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

I. Particulate Systems[1]

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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 particulate preparations from rat CNS. About 30 distinct phosphoproteins were observed. These 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 show large regional variations; and *category C*, phosphoproteins which show a highly restricted regional distribution. We have tentatively interpreted the results on particulate 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 particulate protein kinase activity was also examined. Calcium/calmodulin-dependent protein kinase activity had a marked regional distribution, whereas cyclic AMP-dependent protein kinase activity was more evenly distributed. Calcium/phospholipid-dependent protein kinase activity was barely detectable under the experimental conditions used.

This investigation thus demonstrates striking differences in the regional distribution of particulate 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 cytosolic protein phosphorylation systems are described in the accompanying paper (Walaas, S. I., A. C. Nairn, and P. Greengard (1983) J. Neurosci. *3:* 302-311).

Considerable evidence indicates that several neurotransmitters and neuromodulators may mediate some of their effects through the "second messengers" cyclic AMP, cyclic GMP, and calcium (for reviews, see Bloom, 1975; Nathanson, 1977; Greengard, 1978a, 1981). Other work suggests that all of the physiological actions of the cyclic nucleotides and many of the physiological actions of calcium, both in neuronal and non-neuronal cells, are mediated through regulation of various protein phosphorylation systems (for reviews, see Greengard, 1978b; Nimmo and Cohen, 1977; Krebs and Beavo, 1979). Protein kinases activated by cyclic AMP, cyclic GMP, or calcium, as well as endogenous substrates for these protein kinases, have been found in various preparations from the mammalian CNS (Miyamoto et al., 1969; Greengard and Kuo, 1970; Ueda et al., 1973; DeLorenzo, 1976; Krueger et al., 1977; Takai et al., 1977; Schulman and Greengard, 1978; Schlichter et al., 1978; DeBlas et al., 1979; Kennedy and Greengard, 1981). However, only a restricted number of brain protein kinase substrates have been characterized in any detail. Studies of two of these phosphoproteins, protein I and G-substrate, have re-

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vealed that they have strikingly different regional and subcellular distributions. Protein I, a synaptic vesicle-associated protein which is a major substrate for both cyclic AMP- and calcium/calmodulin-dependent protein kinases (Ueda and Greengard, 1977; Krueger et al., 1977; Huttner et al., 1981), appears to be present in most and possibly all nerve terminals in the mammalian brain (Goelz et al., 1981; De Camilli et al., 1980). In contrast, G-substrate, a cytosolic protein which is a substrate for cyclic GMP-dependent protein kinase (Schlichter *et al.*, 1978; Aswad and Greengard, 1981), appears to be highly concentrated in only one type of neuron; that is, cerebellar Purkinje cells (Schlichter et al., 1980; J. Detre and A. C. Nairn, unpublished observation).

The interesting differences between these two phosphoproteins have led us to undertake a more systematic investigation of the distribution of protein kinases and their substrates in the rat CNS. The present paper reports the results of a study of protein phosphorylation systems regulated by calcium and by cyclic AMP in particulate fractions prepared from various regions from rat brain. In the accompanying paper (Walaas et al., 1983), we report the results of similar studies of soluble protein phosphorylation systems.

Materials and Methods

Materials. Calmodulin was purified from bovine brain by the method of Grand et al. (1979). The inhibitor of cyclic AMP-dependent protein kinase was purified from rabbit skeletal muscle by a modification of the method of McPherson et al. (1979). Phosphatidylserine in chloroform/methanol (Sigma Chemical Co.) was dried under a stream of N_2 and resuspended in 20 mM Tris-HCl (0.5 mg/ml, pH 7.6) by sonication for 5 min at 0°C using a Sonifier Cell Disrupter (model W40). [γ-^{32}P]ATP (5 to 10 × 10^7 cpm/nmol) was prepared by the method of Glynn and Chappell (1964) from ATP (Sigma) and carrier-free [^{32}P]orthophosphate (New England Nuclear). *Staphylococcus aureus* protease V8 was obtained from Miles Biochemicals and was used in peptide mapping experiments with incomplete proteolytic digestion (Cleveland et al., 1977) as described by Huttner and Greengard (1979).

Sample dissection. Male Sprague-Dawley rats (150 to 200 gm body weight) were stunned and decapitated, and their brains and spinal cords were removed and placed in ice-cold "standard buffer" containing 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM dithiothreitol, and the protease inhibitors Trasylol (50 units/ml; Mobay Chemical Corp.), Pepstatin A (2 μg/ml; Sigma), and phenylmethylsulfonyl fluoride (0.1 mM; Sigma).

One set of brain regions was dissected from coronal slices (0.8 mm), which were prepared with a McIlwain tissue chopper after brains had been embedded in low melting temperature agar (Sigma). The slices were transferred immediately back into ice-cold standard buffer and dissected with razor blade splints, following the atlas of König and Klippel (1963). The caudatoputamen, the nucleus accumbens, the olfactory tubercle, the septum, the globus pallidus, and the amygdala were taken from the forebrain, while the diencephalon was divided into the thalamus and hypothalamus. The mesencephalon was divided into a dorsal part, consisting of the superior and posterior colliculi, a middle part which included the medial geniculate body, the periaqueductal gray, and the nucleus ruber, and a ventral part, which consisted primarily of the substantia nigra. Whole mesencephalon was used for protein kinase activity analysis, since initial experiments indicated that the different parts of this region exhibited similar cyclic AMP- and calcium-dependent protein kinase activities (not shown).

A second set of brain regions was dissected freehand. The olfactory bulb, cerebellum, and pons/medulla were freed of superficial blood vessels and put into ice-cold standard buffer. The remaining part of the brain was split along the midline, and the cortex was cut free from the diencephalon and the olfactory tubercle. The hippocampus was removed from the cortical slab and put into ice-cold standard buffer, and the caudatoputamen and the basal forebrain were "scooped" away to give a cortical preparation. In some experiments the cortical preparation was divided into neocortex and piriform cortex by a cut along the rhinal fissure. A schematic drawing of the approximate localization of the dissected samples is shown in Figure 1.

Preparation of particulate fractions. Samples from four to six animals were pooled, weighed, and homogenized in 10 vol of ice-cold standard buffer with 20 strokes in a glass-Teflon homogenizer rotating at 2,100 rpm. One- to two-milliliter aliquots were then separated into particulate and soluble fractions by centrifugation at 150,000 × *g* for 30 min in a Beckman Ultracentrifuge model L2 kept at 4°C. The supernatant was collected on ice, and the pellet was resuspended in the original volume of cold standard buffer by vigorous vortexing for 20 to 40 sec and subjected to a second centrifugation at 150,000 × *g* for 30 min. The pellet was resuspended in twice the original volume of ice-cold standard buffer by means of four to six strokes in a glass-Teflon homogenizer rotating at 900 rpm. This resuspended sample was then kept on ice until analysis. Parts of each sample were used for analysis of

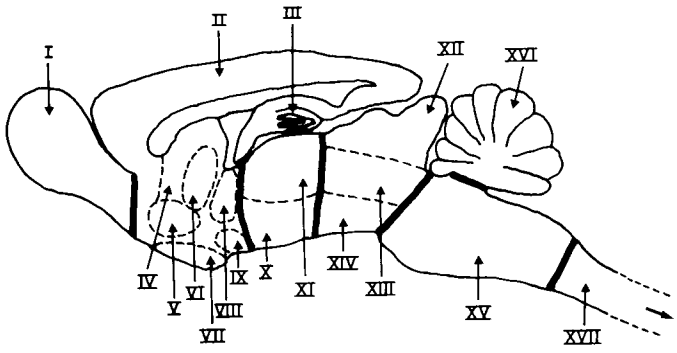

Figure 1. Schematic drawing of parasagittal section of rat CNS. *Solid lines* outline the major subdivisions of the CNS and *dashed lines* indicate the approximate localization of regions studied. *I*, olfactory bulb; *II*, neocortex; *III*, hippocampus; *IV*, caudatoputamen; *V*, nucleus accumbens; *VI*, septum; *VII*, olfactory tubercle; *VIII*, globus pallidus; *IX*, amygdala; *X*, hypothalamus; *XI*, thalamus; *XII*, dorsal mesencephalon; *XIII*, middle mesencephalon; *XIV*, ventral mesencephalon; *XV*, pons/medulla; *XVI*, cerebellum; *XVII*, spinal cord. Localization of the piriform cortex is not indicated.

protein kinase activity and for analysis of endogenous protein kinase substrates. Another aliquot of the sample 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).

Demonstration of endogenously phosphorylated proteins. Endogenous phosphorylation was assayed at 30°C in a reaction mixture (final volume 0.1 ml) containing 25 mM Tris-HCl (pH 7.4), 6 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 μM [γ-^{32}P]ATP, and 20 to 150 μg of protein, in the absence or presence of 2 μM cyclic AMP plus 1 mM IBMX (isobutylmethylxanthine; Sigma), 1.5 mM $CaCl_2$, 1.5 mM $CaCl_2$ plus 1 μg of calmodulin, or 1.5 mM $CaCl_2$ plus 5 μg of phosphatidylserine.

Addition of diacylglycerol has been found to decrease the K_a for calcium and to increase the V_{max} of the purified calcium/phospholipid-dependent protein kinase (Takai et al., 1979a). However, in agreement with results obtained on crude preparations by Kuo et al. (1980), the calcium/phosphatidylserine-dependent increase in endogenous protein phosphorylation observed in our standard preparations was not affected by addition of diacylglycerol (data not shown). Therefore, diacylglycerol was not included in our standard reaction mixture.

After 90 sec of preincubation at 30°C, the reaction was initiated by the addition of [γ-^{32}P]ATP and was terminated after 10 sec by the addition of 50 μl of SDS-containing "stop solution" (Ueda and Greengard, 1977). This incubation time was found to give optimal incorporation into most phosphoprotein bands when compared to background labeling of the interband regions. The samples were boiled immediately for 2 min, and 60- or 100-μl aliquots were subjected to discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli and Favre, 1973). The gels were fixed, stained, destained and dried, and subjected to autoradiography, as described by Ueda and Greengard (1977). The films were exposed for variable time periods, in order to demonstrate phosphoproteins with both low and high ^{32}P content. Therefore, although the relative amount of radioactivity incorporated into various protein bands can be estimated by comparing the darkness of the different protein bands within one autoradiogram, such comparisons cannot be made between different autoradiograms. When it was desirable to compare ^{32}P incorporation into protein bands on different gels, the bands were cut out from the dried gels with the autoradiograms as a guide, and ^{32}P content was quantitated by liquid scintillation spectrometry. Every radioactive band discussed in the text was subjected to peptide mapping by limited proteolysis in at least two separate experiments, using *S. aureus* protease V8 (Huttner and Greengard, 1979). These mapping studies demonstrated that all of the bands examined were protein in nature. Moreover, distinct peptide maps were observed for the individual phosphoproteins, indicating that lower molecular weight phosphoproteins were not proteolytic products of higher molecular weight phosphoproteins.

A number of experiments were carried out in which particulate preparations from various brain regions were mixed, and the protein phosphorylation patterns were compared to those seen using the individual components of the mixture. In all cases, the results indicated, both qualitatively and quantitatively, that the marked regional differences observed in protein phosphorylation were not due either to differences in amounts of protein kinases or protein phosphatases, themselves, or to differences in the amounts of various activating or inhibiting factors. However, we cannot exclude the possibility that components present in different particles were unable to interact.

Molecular weights of the endogenous phosphoproteins were estimated by comparison with the protein standards myosin (200,000 daltons), phosphorylase b (94,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (29,000 daltons), and myoglobin (17,000 daltons), which were run on the same gels. The autoradiograms presented are representative of at least 3 to 10 separate tissue preparations.

Cyclic AMP-dependent and calcium-dependent protein kinase assays. Cyclic AMP-dependent and calcium/calmodulin-dependent protein kinase activities were measured at 30°C in a reaction mixture (final volume, 0.1 ml) containing 50 mM HEPES (pH 7.4), 10 mM magnesium acetate, 1 mM EGTA, 1 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (200 to 1000 cpm/pmol), 2 to 4 μg of particulate protein, and 20 μg of protein I (purified from bovine brain (Ueda and Greengard, 1977)) as substrate. Protein I was found to be much better than any other protein tested, including a variety of histones, as substrate for cyclic AMP-dependent and calcium/calmodulin-dependent protein kinase activity (data not shown). For this reason, protein I was used as substrate for protein kinase assays. It should be emphasized that the observed distributions of protein kinase activity may not reflect the distribution of protein kinases for other substrates. Basal protein kinase activity was measured with no further additions. Cyclic AMP-dependent protein kinase activity was measured by addition of 1 μM cyclic AMP plus 1 mM IBMX. Calcium/calmodulin-dependent protein kinase activity was measured by the separate addition of 1.5 mM $CaCl_2$, or 1 μg of calmodulin, or 1.5 mM $CaCl_2$ plus 1 μg of calmodulin. The results obtained in the presence of calmodulin alone and in the presence of calcium alone were not significantly different from the basal protein kinase activities.

Calcium/phospholipid-dependent protein kinase was measured using the same basal conditions, except that 20 μg of lysine-rich histone type III (Sigma) replaced protein I as substrate. This histone has been found to be the best substrate of those tested for this kinase by Takai et al. (1979b). Basal protein kinase activity was measured with no further additions. Calcium/phospholipid-dependent protein kinase activity was measured by separate addition of 1.5 mM $CaCl_2$, or 5 μg of phosphatidylserine, or 1.5 mM $CaCl_2$ plus 5 μg of phosphatidylserine. Diacylglycerol was not included in the standard assay mixture since initial experiments indicated that it did not affect this protein kinase activity under the experimental conditions employed. The results obtained in the presence of phosphatidylserine alone and in the presence of calcium alone were not significantly different from the basal protein kinase activities (data not shown). The calcium/phospholipid-dependent protein kinase activity

is presented as the difference between the results obtained in the presence of calcium plus phosphatidylserine and those obtained in the presence of calcium alone.

For all protein kinase assays, the reaction mixture was preincubated for 60 sec at 30°C, and then the reaction was initiated by the addition of [γ-^{32}P]ATP. Incubation times of 30 and 60 sec were used for calcium/calmodulin-dependent protein I phosphorylation. Incubation times of 2 and 5 min were used for cyclic AMP-dependent protein I phosphorylation and calcium/phospholipid dependent histone phosphorylation. Under these conditions, linear rates were obtained for basal, cyclic AMP-dependent, and calcium/phospholipid-dependent protein kinase activities. Linear rates for calcium/calmodulin-dependent protein kinase activity could only be measured for up to 1 min following initiation of the reaction, irrespective of the amount of kinase used in the assay.

The reaction was terminated, in some experiments, by the addition of 20 μl of an SDS-containing stop solution and immediate boiling for 2 min, followed by SDS-PAGE as described above. The dried gels were subjected to autoradiography, and the radioactive bands were cut from the gel and counted using liquid scintillation spectrometry. The reaction was terminated in other experiments by addition of 10 μl of 250 mM EDTA (pH 7.0), immediately following which 25- to 50-μl aliquots were applied to 1 × 3 cm strips of P 81 phosphocellulose paper (Whatman), and the papers were washed by the continuous flow of water for 20 to 30 min. Protein I, as well as histone (for which this method had previously been used (Witt and Roskoski, 1975)), was found to be retained quantitatively by phosphocellulose paper, presumably because of its highly positive charge at neutral pH. Following washing, the paper strips were dried and ^{32}P incorporation was determined by liquid scintillation spectrometry. The two methods gave similar results.

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

In most cases, the nature of the protein substrates for the various protein kinases is as yet unknown. For convenience, we have designated these proteins in terms of their molecular weight values based on SDS-PAGE. However, we wish to emphasize that an individual phosphoprotein band may represent more than one protein, that bands of similar molecular weight from different sources may represent different proteins, and that the phosphorylation of any given phosphoprotein may be due to one or to more than one protein kinase. These caveats are applicable both to this and to the accompanying (Walaas et al., 1983) paper.

Results

Cyclic AMP-dependent protein phosphorylation

Category A phosphoproteins. Addition of cyclic AMP plus IBMX stimulated the phosphorylation of 280,000-, 86,000-, 80,000-, 74,000-, 66,000-, and 55,000-dalton phosphoproteins in all regions examined. Results for cortex, cerebellum, and caudatoputamen are shown in Figure 2. Considerable information is available about the identity of certain of these proteins (Lohmann et al., 1980). Thus, the 280,000-dalton phosphoprotein is believed to represent the microtubule-associated protein MAP_2 (Sloboda et al., 1975; Vallee, 1980; Lohmann et al., 1980). On gels containing 6% rather than 7% acrylamide, this band was resolved into two closely spaced phosphoprotein bands in all brain regions except the olfactory bulb, where only one band was seen (data not shown). The 86,000/80,000-dalton phosphoprotein doublet represents protein I. One-dimensional peptide mapping of the 74,000-dalton protein with *S. aureus* protease V8 indicated that this protein probably represents protein IIIa (Huang et al., 1982). The 55,000-dalton phosphoprotein band partly represents the regulatory subunit of type II cyclic AMP-dependent protein kinase (Lohmann et al., 1980). However, one-dimensional peptide mapping of the 55,000-dalton band with *S. aureus* V8 protease indicated that a second phosphoprotein contained in this band is protein IIIb, a neuronal phosphoprotein which recently has been extensively purified and characterized (Huang et al., 1982): the proteolytic digest contained a major phosphopeptide fragment with an M_r of 18,000, as has also been found for purified protein IIIb (Huang et al., 1982).

Category B phosphoproteins. Category B phosphoproteins consisted of a 265,000-dalton phosphoprotein and of a major 52,000-dalton phosphoprotein which probably is identical to protein IIb (Lohmann et al., 1978). Both of these proteins were prominent in the cortex (Fig. 2*B*) and in most subcortical forebrain regions (not shown), but were almost absent from the cerebellum (Fig. 2*B*), pons/medulla, and spinal cord (not shown).

Category C phosphoproteins. The cerebellum contained a major 260,000-dalton phosphoprotein which was not seen in any other brain region examined (Fig. 2*B*). This band co-migrated with a Coomassie blue-stained protein band which also was restricted to the cerebellum. This 260,000-dalton phosphoprotein did not co-migrate on gels containing 6% acrylamide with the category B 265,000-dalton phosphoprotein described above (data not shown). The caudatoputamen (Fig. 2*C*), nucleus accumbens, and olfactory tubercle, that is, the dopamine-innervated dorsal and ventral parts of the neostriatum (Heimer, 1978; Björklund and Lindvall, 1978; Nauta, 1979), but no other region examined, contained a 39,000-dalton category C phosphoprotein.

In some experiments we studied the effect of 2μM cyclic GMP on protein phosphorylation. A slight stimulation of the phosphorylation of some proteins, including protein I, was observed (not shown). The molecular weight of the phosphoproteins and the very slight increase in phosphorylation observed indicated, however, that this concentration of cyclic GMP induced a partial activation of cyclic AMP-dependent protein kinase, since no specific cyclic GMP-stimulated phosphorylation was seen. Previous work (Schlichter et al., 1980) has, in fact, shown that cyclic GMP-dependent protein kinase activity is extremely low in most regions of the rodent brain. Therefore, studies with cyclic GMP were not continued.

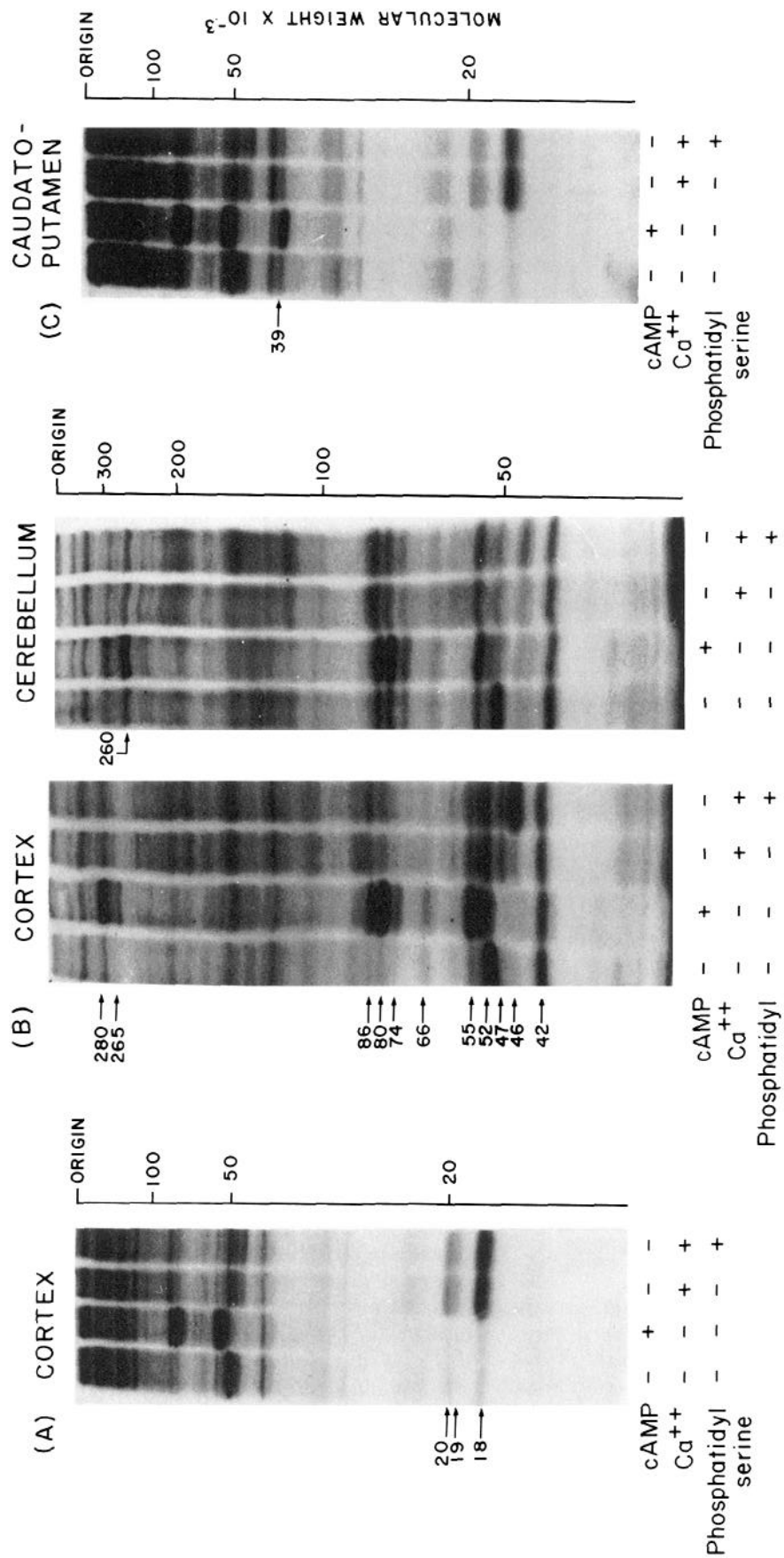

Figure 2. Autoradiogram illustrating cyclic AMP-dependent and calcium-dependent phosphorylation of particulate proteins from rat cerebral cortex, cerebellum, and caudatoputamen. Aliquots containing 100 μg of protein were incubated under standard assay conditions in the absence or presence of either 2 μM cyclic AMP plus 1 mM IBMX, 1.5 mM $CaCl_2$, or 1.5 mM $CaCl_2$ plus 50 μg/ml of phosphatidylserine. Aliquots containing 50 μg of protein were separated by SDS-PAGE, and the phosphoproteins were visualized by autoradiography. *A*, Cortical phosphoproteins separated on 12% polyacrylamide gels, illustrating low molecular weight proteins. *B*, Cortical and cerebellar phosphoproteins separated on 7% polyacrylamide gels, illustrating high molecular weight phosphoproteins. *C*, Caudatoputamen phosphoproteins separated on 12% polyacrylamide gels, illustrating low molecular weight phosphoproteins. *Arrows* indicate major phosphoproteins described in the text.

Calcium-dependent protein phosphorylation

Category A phosphoproteins. Addition of calcium alone greatly stimulated the phosphorylation of 20,000-, 19,000-, and 18,000-dalton phosphoproteins in all CNS regions (e.g., Fig. 2, *A* and *C*). These phosphoproteins may represent myelin basic protein (Sulakhe et al., 1980; Agrawal et al., 1982). An 87,000-dalton protein whose phosphorylation was increased by the addition of calcium was also a category A phosphoprotein (Fig. 2).

Category B phosphoproteins. A 47,000-dalton protein, whose phosphorylation seemed totally dependent on the presence of calcium, was seen in all parts of the forebrain (Fig. 2) but was less concentrated in the diencephalon and ventral mesencephalon and appeared to be absent from the dorsal mesencephalon, pons/medulla, cerebellum (Fig. 2*B*), and spinal cord. Calcium also caused a variable increase in ^{32}P incorporation into a number of other, less well defined bands between 100,000 and 250,000 daltons (Fig. 2*B*). These phosphoproteins appeared to be different in cortex and cerebellum and may represent category B phosphoproteins.

Category C phosphoproteins. None were observed.

Further work will be required to determine whether the stimulation of protein phosphorylation observed by the addition of calcium alone to these particulate preparations is mediated through a calcium/calmodulin-dependent system, a calcium/phospholipid-dependent system, or some other as yet uncharacterized calcium-dependent system. However, a comparison with soluble preparations (Walaas et al., 1983) suggests an involvement of calcium/phospholipid-dependent protein kinase in several of these phosphorylation reactions.

Calcium/phospholipid-dependent protein phosphorylation

Category A phosphoproteins. None were observed.

Category B phosphoproteins. A 46,000-dalton phosphoprotein was observed in the cortex, cerebellum (Fig. 2*B*), and in most telencephalic regions except the globus pallidus, but could not be found in the diencephalon, mesencephalon, pons/medulla, or spinal cord. Calcium plus phosphatidylserine also caused a slight and inconsistent stimulation of the phosphorylation of some category B phosphoproteins between M_r 100,000 and 200,000 (Fig. 2*B*).

Category C phosphoproteins. None were observed.

Calcium/calmodulin-dependent protein phosphorylation

Addition of calmodulin together with calcium greatly stimulated the phosphorylation of a number of protein bands. In cortical samples, 10 to 15 times more ^{32}P was usually incorporated into total protein in the combined presence of these cofactors than in the presence of cyclic AMP, a difference compatible with the difference in protein kinase activities (see below). This difference made it necessary to analyze calmodulin-stimulated protein phosphorylation on separate gels. Figure 3 demonstrates the dramatic effect of calmodulin on ^{32}P incorporation into various proteins in calcium-containing medium using particulate preparations from 17 different regions of the CNS.

Category A phosphoproteins. Phosphorylation of the 86,000/80,000-dalton phosphoprotein doublet (protein I) was stimulated by calmodulin in all regions examined (Fig. 3).

Category B phosphoproteins. The 160,000-, 140,000-, and 48,000-dalton phosphoprotein bands (Fig. 3) were concentrated in cortical regions, including the hippocampus, and were also present in subcortical forebrain regions, but were less prominent in the thalamus, hypothalamus, and mesencephalon, and appeared as minor phosphoproteins in the cerebellum, pons/medulla, and spinal cord. A 62,000-dalton phosphoprotein had a distribution identical to that of these three phosphoproteins, with the exception that this band also was prominent in the cerebellum (Fig. 3). A 58,000-dalton phosphoprotein was concentrated in the hypothalamus, the dorsal and middle mesencephalon, the pons/medulla, cerebellum, and spinal cord. A 49,000-dalton phosphoprotein was found in the thalamus, hypothalamus, the dorsal and middle mesencephalon, and faintly in the pons/medulla, but not in the cerebellum.

Category C phosphoproteins. The 200,000- and 180,000-dalton phosphoproteins were seen exclusively in the cerebellum (Fig. 3).

Basal protein phosphorylation

A number of phosphoproteins whose phosphorylation was stimulated by cyclic AMP were found to be partially phosphorylated under basal conditions. This basal phosphorylation was most prominent in certain brain regions, for example, cerebellum (Fig. 2), pons/medulla, and spinal cord (not shown), and resulted in apparently unequal regional distributions of these proteins. Addition of the purified inhibitor of cyclic AMP-dependent protein kinase abolished the basal phosphorylation of these proteins (not shown), indicating that it resulted from a partial activation of cyclic AMP-dependent protein kinase. The variations in the amounts of these phosphoproteins in different brain regions may have been due to regional variations in postmortem cyclic AMP levels (Schmidt et al., 1971). In support of this interpretation, the regional distribution of these phosphoproteins correlated with the basal phosphorylation of protein I, a well documented substrate for cyclic AMP-dependent protein kinase (Ueda and Greengard, 1977; Huttner et al., 1981). (The only exception to this pattern was the 260,000-dalton category C phosphoprotein specific to the cerebellum.)

Several other phosphoproteins were observed when particulate fractions were incubated under basal conditions in the presence of the purified inhibitor of cyclic AMP-dependent protein kinase. These phosphoproteins were similar in all brain regions. They, therefore, belong to category A. Some of these phosphoproteins, particularly the 50,000- and 42,000-dalton phosphoprotein bands, incorporated markedly less ^{32}P when IBMX or calcium was included in the medium (Fig. 2). Based on the apparent molecular weight and on the decreased ^{32}P incorporation seen in the presence of calcium (Fig. 2, *A* and *B*), the 42,000-dalton band probably represents the α subunit of pyruvate dehydrogenase (Denton et al., 1972; Browning et al., 1981).

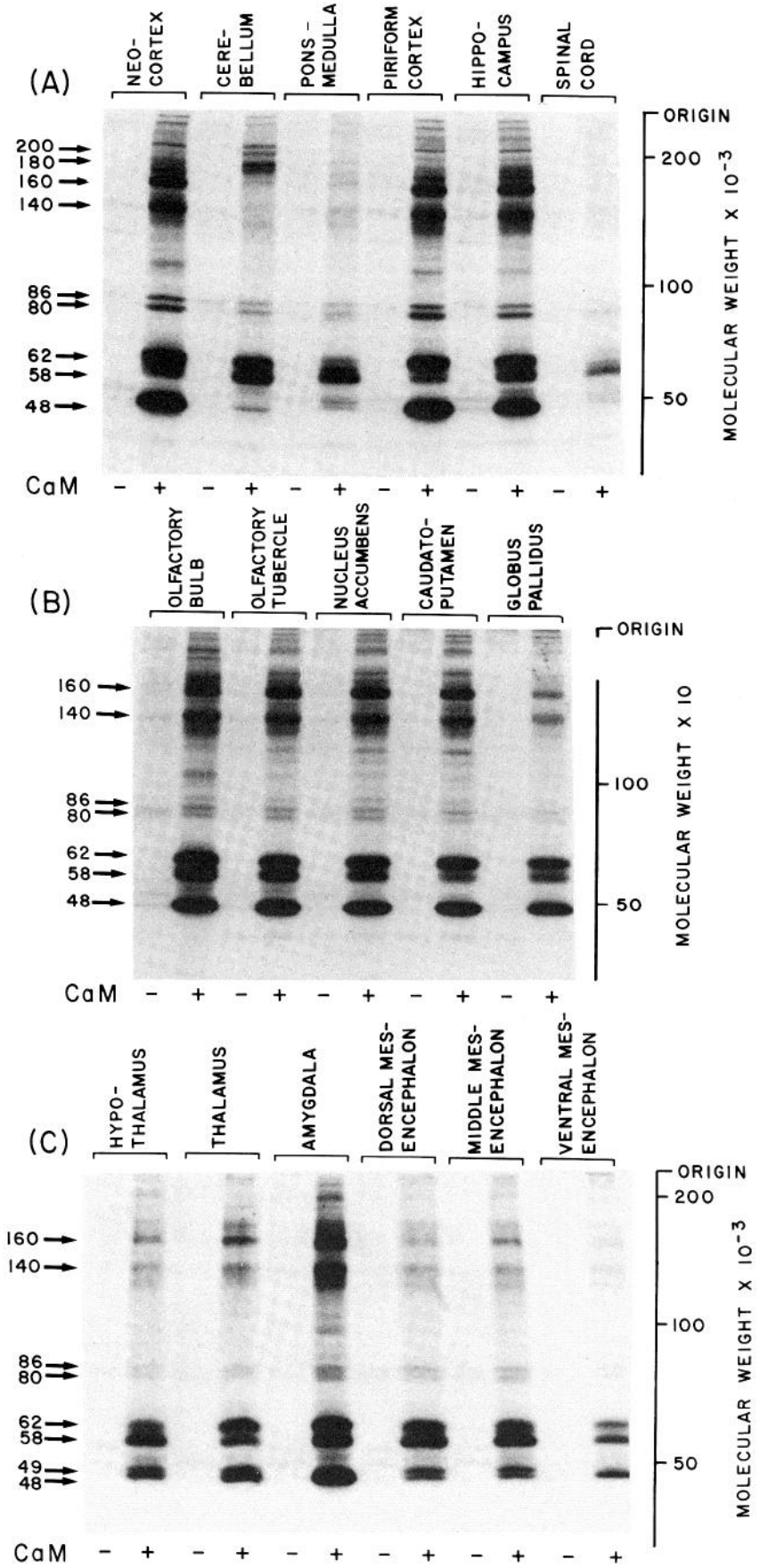

Figure 3. Autoradiogram illustrating calcium/calmodulin-dependent phosphorylation of particulate proteins in various brain regions. Aliquots containing 100 μg of protein were incubated under standard assay conditions in the presence of 1.5 mM $CaCl_2$, in the absence or presence of 10 μg/ml of calmodulin (*CaM*) as indicated. Aliquots containing 50 μg of protein were separated by SDS-PAGE on 8% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. *Arrows* indicate major phosphoproteins described in the text.

Comparison of endogenous protein phosphorylation in brain and non-nervous tissues

Particulate or soluble fractions prepared by our standard procedure from non-nervous tissues including skeletal muscle, heart, liver, kidney, and anterior pituitary showed much lower calcium-stimulated and cyclic AMP-stimulated phosphorylation of endogenous proteins than did preparations from various brain regions. Moreover, phosphoproteins in peripheral tissues appeared in virtually all cases to be distinct from the major phosphoproteins in the brain samples (data not shown). Therefore, it would appear that most of the brain phosphoproteins described in the present study are greatly enriched in, and may be specific to, the nervous system.

Cyclic AMP-dependent and calcium-dependent protein kinase activities

Comparison of cyclic AMP-dependent protein kinase activities in particulate preparations from various regions of the CNS showed a rather even distribution in the different brain regions (Table I). However, regions with a high content of white matter such as the pons/medulla (Table I) and spinal cord (not shown) displayed low cyclic AMP-dependent protein kinase activity.

A large stimulation of protein kinase activity was observed when samples were assayed in the presence of calcium plus calmodulin (Table I). The highest activities were observed in the cortical regions and the lowest in the brain stem. Qualitatively, the distribution of calcium/calmodulin-dependent protein kinase activity paralleled the regional distribution of the category B 160,000-, 140,000-, and 48,000-dalton phosphoproteins which were endogenously phosphorylated upon addition of calcium plus calmodulin.

TABLE I

Regional distribution of particulate 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 ± SEM. Protein I (20 μg) 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 μM 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 μg/ml of calmodulin.

Region	N^a	Basal Activity	Cyclic AMP-dependent Activity	Calcium/calmodulin-dependent Activity
		nmol/min/mg protein		
Neocortex	3	0.15 ± 0.08	0.96 ± 0.15	8.2 ± 0.7
Hippocampus	4	0.39 ± 0.05	0.78 ± 0.13	10.9 ± 0.8
Amygdala	3	0.21 ± 0.04	0.77 ± 0.22	6.5 ± 1.9
Olfactory bulb	3	0.43 ± 0.06	0.90 ± 0.16	4.6 ± 0.3
Nucleus accumbens	3	0.25 ± 0.08	1.2 ± 0.6	4.7 ± 1.3
Caudatoputamen	3	0.36 ± 0.04	1.2 ± 0.3	5.8 ± 0.8
Septum	2	0.22 ± 0.02	1.0 ± 0.1	5.8 ± 0.2
Globus pallidus	4	0.25 ± 0.07	0.58 ± 0.15	2.4 ± 0.3
Thalamus	2	0.37 ± 0.01	1.1 ± 0.2	3.6 ± 0.7
Hypothalamus	3	0.25 ± 0.08	0.60 ± 0.14	2.2 ± 0.6
Mesencephalon	4	0.33 ± 0.05	0.83 ± 0.16	2.3 ± 0.5
Cerebellum	4	0.43 ± 0.10	0.65 ± 0.09	3.2 ± 0.3
Pons/medulla	3	0.20 ± 0.10	0.42 ± 0.04	1.5 ± 0.3

[a] *N*, number of pooled tissue preparations examined.

Calcium/calmodulin-stimulated phosphorylation of protein I in brain is catalyzed by at least two different protein kinases, which incorporate phosphate into two regions of the protein I molecule, designated the "10,000-dalton region" and the "30,000-dalton region," respectively (Kennedy and Greengard, 1981). In the present study, peptide map analysis of phosphorylated endogenous protein I, observed in particulate fractions incubated in the presence of calcium/calmodulin (e.g., Fig. 3), or of exogenously added protein I, used in the calcium/calmodulin-dependent protein kinase activity measurements, showed that only the 30,000-dalton region of the protein I molecule had been phosphorylated (data not shown). This was also found to be the case in experiments with soluble preparations of calcium/calmodulin-dependent protein kinase described in the accompanying paper (Walaas et al., 1983). Thus, the calcium/calmodulin-dependent protein kinase activity we describe appears to be due primarily to the enzyme which catalyzes the phosphorylation of the 30,000-dalton region of protein I.

A small but statistically significant stimulation of histone III phosphorylation could be detected in the presence of phosphatidylserine plus calcium compared to calcium alone. This activity, which was not affected by the addition of diacylglycerol, ranged from 0.02 to 0.06 nmol/min/mg and represented about 1 to 2% of that obtained in comparable soluble preparations (Walaas et al., 1983). This protein kinase activity did not display a distinct regional distribution in our particulate preparations (not shown).

Discussion

The particulate protein phosphorylation systems in mammalian brain which are regulated by cyclic AMP or by calcium can be conveniently divided into three categories according to their patterns of regional distribution. *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. Further, our results indicate that the calcium/calmodulin-stimulated system dominates quantitatively, regarding both protein kinase activity and the number of substrate proteins observed, in these particulate preparations. The cyclic AMP-stimulated system is less active, and the systems stimulated by calcium or by calcium plus phospholipid are relatively inactive in our EDTA-treated particulate preparations.

The category A distribution observed for protein I, MAP_2, and the 20,000-, 19,000-, and 18,000-dalton proteins which may represent myelin basic protein is in agreement with results obtained by other methods. Thus, the distribution of protein I is similar to the regional distribution found by radioimmunoassay (Goelz et al., 1981). The widespread presence of MAP_2 agrees with the widespread distribution of this microtubule-bound protein throughout the CNS (P. De Camilli, personal communication). The ubiquitous presence of myelin basic protein similarly fits with the distribution of myelin-generating oligodendrocytes throughout the CNS (Norton, 1976).

The widespread distribution of category A phosphoproteins throughout the nervous system can be explained

by assuming that these phosphoproteins are present in most or all neurons and/or in glial cells. The particulate category B phosphoproteins, in contrast, have striking regional differences. Most of the observed category B phosphoproteins are substrates for calcium/calmodulin-stimulated protein kinases, and certain of them appear to be concentrated in developmentally distinct parts of the CNS. For example, the 160,000-, 140,000-, 62,000-, and 48,000-dalton phosphoproteins are clearly concentrated in the forebrain and in regions with important connections to the forebrain, such as the substantia nigra in the ventral mesencephalon (data not shown). In contrast, the 49,000-dalton protein is concentrated in the upper brain stem, while the 58,000-dalton protein appears to be concentrated in the cerebellum, in parts of the brain stem, and in the spinal cord.

Particulate category C phosphoproteins were observed in two locations only. The cerebellum contains a major 260,000-dalton substrate for cyclic AMP-dependent protein kinase and two relatively minor 180,000- and 200,000-dalton substrates for calcium/calmodulin-dependent protein kinase, while the neostriatum (caudatoputamen, nucleus accumbens, and olfactory tubercle) contains a 39,000-dalton substrate for the cyclic AMP-dependent protein kinase. It is interesting to note that the cerebellum and the neostriatum are among the brain regions where strong evidence links neurotransmitter (norepinephrine and dopamine) actions to cyclic AMP synthesis (Bloom, 1975; Siggins et al., 1974; Kebabian, 1977). Some of these substrates for cyclic AMP-dependent protein kinase might, therefore, be involved in norepinephrine or dopamine actions.

A considerable number of distinct phosphoproteins with different patterns of regional distribution in the rat CNS were found in this study. There is a variety of reasons to believe that these phosphoproteins represent only a portion of the total number of brain-specific phosphoproteins: certain proteins are probably present in concentrations too low to be detected by the experimental procedures which we have employed, and other phosphoproteins may be hidden by more prominent bands with similar molecular weight values; some phosphoproteins may be particularly effective substrates for endogenous protein phosphatases; some phosphoproteins might be present in our preparations in a fully phosphorylated form, so that incorporation of ^{32}P *in vitro* would be impossible.

In support of the conclusion that some phosphoproteins would not be found by the procedures used, the well known phosphoprotein tyrosine hydroxylase (Joh et al., 1978) was not found in either the particulate or the soluble fractions from the caudatoputamen and was only detected following partial purification from a detergent-extracted preparation (data not shown). Similarly, several other phosphoproteins studied in our own laboratory are observed only after appropriate purification procedures. Such purification procedures include isolation of the subcellular organelle in which the phosphoprotein is located (Ueda et al., 1973), acid extraction of the phosphoprotein of interest from total homogenates (Forn and Greengard, 1978), and immunoprecipitation of the phosphoprotein of interest from detergent-solubilized tissue extract (Nestler and Greengard, 1980).

The fractionation procedures used can also separate kinases and/or activators from their substrates. As one example, although a considerable amount of protein kinase activity activatable by calcium plus calmodulin is retained in particulate fractions prepared without calcium (this paper), most of the calmodulin is removed from the membranes by this treatment (Watterson et al., 1976; Sobue et al., 1981). Addition of calcium plus calmodulin is apparently sufficient to maximally phosphorylate several particulate proteins which are known to be substrates for the calcium/calmodulin-stimulated system (this paper; Schulman and Greengard, 1978).

Most of the calcium/phospholipid-dependent protein kinase was released into the soluble fraction by our procedure (Walaas et al., 1983). However, incubation of total homogenates from several regions with calcium plus phosphatidylserine did not reveal major phosphoproteins in addition to those already observed in the particulate or soluble fractions incubated under the same conditions (data not shown). It is, therefore, unlikely that major particulate substrates for this enzyme were overlooked as a result of the fractionation procedure.

In conclusion, the phosphoproteins seen in the particulate (this paper) and in the soluble (Walaas et al., 1983) fractions probably represent the quantitatively major phosphoprotein systems and their regional distribution in brain. The particulate phosphoproteins are further compared to the soluble phosphoproteins and their regional distribution patterns in the accompanying paper (Walaas et al., 1983).

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