.73 $\pm$ 0.06	3.9 $\pm$ 0.5	3.7 $\pm$ 2.6
Nucleus accumbens	3	0.81 $\pm$ 0.06	4.3 $\pm$ 0.4	5.0 $\pm$ 1.9
Caudatoputamen	4	0.67 $\pm$ 0.05	3.0 $\pm$ 0.4	2.6 $\pm$ 1.0
Globus pallidus	2	0.58 $\pm$ 0.03	2.3 $\pm$ 0.6	2.1 $\pm$ 0.3
Thalamus	2	0.48 $\pm$ 0.01	1.9 $\pm$ 0.4	0.72 $\pm$ 0.07
Hypothalamus	2	0.39 $\pm$ 0.09	1.8 $\pm$ 0.6	1.5 $\pm$ 0.9
Mesencephalon	4	0.56 $\pm$ 0.04	2.4 $\pm$ 0.2	1.3 $\pm$ 0.4
Cerebellum	4	0.44 $\pm$ 0.13	1.2 $\pm$ 0.3	1.8 $\pm$ 0.5
Pons/medulla	3	0.33 $\pm$ 0.10	1.6 $\pm$ 0.4	1.4 $\pm$ 0.5

^a N, number of pooled tissue preparations examined.

C phosphoprotein substrates for the cyclic AMP-dependent protein kinase seen in these regions (Figs. 2 and 3).

Calcium/calmodulin-dependent protein kinase activity, also assayed with protein I as substrate (Kennedy and Greengard, 1981; Huttner et al., 1981), was found to be present in all regions, but displayed a more pronounced regional variability than did the cyclic AMP-dependent enzyme (Table I). (Addition of calcium alone to the standard assay mixture caused a slight increase in protein I phosphorylation, presumably due to small amounts of endogenous calmodulin (not shown)). High calcium/calmodulin-dependent protein kinase activity was found in the cortical regions, particularly in the hippocampus and in most subcortical forebrain regions. Relatively low activity was found in the cerebellum, diencephalon, mesencephalon, pons/medulla, and spinal cord. This distribution in general parallels the distribution of the particulate calcium/calmodulin-dependent protein kinase (Walaas et al., 1983). The subcellular distribution of the calcium/calmodulin-dependent protein kinase activity was much more variable between experiments than that of the cyclic AMP-dependent protein kinase activity. Thus, in several experiments, we observed that, although the sum of the activities of the soluble and particulate fractions remained constant, the proportion of the activities in the two fractions was highly variable.

Calcium/phospholipid-dependent protein kinase activity displayed a widespread distribution, with particularly high activity in cortical regions and in the cerebellum, intermediate activity in subcortical telencephalic regions, and low activity in the diencephalon, mesencephalon,

rhombencephalon (except for the cerebellum), and spinal cord (Table II). (Addition of calcium alone or phosphatidylserine alone caused only a slight increase in histone phosphorylation under the experimental conditions used (not shown)). The distribution of calcium/phospholipid-dependent histone kinase activity paralleled that of the phosphoproteins with apparent M_r above 40,000 that were substrates for this enzyme, but was clearly different from that of the 20,000- and 18,000-dalton substrates.

Discussion

The results presented in this report demonstrate the presence of soluble protein phosphorylation systems regulated by cyclic AMP or by calcium throughout the rat CNS. It is convenient, as was done for the particulate preparations (Walaas et al., 1983), to divide these phosphoproteins into three different categories based on their regional distribution. Thus, category A phosphoproteins are found throughout the CNS, category B phosphoproteins are highly enriched in certain subdivisions, and category C phosphoproteins are clearly observed only in specific regions or nuclei. The relative activities of the cyclic AMP-stimulated and calcium-stimulated phosphorylation reactions (Fig. 1) indicate that the calcium/phospholipid-stimulated system is the most active in most regions, the calcium/calmodulin-stimulated system is intermediate, and the cyclic AMP-stimulated system is least active. However, several of the cyclic AMP-regulated phosphoproteins did show interesting category C regional distributions, which suggests a prominent functional role for this system in these particular regions.

A comparison between the various categories of phosphoproteins in the soluble and particulate preparations is interesting. The main phosphoproteins found in the particulate and soluble fractions and their regional distribution patterns are summarized in Table III. The main category A phosphoproteins phosphorylated upon addition of cyclic AMP to either the soluble or particulate preparations are the 280,000- (MAP_2) and 55,000-dalton phosphoprotein bands. In the particulate fraction the 55,000-dalton band was found to consist of at least two proteins, one being the phosphorylated regulatory subunit (R-II) of type II cyclic AMP-dependent protein kinase, and the other the neuronal phosphoprotein protein IIIb (Huang et al., 1982). Protein IIIb is not solubilized by the procedure used in this study (data not shown), while type II cyclic AMP-dependent protein kinases are known to be present in both membranes and cytosol in the CNS (Uno et al., 1977; Walter et al., 1978; Rubin et al., 1979). Therefore, the soluble 55,000-dalton phosphoprotein consists, at least partly, of phosphorylated R-II. Similarly, phosphorylatable MAP_2 has previously been found in both cytosol (Sloboda et al., 1975) and membrane fractions (Lohmann et al., 1980).

The soluble 280,000-dalton category A protein, which co-migrates with and probably is identical to MAP_2 , was also found to be a substrate for calcium/calmodulin-dependent and calcium/phospholipid-dependent protein kinases. Preliminary peptide mapping experiments indicate that the cyclic AMP-dependent, calcium/calmodulin-dependent, and calcium/phospholipid-dependent protein kinases phosphorylate three distinct sites on the MAP_2 molecule (data not shown). Calcium is known to

TABLE II

Regional distribution of cytosolic calcium/phospholipid-dependent histone III kinase activity in rat CNS

Results represent nanomoles of ^{32}P incorporated into protein, presented as mean $\pm$ SEM. Histone III (20 μ g) was used as substrate. Basal protein kinase activity was measured under standard conditions (see the text) in the presence of 1 mM EGTA and in the presence of 1 mM EGTA plus 50 μ g/ml of phosphatidylserine. Activities of 0.1 to 0.4 nmol/min/mg of protein were observed in all regions examined under both of these conditions. Calcium/phospholipid-dependent protein kinase activity was measured in the presence of 1.5 mM $CaCl_2$ plus 50 μ g/ml of phosphatidylserine.

Region	N ^a	Calcium/Phosphatidylserine-dependent activity nmol/min/mg of protein
Neocortex	3	7.1 $\pm$ 0.8
Hippocampus	2	9.1 $\pm$ 1.1
Amygdala	3	4.2 $\pm$ 0.6
Olfactory bulb	2	6.0 $\pm$ 0.4
Olfactory tubercle	2	5.4 $\pm$ 0.1
Nucleus accumbens	3	6.1 $\pm$ 0.6
Caudatoputamen	4	6.4 $\pm$ 0.7
Septum	3	4.7 $\pm$ 1.4
Globus pallidus	3	5.7 $\pm$ 0.8
Thalamus	2	2.5 $\pm$ 0.6
Hypothalamus	3	1.7 $\pm$ 0.7
Mesencephalon	4	2.6 $\pm$ 0.7
Cerebellum	3	7.0 $\pm$ 0.4
Pons/medulla	2	2.8 $\pm$ 0.1
Spinal cord	2	1.9 $\pm$ 0.2

^a N, number of pooled tissue preparations examined.

TABLE III

Regional distribution patterns of major particulate and soluble brain proteins phosphorylated by cyclic AMP-dependent, calcium/calmodulin-dependent, and calcium/phospholipid-dependent protein kinases.

Phosphoproteins are identified by molecular mass values (*Kd*, kilodalton). Category A phosphoproteins have a relatively even distribution throughout the CNS, category B phosphoproteins have a fairly widespread but uneven distribution, and category C phosphoproteins have a very restricted distribution.

Substrates for	Particulate Phosphoproteins		
	Category A	Category B	Category C
Cyclic AMP-dependent protein kinase	280	<i>Kd</i>	
	86	265	260
	80	52	39
	74		
	55		
Calcium/calmodulin-dependent protein kinase	86	160	N.O. ^a
	80	140	
		62	
		58	
		49	
Calcium (calcium/phospholipid?)-dependent protein kinase		48	
	87	47	N.O.
	20	46	
	19		
	18		

Substrates for	Soluble Phosphoproteins		
	Category A	Category B	Category C
Cyclic AMP-dependent protein kinase		<i>Kd</i>	
	280	N.O.	98
	130		96
	55		81
	52		40
	43		34
			32
Calcium/calmodulin-dependent protein kinase		21	
	280	62	N.O.
		58	
		48	
Calcium/phospholipid-dependent protein kinase		18	
	280	20	N.O.
	87	18	

^a N.O., none observed.

regulate several aspects of microtubule function, a regulation which may be achieved at least partly by changing the properties of MAP₂ (Schliwa et al., 1981; Marcum et al., 1978). Our evidence suggests that some of these regulatory functions might be mediated through the phosphorylation of MAP₂. Further examination of the phosphorylation of the 280,000-dalton protein is in progress.

A great number of other category A proteins are phosphorylated upon addition of calcium plus phospholipid to the soluble preparations. A major protein in this category with an apparent *M_r* of 87,000 is similar to the minor 87,000-dalton phosphoprotein seen in particulate preparations, not only with respect to its molecular weight but also in regional distribution and in peptide maps using *Staphylococcus aureus* V8 protease (not

shown). This phosphoprotein may, therefore, be present in both soluble and particulate preparations.

Similarly, the soluble 20,000- and 18,000-dalton category B phosphoproteins migrated on 15% polyacrylamide gels close to the 20,000- and 18,000-dalton particulate phosphoproteins which are phosphorylated upon addition of calcium alone (not shown). The latter proteins were suggested to represent myelin basic protein (Walaas et al., 1983). The regional distribution of the soluble 20,000- and 18,000-dalton proteins phosphorylated upon addition of calcium plus phosphatidylserine is presented schematically in Figure 7. These two category B proteins were found only in myelin-rich regions such as the pons/medulla and spinal cord, suggesting that they may represent partly solubilized myelin basic protein. In contrast, the soluble 19,000-dalton protein is present in all regions, irrespective of the amount of white matter present. Further characterization of these proteins is in progress.

Other major category B phosphoproteins seen in the soluble fractions were found to be substrates for calcium/calmodulin-dependent protein kinases, the most prominent being 62,000-, 58,000-, and 48,000-dalton proteins. These phosphoproteins display molecular weight values, regional distributions, and peptide maps using *S. aureus* protease V8 similar to three of the main particulate substrates for calcium/calmodulin-dependent protein kinase. Therefore, they are probably identical to the particulate phosphoproteins. Thus, some of the major substrates for calcium/calmodulin-dependent protein kinase in brain appear to be present in both particulate and soluble cell fractions as has also been described for other components of protein phosphorylation systems; for example, cyclic AMP-dependent protein kinase (Walter et al., 1978; Rubin et al., 1979), MAP₂ (Sloboda et al., 1975; Lohmann et al., 1980), and calmodulin (Sobue et al., 1981). The regional distributions of the major soluble and particulate substrates for calcium/calmodulin-dependent protein kinase are presented schematically in Figure 8, where their category B distribution pattern is shown in relation to the main CNS subdivisions.

The main category C phosphoproteins seen in the

Figure 7. Schematic illustration of the regional distribution of the 20 Kd (kilodalton) and 18 Kd soluble proteins phosphorylated by calcium/phospholipid-dependent protein kinase in rat brain. These proteins belong to category B. Symbols and abbreviations are the same as in the legend to Figure 8.

soluble fractions were found in the cyclic AMP-stimulated preparations. A well defined group of such proteins is present in the basal ganglia, where at least seven region-specific soluble substrate proteins for cyclic AMP-dependent protein kinase can be found. Dopamine is believed to be the main neurotransmitter influencing cyclic AMP synthesis in these parts of the brain (Forn et al., 1974; Iversen, 1977; Keibadian and Calne, 1979), where a dense population of dopamine nerve cells or terminals is found (Björklund and Lindvall, 1978). The state of phosphorylation of at least one of these basal ganglia phosphoproteins (32,000 daltons) has been found to be regulated by dopamine (S. I. Walaas and P. Greengard, manuscript in preparation). Thus, some of these phos-

Figure 8. Schematic illustration of the regional distribution of proteins phosphorylated by calcium/calmodulin-dependent protein kinase in rat brain. These proteins belong to category B. Phosphoproteins are identified by molecular mass in kilodaltons. The 62-, 58-, and 48-Kd phosphoproteins were found in both particulate and soluble fractions. The 160-, 140-, and 49-Kd phosphoproteins were seen clearly only in particulate preparations. B, olfactory bulb; CO, cortex; NS, neostriatum; GP, globus pallidus; A, amygdala; HI, hippocampus; TH, thalamus; HY, hypothalamus; DM, dorsal mesencephalon; MM, middle mesencephalon; VM, ventral mesencephalon; CBL, cerebellum; P/M, pons/medulla; SP, spinal cord. Higher concentrations of phosphoproteins are indicated by higher numbers of *plus signs* and higher density of *parallel lines*, with the exception of the 62 Kd* phosphoprotein, which is high(+++) in cerebellar particulate preparations.

Figure 9. Schematic illustration of the regional distribution of proteins phosphorylated by cyclic AMP-dependent protein kinase in rat brain. These proteins belong to category C. Symbols and abbreviations are the same as in the legend to Figure 8. The 260 Kd and 39 Kd phosphoproteins were found only in particulate preparations. The 98-, 96-, 81-, 40-, 32-, and 21-Kd phosphoproteins were seen only in soluble preparations.

phoproteins may be involved in physiological or pharmacological effects of dopamine mediated through dopamine-sensitive adenylate cyclase. Furthermore, the distribution of these phosphoproteins, together with that of the 39,000-dalton particulate phosphoprotein, which was phosphorylated upon addition of cyclic AMP to a particulate neostriatal preparation (Walaas et al., 1983), suggests that the proteins may belong to two groups. One group would consist of the soluble 98,000-, 96,000-, 81,000-dalton proteins and the particulate 39,000-dalton protein, all of which were concentrated in the forebrain basal ganglia (the caudatoputamen, nucleus accumbens, and olfactory tubercle), where the density of dopamine

nerve terminals is high (Björklund and Lindvall, 1978). The other group would consist of the 40,000-, 32,000-, and 21,000-dalton proteins (and possibly the faint 34,000-dalton protein), which were present not only in these forebrain regions but also in those nuclei where the main efferent tracts from these forebrain regions terminate; that is, the globus pallidus and the substantia nigra (Nauta and Domesick, 1979). The anatomical basis for the two types of distribution represents an interesting subject for future investigation. Preliminary results show, indeed, that the 32,000-dalton phosphoprotein is highly concentrated in the striatonigral neurons and fibers (S. I. Walaas, C. Ouimet, and P. Greengard, unpublished results). The regional distribution of the major soluble and particulate phosphoprotein substrates for cyclic AMP-dependent protein kinase is presented schematically in Figure 9, where the distribution pattern of these category C proteins is shown in relation to the main CNS subdivisions.

The results described in this and the preceding (Walaas et al., 1983) paper demonstrate the existence of a rich variety of phosphoproteins in the mammalian CNS. In addition, numerous other brain-specific phosphoproteins undoubtedly exist which we have been unable to study in the present investigation. (Some of the latter phosphoproteins can be seen as weak bands in the various autoradiograms.) It seems likely that certain of these brain phosphoproteins (e.g., protein I) are involved in regulating functions in most and possibly all neurons. Other phosphoproteins (e.g., those associated with the dopamine-enriched regions of the brain) presumably have functional roles in relation to specific neurotransmitter systems. Still other phosphoproteins (e.g., G-substrate (Nairn et al., 1982) and the 260,000-dalton substrate for cyclic AMP-dependent protein kinase which may be present only in cerebellar Purkinje cells (unpublished results)) appear to be associated with a single type of neuron, presumably regulating some highly specific function of such neurons. A clarification of the functional roles of these various phosphoproteins should lead to a significant increase in our understanding of the molecular basis of brain function.

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