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Neuronal Protein Phosphorylation: Recent Studies Concerning Protein I, A Synapse-Specific Phosphoprotein

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DOLPHIN, A. C., S. E. GOELZ AND P. GREENGARD. *Neuronal protein phosphorylation: Recent studies concerning Protein I, a synapse-specific phosphoprotein.* PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 169–174, 1980.—Recent studies are described concerning the physiological role and tissue distribution of Protein I, a synapse-specific phosphoprotein. This protein is present in both the central and the peripheral nervous system and appears to be enriched in synaptic vesicles. It is a major substrate for both Ca^{2+}- and cyclic AMP-dependent phosphorylation in the brain. In slices of the facial motor nucleus from rat brainstem, serotonin (5-HT) added to the incubation medium stimulates the phosphorylation of Protein I. This effect is potentiated by low concentrations of isobutylmethylxanthine and is prevented by the serotonin antagonist mianserin.

Brain Protein phosphorylation Protein I Cyclic AMP Serotonin

WORK by many investigators has led to the hypothesis that protein phosphorylation is involved in mediating not only the actions of cyclic AMP and those hormones and neurotransmitters whose effects are acheived through altering cyclic AMP levels, but the actions of a large number of other regulatory agents as well (Fig. 1). In nervous tissue alone, three distinct classes of protein kinases have been discovered, activated by the three known types of intracellular second messengers, namely cyclic AMP, cyclic GMP and calcium [5, 7, 8, 10]. To varying degrees these protein kinases have been purified and characterized. Moreover, for each of these three classes of protein kinases several endogenous substrate proteins have been found ([9, 10, 13] D. W. Aswad and P. Greengard, unpublished results). The results of recent studies of one of these substrate proteins will be described here. This is a very prominent substrate not only for cyclic AMP-dependent protein kinase, but also for calcium-dependent protein kinases [4,13], and is referred to as Protein I. In the experiment illustrated in Fig. 2 (modified from ref. [11]), a synaptic membrane fraction was incubated with [γ-^{32}P]ATP in the absence or presence of cyclic AMP. The reaction was then terminated by the addition of the detergent sodium dodecyl sulfate (SDS) which also solubilized the membrane proteins. These proteins were then separated from one another by SDS-polyacrylamide gel electrophoresis and were analyzed by protein staining and autoradiography. Of the many protein bands present in this synaptic fraction, it can be seen that the phosphorylation of only a few was markedly affected by the presence of cyclic AMP. Two of these proteins, referred to as Proteins Ia and Ib, have very similar properties and this doublet is collectively called Protein I. Protein I has been purified to homogeneity from bovine brain by Tetsufumi Ueda [14]. Proteins Ia and Ib maintained a constant molar proportion of 1:2 throughout purification to homogeneity. Both polypeptides are extremely basic, having isoelectric points of greater than 10, and they have an unusual chemical structure. A variety of physico-chemical studies indicate that each of the polypeptides has a globular region which is insensitive to collagenase as well as an elongated proline-rich tail which is rapidly degraded even by highly purified collagenase [14].

Our knowledge of the tissue distribution of Protein I is summarized in Table 1. Recently, a sensitive and precise radioimmunoassay has been developed for determining the amounts of Protein I in different tissues (S. E. Goelz, unpublished data). The application of this radioimmunoassay to a study of the amount of Protein I in various regions of the nervous system is presented in Table 2. The preliminary results of a subcellular fractionation study of rat cerebral cortex (W. B. Huttner, P. De Camilli, S. E. Goelz and P. Greengard, unpublished experiment) suggest that Protein I makes up approximately 0.5% of the total protein present in a rat cortical homogenate. From these preliminary results it appears that about 4% of the total protein of the crude synaptic vesicle fraction can be accounted for by Protein I. This high concentration of Protein I in the vesicle fraction supports the idea that Protein I is important in some aspect of the function of synaptic vesicles.

Several studies have been concerned with investigating the state of phosphorylation of Protein I under physiological conditions [2,12]. A similar approach has been used in all the studies to be described, that is to extract Protein I from brain tissue in the presence of 5 mM Zn^{2+}, a procedure which inhibits phosphatase activity, thereby allowing the proportion of Protein I which is present in the phosphorylated form to be estimated. This is performed by "back phosphoryla-

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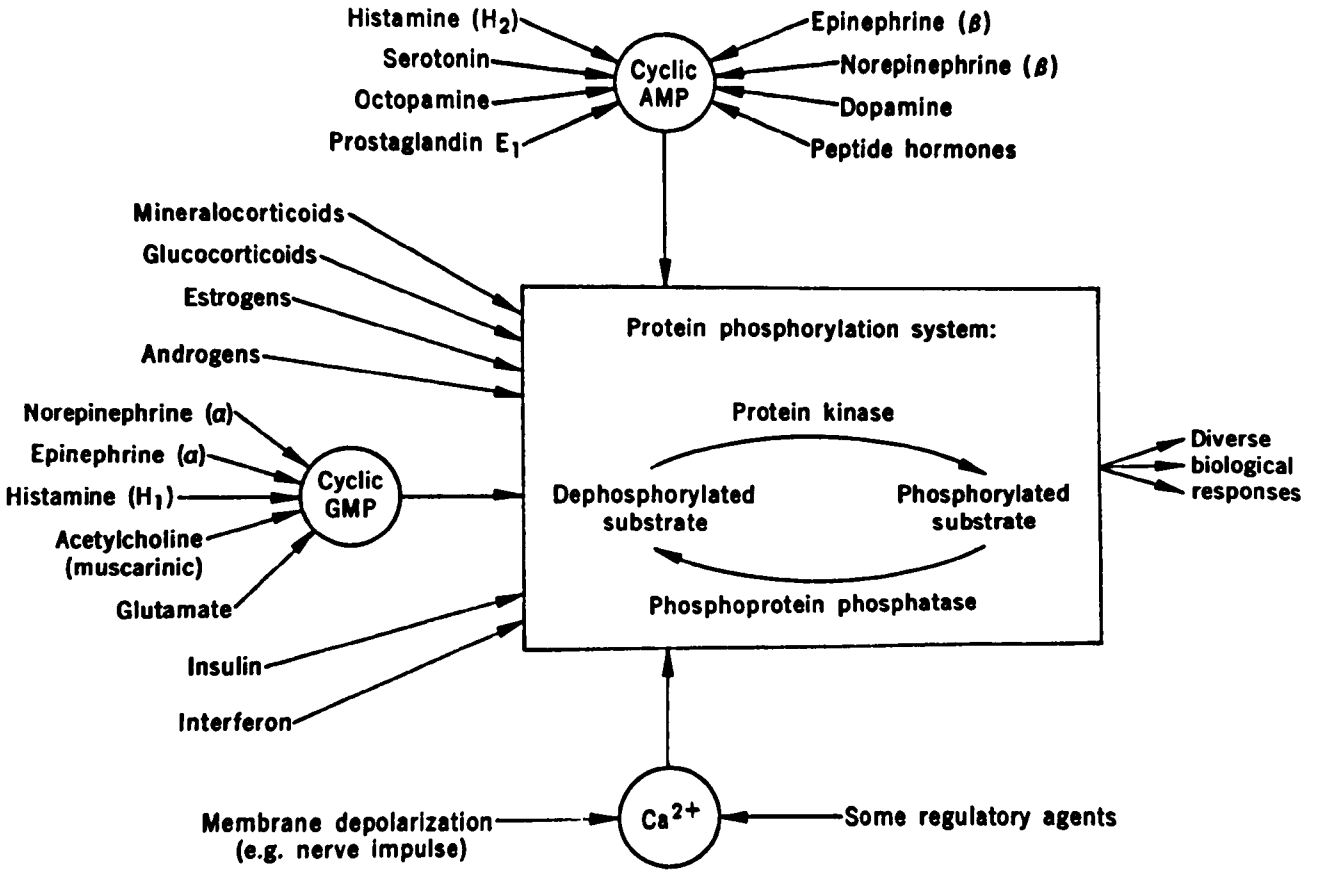

FIG. 1. Schematic diagram of postulated role played by protein phosphorylation in mediating some of the biological effects of a variety of regulatory agents. The diagram gives examples of regulatory agents, some of whose effects may be mediated through regulation of the phosphorylation of specific proteins, and is not intended to be complete. In addition to cyclic AMP and a variety of neurotransmitters and hormones whose effects are mediated through cyclic AMP, these regulatory agents include cyclic GMP, a variety of neurotransmitters and hormones whose effects are mediated through cyclic GMP, Ca^{2+}, and agents whose effects are mediated through Ca^{2+}, as well as several classes of steroid hormones, insulin, and inteferon. For brevity, numerous peptide hormones whose effects are known to be mediated through cyclic AMP, and the various regulatory agents believed to act through translocation of Ca^{2+}, are not listed individually. It seems likely that some, but not necessarily all, of the biological responses elicited by any given regulatory agent are mediated through the protein phosphorylation system.

tion" of the dephosphorylated form using either cyclic AMP-dependent protein kinase in the presence of cyclic AMP or the catalytic subunit of cyclic AMP-dependent protein kinase, with [γ-^{32}P]ATP as substrate. In all these studies, a sufficient amount of kinase has been used (unpublished results) to phosphorylate only phosphorylation site 1 on Protein I [3]. This is the only peptide whose phosphorylation appears to be physiologically regulated by cyclic AMP as well as by Ca^{2+} [3]. In brain *in vivo*, it would appear that Protein I exists partially (about 33%) in the phosphorylated form, and partially (about 67%) in the dephosphorylated form on site 1 [12]. In contrast, in slices of rat cerebral cortex incubated *in vitro* under resting conditions, virtually all of the Protein I exists in the dephosphorylated form on site 1; less than 1% of Protein I would appear to be in the phosphorylated form under these circumstances. Incubation of cerebral cortex slices for 30 sec in 60 mM potassium resulted in approximately two-thirds of Protein I being con-

TABLE 1

DISTRIBUTION OF PROTEIN I

A)	Present only in nervous system (both central and peripheral)
B)	Within nervous system, present only in neurons
C)	Within neurons, concentrated in synaptic region
D)	Within synaptic region, present primarily in synaptic vesicles
E)	Present at most, and possibly at all, synapses
F)	Appears simultaneously with synapse formation during development

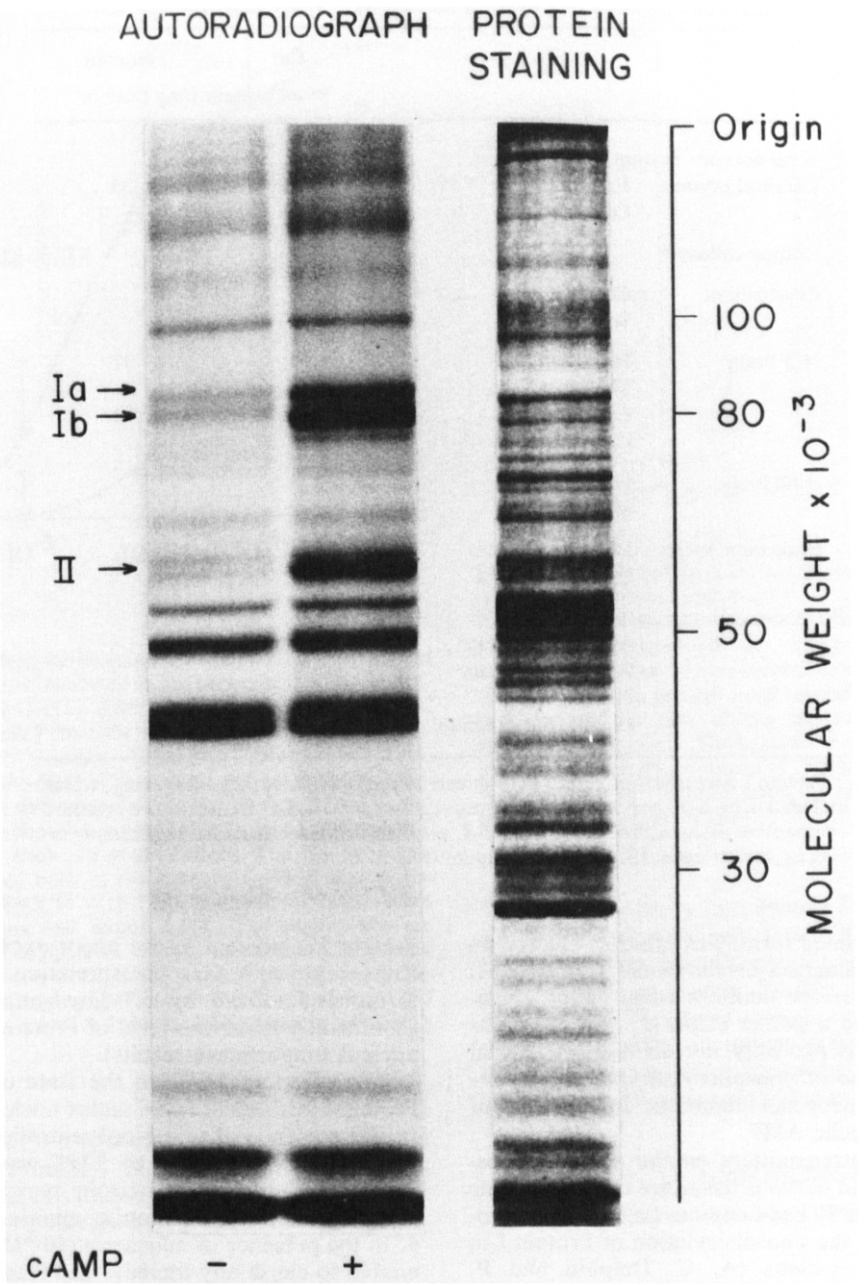

FIG. 2. Effect of cyclic AMP on endogenous protein phosphorylation in a synaptic membrane fraction from rat caudate nucleus. The synaptic membrane fraction was incubated with 7 μM [γ-^{32}P]ATP for 15 seconds at 30°C in the absence (−) or presence (+) of 10 μM cyclic AMP. The reaction was terminated by the addition of SDS, and the mixture was then subjected to SDS-polyacrylamide gel electrophoresis in order to separate the membrane proteins from one another. The separated proteins were located in the gel by standard procedures of protein staining. Autoradiography was then carried out to determine those protein bands into which radioactive phosphate had become incorporaated. (Left) Phosphorylation of endogenous protein in the absence or presence of cyclic AMP; (right) protein staining, (Modified from ref. [11]).

TABLE 2
PROTEIN I LEVELS IN VARIOUS REGIONS OF THE NERVOUS SYSTEM

		Cat	Human
		pmol protein I/mg protein	
Central nervous system			
Cerebral cortex:	Frontal	51	33
	Occipital	36	
Corpus callosum		4	
Cerebellum:	Cortex	28	
	White matter	2	
Mid brain:	Hippocampus	54	37
	Thalamus	50	
	Caudate	31	31
	Hypothalamus	32	
Hind brain:	Pons	5	
	Medulla	7	
Brainstem nuclei:	Locus coeruleus	14	
	Substantia nigra	30	
Peripheral nervous system			
	Anterior pituitary	0	
	Posterior pituitary	9	
	Pineal	2	
	Dorsal root ganglion	0.3	

Protein I concentrations were determined by radioimmunoassay. The assay, which included 0.1% SDS and 0.5% NP40 to prevent nonspecific adherence, was a standard competitive assay utilizing ^{125}I-Protein I, purified Protein I standards and *Staphylococcus aureus* cells. (S. E. Goelz, unpublished results.)

verted to the phosphorylated form. Ten minutes incubation with the cyclic AMP analogue 8-bromo-cyclic AMP (4 μM) or with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; 1 mM) had a similar effect [2]. These results indicate that Protein I is probably not an inert structural protein, but that its state of phosphorylation is rapidly attuned to the state of the neuronal membrane and the level of intracellular Ca^{2+} and cyclic AMP.

The effects of neurotransmitters on the state of phosphorylation of Protein I in nervous tissue are currently being investigated. It has recently been demonstrated that serotonin (5-HT) brings about the phosphorylation of Protein I in slices of facial motor nucleus (A. C. Dolphin and P. Greengard, manuscript in preparation). This is a relatively homogeneous brainstem nucleus of the central nervous system which has been studied extensively by Dr. George K. Aghajanian. Virtually the only neuronal cell body present in the facial motor nucleus is that of the motor neuron that sends axons to innervate the facial musculature [6]. The nucleus appears to be devoid of any interneurons, which greatly simplifies the task of analysis [6]. There are two major types of input onto the motor neurons. The major afferent input consists of excitatory fibers which are believed to utilize an amino acid, possibly glutamate, as their neurotransmitter. A minor input (approximately 2% of the afferent fibers) is composed of serotonergic nerve fibers from the nucleus raphé magnus [6]. Cytochemical studies (unpublished results in collaboration with Dr. P. De Camilli) have suggested that almost all of the cytochemically demonstrable Protein I is present in the major excitatory afferent input. Consistent with this interpretation, destruction of the serotonergic fibers by 5,7-dihydroxytryptamine does not significantly lower the level of Protein I in the facial motor nucleus (unpublished results).

The effect of 5-HT on the state of phosphorylation of Protein I in slices of facial motor nucleus is shown in Fig. 3. In the presence of a low concentration of IBMX (4×10^{-5} M), the maximal effect of 5-HT was to convert approximately 30% of Protein I to the phosphorylated form. The effects of the 5-HT antagonist, mianserin, are shown in Fig. 4. In the presence of mianserin (10^{-5}M), 5-HT (10^{-4} M) was unable to cause any increase in Protein I phosphorylation. However, mianserin alone produced a slight phosphorylation of Protein I, which may possibly be accounted for by its previously described partial agonist characteristics on cerebral 5-HT stimulated adenylate cyclase [1].

We tentatively interpret the results obtained with 5-HT in the facial motor nucleus as indicating that 5-HT is altering the state of phosphorylation of Protein I in the afferent terminals. This effect of 5-HT may reflect the functional equivalent of an axo-axonic communication between the serotonergic fibers and the main afferent excitatory pathway. However, we cannot rule out the possibility, at the present time, that 5-HT is affecting the state of phosphorylation of Protein I in the motor neuron somata, even though cytochemical evidence suggests that the concentration of Protein I is low in neuronal cell bodies.

Since both calcium and cyclic AMP stimulate the phos-

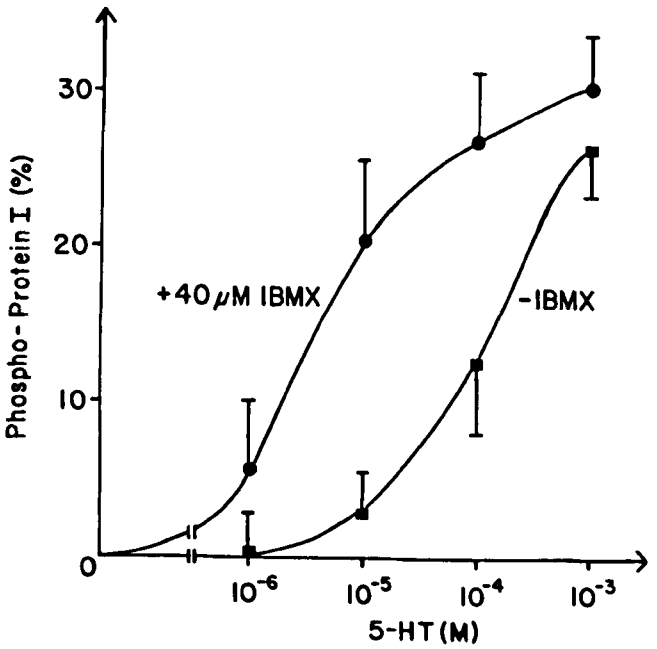

FIG. 3. Formation of phospho-Protein I from dephospho-Protein I in slices of rat facial nucleus incubated in the presence of various concentrations of serotonin (5-HT). After termination of the incubation and extraction of Protein I from the slices, the extracts were incubated with [γ-^{32}P]ATP, phosphorylating the Protein I which remained in the dephospho-form. The amount of phosphate incorporated into Protein I was determined as previously described [2], and the % conversion of dephospho- to phospho-Protein I due to 5-HT was then calculated for each pair of incubations. The dose-response curves were determined both in the absence (—■—) and in the presence (—●—) of IBMX (4×10^{-5} M). In the latter case, IBMX was included both plus and minus 5-HT. The results are the means ± SEM of 5–10 samples all assayed in duplicate. The basal level of phosphorylation was 7.9 ± 0.8 pmol of phosphate/mg Zn-acetate precipitable protein (n=25) in the absence, and 7.4 ± 0.7 pmol phosphate/mg protein (n=25) in the presence of IBMX (4×10^{-5} M).

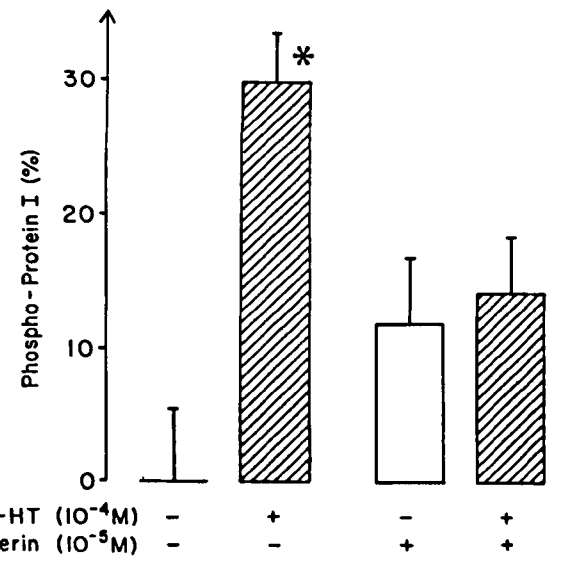

FIG. 4. The effect of the 5-HT antagonist mianserin on the formation of phospho-Protein I induced by 5-HT in rat facial nucleus slices. Each pair of facial nuclei was incubated plus or minus 5-HT (10^{-4} M) for 10 min. Mianserin (10^{-5} M), where included, was added to each pair of facial nuclei 15 min before the end of the incubation. IBMX (4×10^{-5} M) was present in all incubations and was added 12 minutes before the end of the incubation. The results are the means ± SEM (n=5). The basal level of incorporation of phosphate into Protein I in the presence of IBMX (4×10^{-5} M) was 6.7 ± 0.4 pmol/mg protein. *$p<0.01$ when the effect of 5-HT is compared to basal level (Student's t-test).

phorylation of Protein I, it is attractive to speculate that the phosphorylation of Protein I might be involved in certain physiological mechanisms underlying potentiation or inhibition of neurotransmitter release by neuromodulatory agents.

It is highly likely that Protein I is present in most, if not all, presynaptic terminals. It would thus appear to be a useful biochemical marker of terminals, in both the central and peripheral nervous system. Studies are in progress to investigate Protein I levels in various types of experimentally induced neuropathy, including fetal alcohol syndrome, and in different disease states including Huntington's chorea and Alzheimer's Disease using human post-mortem tissue.

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