Neuronal Phosphoproteins

Mediators of Signal Transduction

Paul Greengard

The Laboratory of Molecular and Cellular Neuroscience
The Rockefeller University
1230 York Avenue
New York, NY 10021

Abstract

This article summarizes some of our knowledge concerning intracellular protein phosphorylation pathways in nerve cells. It also summarizes, very briefly, recent direct experimental evidence involving intracellular injection of protein kinases, protein kinase inhibitors, and substrates, indicating that protein phosphory-

lation mediates the actions of a variety of neurotransmitters on their target cells. Finally, it summarizes in somewhat greater detail the results of studies of three different types of substrate proteins that appear to regulate different types of biological responses in nerve cells: synapsin I, a substrate protein present in virtually all nerve terminals, which appears to regulate neurotransmitter release from those nerve terminals; the acetylcholine receptor, the phosphorylation of which regulates its rate of desensitization in the presence of acetylcholine; and DARPP-32, the phosphorylation of which converts it into a very potent phosphoprotein phosphatase inhibitor that may be involved in the regulation by the neuromodulator dopamine of the effects of the neurotransmitter glutamate. The identification and characterization of additional neuronal phosphoproteins can be expected to lead to the clarification of numerous additional molecular mechanisms by which signal transduction is carried out in nerve cells.

Index Entries: Neuronal phosphoproteins; phosphoproteins, neuronal; signal transduction, neuronal phosphoproteins as mediators of; mediators, of signal transduction by neuronal phosphoproteins; neurotransmitter actions; synapsin I; nicotinic acetylcholine receptor; DARPP-32, regulation of; cAMP regulation of DARPP-32; dopamine regulation, of DARPP-32.

Phosphoproteins and Neuronal Function

There is now convincing evidence, based on studies in many laboratories, that protein phosphorylation represents a final common pathway through which a large number of extracellular regulatory agents produce their biological effects in target cells, both neuronal and nonneuronal. Figure 1 illustrates the protein phosphorylation reaction. All protein phosphorylation systems have three components in common: (a) a substrate protein that can exist in either the dephospho or the phospho form; (b) a protein kinase that, in the presence of ATP, catalyzes the phosphorylation of the substrate protein; and (c) a protein phosphatase that catalyzes the reverse reaction. It is now clear that this reaction appeared very early in the course of evolution, since it has been found in cells and tissues throughout the animal kingdom, in which it has been adapted for the specific regulatory purposes of the organism in question.

The manner in which those hormones and neurotransmitters that use cAMP as a second messenger have adapted the protein phosphorylation system is illustrated in Fig. 2. Such hormones and neurotransmitters activate the enzyme adenylate cyclase, causing the conversion of ATP to cAMP. The newly formed cAMP activates a specific protein kinase, known as cAMP-dependent protein kinase, which catalyzes the phosphorylation of the substrate protein. The phosphorylated substrate protein, then, through one or more biochemical steps, produces the metabolic or physiological response characteristic of the hormone or neurotransmitter and cell type in question. The physiological response to this stimulus is terminated when the cAMP is hydrolyzed by a phosphodiesterase and the phosphoprotein is dephosphorylated by a protein phosphatase.

The current status of our knowledge concerning intracellular protein phosphorylation pathways in the nervous system is illustrated in Fig. 3. It is now clear that a variety of extracellular signals or first messengers that, in the case of the nervous system, can be hormones, neurotransmitters, or the nerve impulse itself, produce a variety of biological responses by one or another of the pathways indicated in this figure. We now know of five types of intracellular second messengers that are utilized by nerve cells. Four of these second messengers, namely, cAMP, cGMP, calcium,

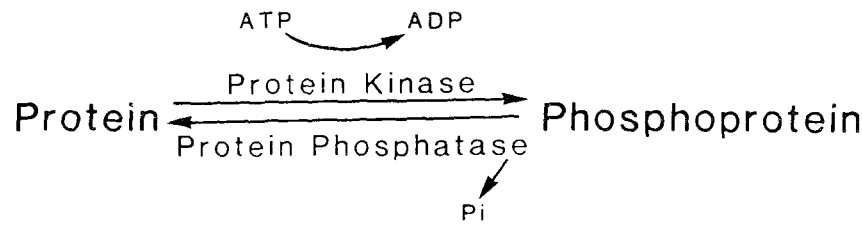

Fig. 1. Protein phosphorylation/dephosphorylation reaction.

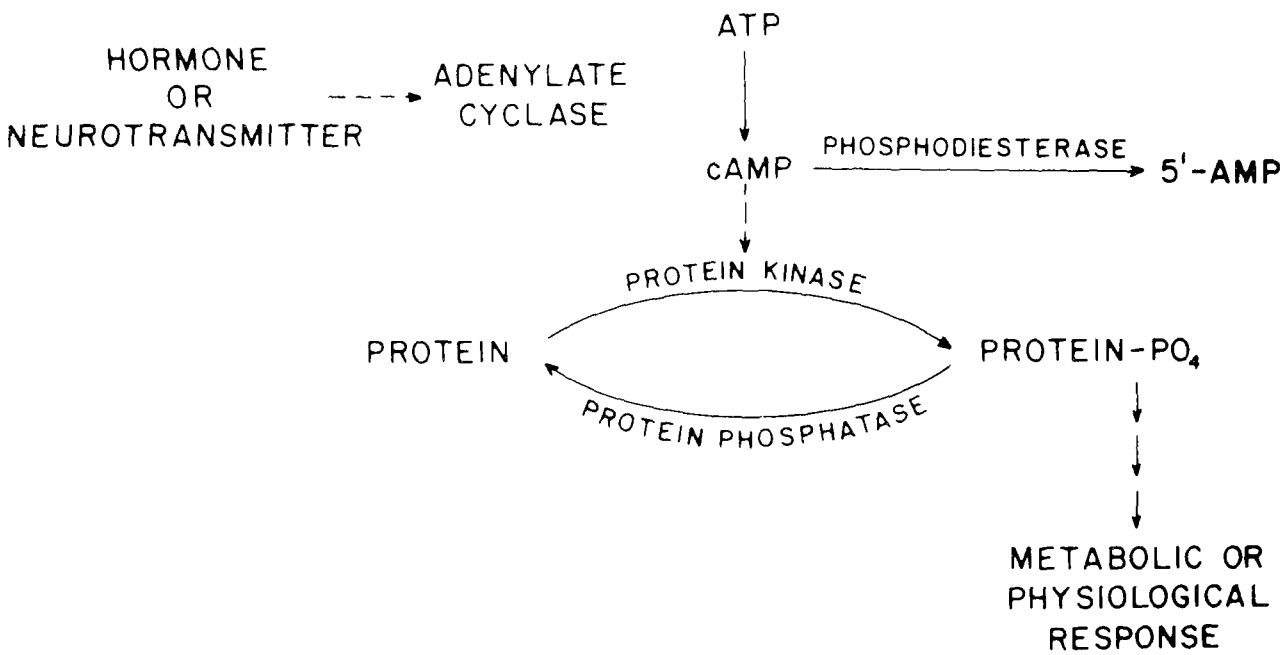

Fig. 2. Schematic diagram of the apparent role played by protein phosphorylation in mediating the biological effects of those hormones and neurotransmitters that act through cyclic AMP. The appropriate hormone or neurotransmitter stimulates a specific adenylate cyclase present in the target tissue, causing an increased conversion of ATP to cyclic AMP; the newly formed cyclic AMP is either hydrolyzed by a phosphodiesterase to 5' -AMP, or else it activates a protein kinase, leading to the phosphorylation of a substrate protein; the substrate protein, through one or more steps, controls the rate of some metabolic or physiological response; the phosphorylation of this substrate protein leads to an increase or decrease in the rate of this response. From Greengard, 1981.

and diacylglycerol, achieve many of their affects through activation of one or another specific protein kinase. The fifth intracellular second messenger, inositol triphosphate, may or may not work through a protein kinase—that situation is not yet clear. Almost all of the effects of cAMP in neurons are achieved through activation of cAMP-dependent pro-

tein kinase, an enzyme with a very broad substrate specificity that phosphorylates a large number of substrates in nerve cells, as indicated symbolically by the multiple arrows in Fig. 3. Some, but not all, of the effects of cGMP are achieved through activation of cGMP-dependent protein kinase. The second messenger actions of calcium are somewhat more complex.

A few of the second messenger actions of calcium are achieved through regulation of physiological effectors other than protein kinases. However, it appears that most of the second messenger actions of calcium are achieved through activation of one of two subclasses of calcium-dependent protein kinases. The enzymes belonging to one of these subclasses are referred to as calcium/calmod-

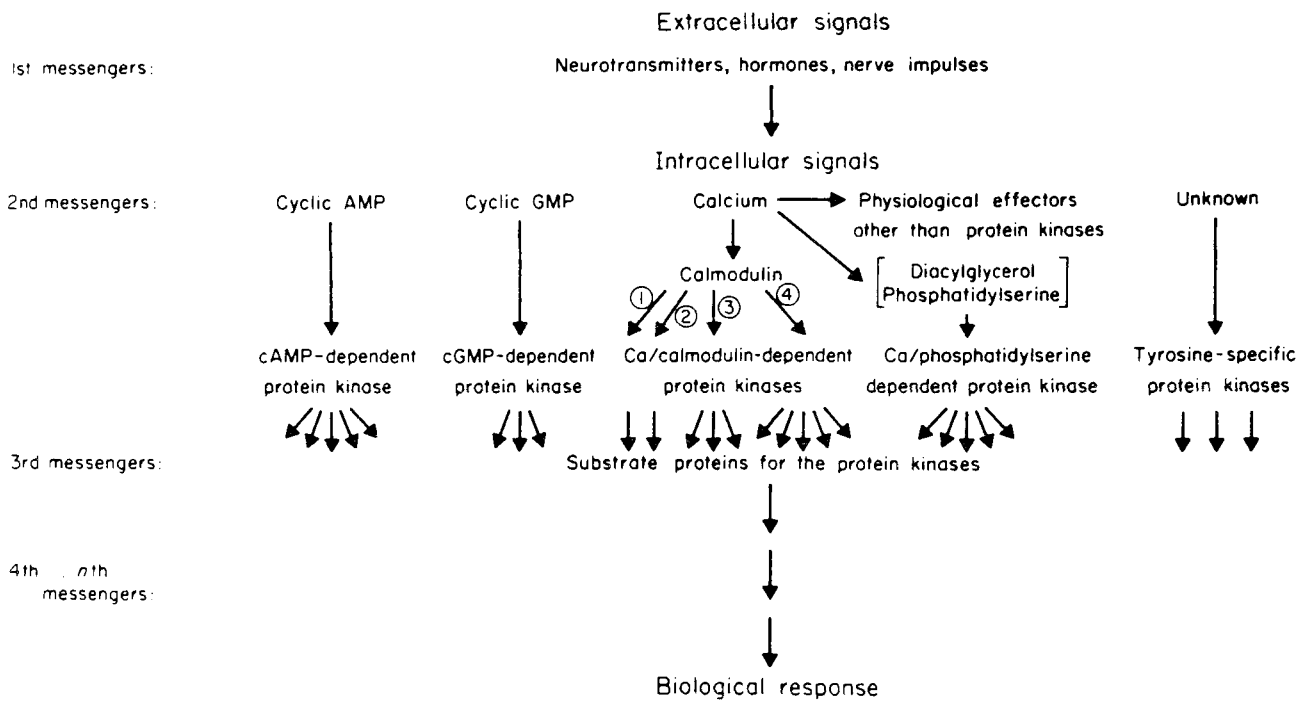

Fig. 3. Signals in the nervous system. Extracellular signals (first messengers) produce specific biological responses in target neurons via a series of intracellular signals (second, third, and other messengers). Second messengers in the brain include cyclic AMP, cyclic GMP, diacylglycerol, and calcium. Cylic AMP and cyclic GMP produce many of their second messenger actions through the activation of virtually one type of cyclic AMP-dependent protein kinase and one type of cyclic GMP-dependent protein kinase, respectively. The former enzyme exhibits a broad substrate specificity and the latter a more restricted specificity. Calcium exerts certain of its second messenger actions through the activation of calcium-dependent protein kinases and other of its actions through a variety of physiological effectors other than protein kinases. Calcium activates protein kinases in conjunction with calmodulin or with diacylglycerol plus phosphatidylserine. These various cyclic nucleotide-dependent and calcium-dependent protein kinases phosphorylate proteins specifically at serine and threonine residues. There is also a class of protein kinases that phosphorylate proteins specifically at tyrosine residues (protein tyrosine kinases). The activation of individual protein kinases causes the phosphorylation of specific substrate proteins in target neurons. In some cases these substrate proteins, or third messengers, represent the immediate effector for the biological response. In other cases they produce the biological response indirectly through fourth, fifth, sixth, and other messengers.

ulin-dependent protein kinases. What appears to happen in the case of these enzymes is that calcium combines with the low molecular weight protein calmodulin, causing a conformational change in the calmodulin and, thereby, exposing a hydrophobic region of the molecule. This hydrophobic region of the molecule then combines with a hydrophobic region on one of the target protein kinases, thereby activating that protein kinase. There are several calcium/calmodulin-dependent protein kinases in the nervous system, and they vary in their substrate specificity from very narrow to very broad. Most of these calcium/calmodulin-dependent protein kinases appear to phosphorylate only one or two substrate proteins. However, one of these calcium/calmodulin-dependent protein kinases has a very broad substrate specificity. It is referred to as calcium/calmodulin-dependent protein kinase II, or multifunctional calcium/calmodulin-dependent protein kinase.

The other subclass of calcium-dependent protein kinase is activated by the second messengers calcium and diacylglycerol and requires the presence of phosphatidylserine. This enzyme, discovered by Nishizuka and his colleagues (Nishizuka, 1984), is usually referred to by the name protein kinase C. Protein kinase C also has a very broad substrate specificity.

Each of these cyclic nucleotide-dependent and calcium-dependent protein kinases phosphorylates its substrate protein(s) on either serine or threonine residues. Serine and threonine phosphorylation appears to represent over 99% of all protein phosphorylation in the brain. During the past few years, a new class of protein kinases has been discovered that phosphorylates substrate proteins on tyrosine residues. These protein kinases are referred to as protein tyrosine kinases. Although they are responsible for less than 1% of all protein phosphorylation in the brain, it seems likely that they are important in the regulation of brain function. However, relatively little is known as yet concerning the signals that activate these protein tyrosine kinases in the nervous system or about their physiological effects.

In contrast to the relatively small number of protein kinases that phosphorylate substrate proteins on serine and threonine residues, there is an enormous number of substrate proteins for these kinases in brain. Thus, more than 70 neuron-specific substrate proteins have been demonstrated in brain tissue (Walaas et al., 1983b,c). By neuron-specific, I refer to substrate proteins that are present in reasonably high concentrations in nervous tissue and are undetectable with currently available methods in nonneuronal tissues.

These substrate proteins represent a very diverse or heterogeneous group of molecules. They differ, for example, with respect to the protein kinases that phosphorylate them. They also differ with respect to their cellular distribution. Some of these substrate proteins are present in every neuron throughout the entire mammalian nervous system, where they appear to be involved in regulation of functions common to all nerve cells. An example of such a substrate protein is synapsin I, a protein that is present in all nerve terminals, where it appears to be involved in the regulation of neurotransmitter release, a physiological property common to all nerve cells. I shall describe synapsin I in greater detail a little later. At the other extreme are substrate proteins that are present in only a single type of neuron throughout the entire mammalian nervous system. One such substrate protein is a protein called G-substrate. G-substrate is a substrate for cGMP-dependent protein kinase and is localized almost exclusively in the Purkinje cells of the mammalian cerebellum (Schlichter et al., 1978; Aswad et al., 1981). Antibodies to this system have been used with great effectiveness by Dr. De Camilli to map the cytoarchitecture of the Purkinje cells (De Camilli et al., 1984). Many of the substrate proteins in

the nervous system seem to have an intermediate distribution, being present in certain classes of nerve cells and not in others. An example of one such substrate protein is DARPP-32, a protein localized to neurons that contain the D-1 subclass of dopamine receptor. I shall describe this protein in greater detail later on in this presentation.

These various substrate proteins also differ with respect to their subcellular distribution. Some of them are cytosolic, whereas others are associated with one or another cellular organelle, such as the plasma membrane, synaptic vesicles, or cytoskeleton. One might predict, based on the known cellular and subcellular distribution of these proteins, that they would also differ with respect to their physiological roles. Recent experimental data indicate that this is indeed the case.

Table 1 lists some of the classes of neuronal proteins that are regulated by phosphorylation. Some of the enzymes that are involved in the biosynthesis of neurotransmitters are regulated by phosphorylation. For example, tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of the catecholamine neurotransmitters, and tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of serotonin, are each phosphorylated and thereby activated by three distinct classes of protein kinases. Some of the enzymes that are involved in the synthesis and degradation of cAMP and cGMP are regulated by phosphorylation. Virtually every known protein kinase has been shown to undergo intramolecular autophosphorylation, and this autophosphorylation usually converts the kinase from an inactive to an active form. Several molecules that act as inhibitors of protein phosphatases have been shown to be regulated by phosphorylation: the phosphorylation of the molecule converts it either from an inactive compound to an active phosphatase inhibitor or the reverse. Several of the proteins that are involved in the regulation of transcription and translation have been shown to be controlled by phosphorylation. Virtually every known cytoskeletal protein in the nervous system has been shown to be a phosphoprotein. Four distinct proteins associated with synaptic vesicles have been shown to be regulated by phosphorylation. Finally, several types of neurotransmitter receptor and several types of ion channel have been shown to be controlled by protein phosphorylation, and it seems increasingly likely that most if not all neurotransmitter receptors and ion channels will ultimately be shown to be regulated by phosphorylation.

Direct Evidence That Protein Phosphorylation Mediates Some Neurotransmitter Actions

For almost 20 years the hypothesis that protein phosphorylation mediates the actions of various neurotransmitters on their target cells was based primarily on evidence showing correlations between the state of phosphorylation of one or another substrate protein and the state of some physiological response. During the past few years, it has been possible to obtain direct evidence for a role of protein phosphorylation in mediating neurotransmitter action.

This evidence has come from studies involving the intracellular injection of protein kinases into nerve cells. Thus, each of the four known major classes of protein kinases that phosphorylate substrate proteins on serine or threonine residues has been demonstrated, upon intracellular injection, to mimic the physiological activation of nerve cells by neurotransmitters (Table 2).

The first protein kinase to be studied in this way was cAMP-dependent protein kinase. It was found in two simultaneous studies, one carried out on bag cell neurons of *Aplysia*

(Kaczmarek et al., 1980) and the other carried out on sensory neurons of *Aplysia* (Castellucci et al., 1980), that intracellular injection of the catalytic subunit of cAMP-dependent protein kinase mimics physiological activation of these two types of nerve cells. With both types of nerve cell preparation the physiological response involved a shutting off of potassium channels, a broadening of the action potential, an increased influx of calcium, and an enhanced physiological response. Conversely, the injection into both types of preparation of a low molecular weight protein that specifically inhibits cAMP-dependent protein kinase abolished the ability of these cells to respond to physiological stimuli (Castellucci et al., 1982; Kaczmarek et al., 1984). The demonstration that the protein kinase itself mimicked physiological activation of these nerve cells and that the protein kinase inhibitor abolished physiological activation of these nerve cells demonstrates conclusively that cAMP-dependent protein phosphorylation is both necessary and sufficient to produce a physiological response in these two types of neurons. Subsequently, a number of other laboratories, working with a variety of other excitable cells, have reported similar results.

A second protein kinase to be studied in this way was the multifunctional calcium/ calmodulin-dependent protein kinase. This enzyme was investigated in the squid giant synapse (Llinas et al., 1985). In these studies it was demonstrated that the injection of the multifunctional calcium/calmodulin-dependent protein kinase enhanced neurotransmitter release in response to depolarization of the presynaptic membrane.

Calcium/diacylglycerol dependent protein kinase has been studied in bag cell neurons of *Aplysia* (DeRiemer et al., 1985). The injection of this kinase enhanced the conductance through calcium channels in the bag cell neurons. More recently, the same enzyme was studied in collaboration with Gerschenfeld and his colleagues (Hammond et al., 1987). In these studies it was shown that the neurotransmitter cholecystokinin (CCK) shuts off voltage-sensitive calcium channels in identified neurons of the snail *Helix aspersa*, and that this effect of CCK is mediated through calcium/diacylglycerol-dependent protein kinase (Hammond et al., 1987). In another collaborative study with Gerschenfeld and his colleagues (Paupardin-Tritsch et al., 1986), it was demonstrated that the ability of serotonin to regulate voltage-sensitive calcium channels in identified ventral neurons of *H. aspersa* was mediated through cGMP-dependent protein kinase. The experimental results indicated that serotonin activates a guanylate cyclase, leading to the production of cGMP, that this cGMP activates cGMP-dependent protein kinase, and that this cGMP-dependent protein kinase phosphorylates either the voltage-sensitive calcium channel or some protein closely associated with the calcium channel. As a result of the phosphorylation, these voltage-sensitive calcium channels showed an enhanced voltage sensitivity.

Thus, direct evidence has been obtained that each of four distinct classes of protein kinases mediate the actions of various neurotransmitters on their target cells. These results make it increasingly important to identify and characterize the various substrate proteins present in nerve cells that produce various physiological responses.

I would like in the remainder of this presentation to describe three such substrate proteins that our research group has been studying. I have chosen these particular substrate proteins because they illustrate three distinct types of biological response that appear to be regulated by protein phosphorylation.

TABLE 1

Classes of Neuronal Proteins Regulated by Phosphorylation[a]

Enzymes involved in neurotransmitter biosynthesis	
Tyrosine hydroxylase	Joh et al. (1978); Edelman et al. (1981);
Tryptophan hydroxylase	Yamauchi and Fujisawa (1981); Kuhn and Lovenberg (1982)
Neurotransmitter receptors	
Nicotinic acetylcholine receptor	Gordon et al. (1977); Teichberg et al. (1977); Huganir et al. (1984)
Muscarinic acetylcholine receptor	Burgoyne (1983)
β-Adrenergic receptor	Stadel et al (1983)
GABA receptor (GABA-modulin)	Wise et al. (1983)
Ion channels	
Sodium channel	Costa et al. (1982); Costa and Catterall (1984a,b);
Potassium channel	Kaczmarek et al. (1980); Castellucci et al. (1980); Adams and Levitan (1982); de Peyer et al. (1982); Alkon et al. (1983); Osterrider et al. (1982); DeRiemer et al. (1985);
Enzymes involved in cyclic nucleotide metabolism	
Adenylate cyclase	Richards et al. (1981); Ehrlich et al. (1982)
Guanylate cyclase	Zwiller et al. (1982)
Phosphodiesterase	Sharma et al. (1980); Marchmont and Houslay (1980)
Autophosphorylated protein kinases	
Cyclic AMP-dependent protein kinase	Rangel-Aldao and Rosen (1976)
Cyclic GMP-dependent protein kinase	de Jonge and Rosen (1977)
Calcium/calmodulin-dependent protein kinases	*see* Nestler and Greengard (1984)
Calcium/phospholipid-dependent protein kinase	Kikkawa et al. (1982)
Tyrosine-specific protein kinase	Barnekow et al. (1982); Cotton and Brugge (1983)
Rhodopsin kinase	Lee et at, (1981); Schichi and Somers (1978)
Proteins involved in regulation of transcription and translation	
RNA polymerase	Hook et al. (1981)
Histones	Langan (1969); Gurley et al. (1981)
Nonhistone nuclear proteins	Johnson (1982)
Ribosomal protein S6	Roberts (1982)
Other ribosomal proteins	Roberts (1982); Thomas (1982)

(Table continued next page)

TABLE 1 (*continued*)

Cytoskeletal proteins	
Map-2	*see* Nestler and Greengard (1984)
Tau	*see* Nestler and Greengard (1984)
Neurofilaments	*see* Nestler and Greengard (1984)
Myosin light chain	Hathaway and Traugh (1982)
Actin	De Maille and Pechere (1983)
Tubulin	Goldenring et al. (1982)

[a]Neuronal proteins regulated by phosphorylation are listed with selected references. Some of the proteins listed are specific to neurons, whereas others are present in many cell types, including neurons. Not included are many phosphoproteins present in a variety of different tissues (including brain) that play roles in cellular processes and are not thought to play roles in neuron-specific phenomena. Modified from Nestler and Greengard (1984).

TABLE 2

Systems in Which Direct Evidence Has Been Obtained for a Role of Protein Phosphorylation in Neuronal Function[a]

Cell, genus	Kinase injected[b]	Inhibitor injected[c]	Conclusion of studies	References
Bag cell neurons *Aplysia*	Cyclic AMP	PKI	Kinase mediates effect of synaptic activation and of exogenous cyclic AMP in producing the afterdischarge; this action appears to be achieved through decreases in the conductance of calcium-dependent potassium channels and of early (I_A) voltage-dependent potassium channels	Kaczmarek et al. (1980) Kaczmarek et al. (1984)
Sensory neurons *Aplysia*	Cyclic AMP	PKI	Kinase mediates effect of synaptic activation and of exogenous serotonin and cyclic AMP in facilitating neurotransmitter release in response to nerve impulses; this action appears to be achieved through decreases in the conductance of novel serotonin-regulated potassium channels	Castellucci et al. (1980) Castellucci et al. (1982) Siegelbaum et al. (1982)
Neuron R 15 *Aplysia*		PKI	Kinase mediates effect of exogenous serotonin and cyclic AMP in inhibiting bursting	Adams and Levitan (1982) Benson and Levitan (1983)

(Table continued next page)

TABLE 2 *(continued)*

Cell, genus	Kinase injected[b]	Inhibitor injected[c]	Conclusion of studies	References
			activity and in enhancing interburst hyperpolarization; this action appears to be achieved through increases in the conductance of novel serotonin regulated, anomalously rectifying potassium channels	de Peyer et al. (1982)
Unidentified neurons *Helix*	Cyclic AMP		Kinase increases the conductance of calcium-dependent rectifying potassium channels	de Peyer et al. (1982)
Unidentified neurons, *Helix*	Cyclic AMP	Tolbutamide[d]	Kinase increases the conductance of voltage-dependent calcium channels	Doroshenko et al. (1984) Chad and Eckert (1984)
Photoreceptor cells, *Hermissenda*	Cyclic AMP		Kinase decreases the conductance of early (I_A) and late (I_B) voltage-dependent calcium channels	Alkon et al. (1983)
Hippocampal (CA1) pyramidal neurons, *Cavia*	Cyclic AMP	PKI	Kinase mediates effect of dopamine and cyclic AMP in producing a long-lasting increase in input resistance	Gribkoff et al. (1984)
Bag cell neurons *Aplysia*	Calcium/ phospholipid		Kinase increases the height of action potentials; this action appears to be achieved through increases in the conductance of calcium channels	DeRiemer et al. (1985)
Terminal digits of giant synapse, *Loligo*	Calcium/ calmodulin II		Kinase facilitates neurotransmitter relase	Llinas et al. (1985)
Ventral neurons *Helix*	Cyclic GMP		Kinase mediates effect of serotonin and cyclic GMP in broadening the action potential; this effect appears to be achieved through increases in the conductance of calcium channels	Paupardin-Tritsch et al. (1986)

[a] Modified from Nestler and Greengard (1983) and Hemmings and Greengard (1986).

[b] Protein kinase abbreviations: cyclic AMP, catalytic subunit of cyclic AMP-dependent protein kinase; cyclic GMP, cyclic GMP-dependent protein kinase holoenzyme; calcium/phospholipid, calcium/phospholipid-dependent protein kinase holoenzyme; calcium/calmodulin II, calcium/ calmodulin-dependent protein kinase II holenzyme.

[c] PKI, specific protein inhibitor of cyclic AMP-dependent protein kinase.

[d] The authors claim that tolbutamide is a specific inhibitor of cyclic AMP-dependent protein kinase in vitro [Doroshenko et al. (1984)].

TABLE 3

Physicochemical Properties of Bovine Synapsin I

	Synapsin Ia	Synapsin Ib
Molar proportion	1	2
Molecular weight (M_r)	86,000	80,000
Isoelectric point	> 10.0	> 10.0
Stokes radius	59 Å	59 Å
Sedimentation coefficient	2.9 *S*	2.9 *S*
Frictional ratio	2.2	2.2
Acid soluble	Yes	Yes
Amino acid composition	Rich in proline and glycine, elongated, highly asymmetric molecule with collagenase-insensitive domain (head region) and highly basic collagenase-sensitive domain (tail region)	

Synapsin I

Synapsin I (previously termed Protein I) was first identified in the early 1970s in studies designed to search for phosphoproteins that might play a role in the regulation of synaptic transmission. In those studies, synapsin I was shown to be a major endogenous substrate for cyclic AMP-dependent protein kinase in particulate synaptic fractions of brain (Johnson et al. 1972; Ueda et al., 1973). Subsequently, synapsin I was shown to be a major endogenous substrate for calcium-dependent protein kinases in particulate synaptic fractions (Krueger et al., 1977; Sieghart et al., 1979). Synapsin I has been purified to homogeneity from bovine brain (Ueda and Greengard, 1977) and rat brain (Huttner et al., 1981) and has been extensively characterized. The available evidence supports the hypothesis that synapsin I is part of the molecular machinery in nerve terminals that regulates neurotransmitter release.

Physicochemical Properties and Protein Kinase Specificity of Synapsin I

Some of the physicochemical properties of synapsin I are summarized in Table 3. Synapsin I consists of two closely related proteins, synapsin Ia and synapsin Ib, with apparent molecular weights of 86,000 and 80,000, respectively. Synapsin I is an extremely basic, highly elongated, and acid-soluble protein. A variety of physicochemical data indicates that synapsin I consists of two domains, each representing roughly half of the molecule: a collagenase-insensitive domain, and a proline-rich domain that is rapidly and specifically degraded by highly purified collagenase (Ueda and Greengard, 1977). Recently, the messenger RNA for synapsin I has been isolated, and it appears that distinct species of messenger RNA exist for synapsin Ia and synapsin Ib (DeGennaro et al., 1983; Wallace et al., 1985).

TABLE 4

Protein Kinase Specificity of Synapsin I

Synapsin I undergoes multisite phosphorylation
1. One serine residue in the head region of synapsin I is phosphorylated by cyclic AMP-dependent protein kinase and by Ca^{2+}/calmodulin-dependent protein kinase I.
2. Two serine residues in the tail region of synapsin I are phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II
3. Not a physiological substrate for cyclic GMP-dependent protein kinase or for Ca^{2+}/diacylglycerol-dependent protein kinase (protein kinase C)

The protein kinase specificity of synapsin I is summarized in Table 4. Synapsin I is an effective substrate protein for at least three distinct protein kinases in brain and undergoes multisite phosphorylation. Synapsin I contains one serine residue (site 1) in the collagenase-insensitive domain of the molecule that is phosphorylated both by cyclic AMP-dependent protein kinase and by calcium/calmodulin-dependent protein kinase I (Huttner et al., 1981; Kennedy and Greengard, 1981; Nairn and Greengard, 1983). Synapsin I also contains two serine residues (sites 2 and 3) in the collagenase-sensitive domain of the molecule that are phosphorylated by calcium/calmodulin-dependent protein kinase II (Huttner et al., 1981; Kennedy and Greengard, 1981; Kennedy et al., 1983). For each of these three protein kinases, synapsin I is one of the best substrate proteins known.

Distribution of Synapsin I

We now have a fairly detailed knowledge of the distribution of synapsin I. This is due in large measure to the studies of my colleague, Pietro De Camilli, initially at Yale University and, subsequently, at the University of Milano. To study the distribution of synapsin I, a variety of techniques has been used, including immunochemical techniques, subcellular fractionation, light microscope immunocytochemistry, and electron microscope immunocytochemistry. The results of such studies are summarized in Table 5. Synapsin I is present only in the nervous system (Ueda and Greengard, 1977) and, within the nervous system, is present only in neurons (Sieghart et al., 1978; De Camilli et al., 1979, 1983a; Bloom et al., 1979; Fried et al., 1982).

TABLE 5

Distribution of Synapsin I

1. Present only in nervous system (both central and peripheral)
2. Within nervous system, present only in neurons
3. Within neurons, concentrated in nerve terminals
4. Within terminals, associated with small (40–60 nm) synaptic vesicles
5. Present at virtually all synapses
6. Appears simultaneously with synapse formation during development

Synapsin I, measured by radioimmunoassay, represents approximately 0.4% of the total protein present in the cerebral cortex of each of five mammalian species studied, indicating that synapsin I is a major protein in mammalian brain (Goelz et al., 1981). The levels of synapsin I in peripheral tissues were found to be much lower than those in cerebral cortex, results attributable to the fact that the density of synaptic elements in peripheral tissues is much lower than in brain (Fried et al., 1982).

Several lines of evidence indicate that, within neurons, synapsin I is concentrated in presynaptic nerve terminals and appears to be present in virtually all presynaptic nerve terminals throughout the nervous system. First, the immunocytochemical staining pattern of synapsin I in hundreds of sections through numerous regions of the central and peripheral nervous system is consistent with a localization of synapsin I to all nerve terminals: synapsin I staining surrounds cell bodies and dendrites (which themselves are unstained) and leaves little space on the surface of the neurons to account for the presence of any unstained synapses (De Camilli et al., 1983a). Second, electron microscope study of rat brain synaptosomes, using ferritin-labeled antibodies to synapsin I, has demonstrated the presence of synapsin I in close to 100% of all synaptosomes examined in hundreds of electron microscope sections (De Camilli et al., 1983b). Third, denervation of peripheral tissues, such as adrenal medulla, results in a virtually complete loss of synapsin I content (Fried et al., 1982). Finally, synapsin I has been shown to be present in nerve terminals of several different neurotransmitter types, including adrenergic, cholinergic, dopaminergic, GABA-ergic, and glutamatergic nerve terminals (*see* Nestler and Greengard, 1984).

Within presynaptic nerve terminals, synapsin I is associated primarily with synaptic vesicles, as shown by electron microscopy (Bloom et al., 1979; De Camilli et al., 1983b) and subcellular fractionation studies (Ueda et al., 1979; Fried et al., 1982; Huttner et al., 1983). In fact, synapsin I appears to be a major protein of synaptic vesicles: it represents approximately 6% of the total protein present in highly purified vesicle preparations (Huttner et al., 1983). Recent electron microscope studies have further suggested that synapsin I is preferentially associated with small (40–60 nm) synaptic vesicles rather than large (greater than 60 nm) dense-core vesicles in nerve terminals of the bovine hypothalamus (Navone et al., 1984). Since the smaller vesicles appear to be restricted to nervous tissue, whereas the larger ones are present in many types of secretory cells in addition to neurons, this observation may explain (Navone et al., 1984) why synapsin I is present in nervous tissue but absent for nonnervous cells— including even those, such as adrenal chromaffin cells, that are developmentally related to neurons and serve a secretory function.

A variety of biochemical studies have demonstrated, in agreement with electron microscope observations, that synapsin I is located on the outer or cytoplasmic surface of vesicle membranes (Huttner et al., 1983). The collagenase-sensitive domain of synapsin I appears to be the region of the molecule that binds to synaptic vesicles (Ueda, 1981; Huttner, 1983). Phosphorylation of synapsin I in this domain (i.e., at sites 2 and 3) by calcium/calmodulin-dependent protein kinase II decreases its ability to bind to synaptic vesicles, at least under some experimental conditions (Huttner et al., 1983; Schiebler et al., 1986). Synaptic vesicles appear to contain a specific, saturable, and high-affinity (K_d = 10 n*M*) binding site for synapsin I (Schiebler et al., 1983, 1986). Possibly, the collagenase-insensitive domain of synapsin I binds to some other component of the nerve terminal, such as the cytoskeleton or plasma membrane, with the phosphorylation of this domain regulating this interaction. If this is so, then synapsin I could be viewed as forming a physical bridge between synaptic vesicles and the cytoskeleton or plasma membrane, with the integrity of this

bridge regulated by the multisite phosphorylation of synapsin I. This idea represents one molecular mechanism through which synapsin I might regulate synaptic vesicle availability and neurotransmitter release. Studies are now under way to identify the putative element of the cytoskeleton or plasma membrane that binds the collagenase-insensitive domain of synapsin I in the nerve terminal.

Synapsin I appears in brain during development at about the time of synaptogenesis (Lohmann et al., 1978). This appearance is associated with a concomitant appearance of messenger RNA for synapsin I (DeGennaro et al., 1983; Wallace et al., 1985). The association of synaptogenesis with the appearance of both synapsin I and messenger RNA for synapsin I has been observed in several brain regions and in brains of several mammalian species (DeGennaro et al., 1983). The mechanism underlying the expression of synapsin I at the time of synaptogenesis remains unknown. Further research is needed to distinguish between two possible interpretations of the data: (1) that the initiation of synapsin I synthesis occurs in the innervating neuron as a result of some interaction between the innervating neuron and its target neuron; or (2) that the initiation of synapsin I synthesis in the innervating neuron does not require this cell–cell interaction, but, rather, is an event that is genetically programmed to occur at roughly the same time as the formation of synaptic contacts with target neurons. Recent observations by Ellis et al. (1985) that synapsin I is present in growth cones isolated from fetal rat brain support the latter interpretation by indicating that synapsin I is already present in brain during axonal sprouting. Nevertheless, the specific temporal association of synapsin I expression with the onset of synaptogenesis has made it possible to use synapsin I as a convenient indicator for synaptogenesis in developmental studies of the nervous system (e.g., *see* Levitt et al., 1984).

Phylogenetic studies of synapsin I have revealed that it is a highly conserved protein. The synapsin I molecules in brain of several mammalian species, including human, are immunochemically indistinguishable and exhibit similar biochemical and physicochemical properties (Goelz et al., 1981). In addition, proteins that cross-react immunologically with mammalian synapsin I have been identified in the nervous systems of species throughout the animal kingdom, including all other vertebrate classes (bird, reptile, amphibian, and fish) and three invertebrate phyla (echinodermata, arthropoda, and mollusca) (Sorensen and Babitch, 1984; Goelz et al., 1985; Llinas et al., 1985). The proteins present in nonmammalian species exhibit different molecular weights from mammalian synapsin I, but resemble it in a number of other ways. For example, four proteins present in fish brain that cross-react with synapsin I antisera are enriched in synaptic fractions of fish brain, are acid soluble, are digested by collagenase, and are substrates for endogenous protein kinases (Goelz et al., 1985). As another example, proteins in *Manduca* moths that cross-react with synapsin I appear in moth-head ganglia as the adult moths emerge from pupation, the time during which synaptogenesis occurs (Goelz et al., 1985). The early evolutionary appearance of synapsin I-like proteins and the highly conserved nature of synapsin I in mammals suggest that the protein plays a vital role in neuronal function.

Regulation of State of Phosphorylation by Physiological and Pharmacological Stimuli

Regulation of the state of phosphorylation of synapsin I has been studied in a variety of nervous tissue preparations. The results of such studies are summarized in Table 6. Consistent with the observations that synapsin I is phosphorylated by cyclic AMP-dependent and calcium-dependent protein kinases in vitro, the state of phosphorylation of synapsin I is stimulated in intact neuronal preparations by a vari-

TABLE 6

Physiological and Pharmacological Regulation of Synapsin I

1. In synaptosomes and in slices of nervous tissue, depolarizing agents and cyclic AMP increase state of phosphorylation
2. In specific anatomical regions of central and peripheral nervous system, the relevant neurotransmitters increase state of phosphorylation
   (a) Serotonin and adenosine in facial motor nucleus
   (b) Dopamine in superior cervical ganglion, posterior pituitary, caudatoputamen, and substantia nigra
   (c) Norepinephrine in frontal cortex
3. In isolated superior cervical ganglion and in posterior pituitary, impulse conduction under physiological conditions increases state of phosphorylation
4. In whole animals, convulsants increase and depressants decrease state of phosphorylation in cerebrum
5. In whole animals, neurotransmitters and hormones increase total amount in specific brain regions
   (a) Norepinephrine in pinealocytes
   (b) Corticosterone in hippocampus
   (c) Opiates in striatum

ety of manipulations that increase cyclic AMP or calcium levels in neurons. In synaptosomes (Krueger et al., 1977; Huttner and Greengard, 1979) and slices (Forn and Greengard, 1978) of nervous tissue, depolarizing agents, which increase the flux of calcium into nerve terminals, stimulate a calcium-dependent phosphorylation of synapsin I. In these same preparations, phosphodiesterase inhibitors, which increase cyclic AMP levels, and cyclic AMP analogs stimulate a calcium-independent phosphorylation of synapsin I.

Synapsin I phosphorylation is regulated by a number of specific neurotransmitters in well-defined, relatively homogeneous regions of the nervous system. Serotonin and adenosine have been shown to increase the state of phosphorylation of synapsin I in rat facial motor nucleus (Dolphin and Greengard, 1981a,b), dopamine has been shown to increase the state of phosphorylation of synapsin I in bovine and rabbit superior cervical ganglion (Nestler and Greengard, 1980, 1982b), rat posterior pituitary (Tsou and Greengard, 1982), and rat caudatoputamen and substantia nigra (Walaas and Greengard, unpublished observations), and norepinephrine has been shown to increase the state of phosphorylation of synapsin I in rat frontal cortex (Mobley and Greengard, 1985). In each of these regions, the respective neurotransmitter appears to stimulate the phosphorylation of synapsin I in presynaptic nerve terminals via an increase in cyclic AMP levels and the activation of cyclic AMP-dependent protein kinase.

Since the state of phosphorylation of synapsin I is regulated in nerve terminals by neurotransmitters acting on presynaptic receptors, investigation of synapsin I phosphorylation can be used to study presynaptic receptors in the nervous system. Indeed, in all of the regions so far examined, study of synapsin I phosphorylation has either revealed the existence of previously unidentified presynaptic receptors or confirmed the existence of presynaptic receptors that had been identified by other methods. In most cases, it was found that receptors for the same type of neurotransmitter are present on presynaptic nerve terminals and on postsynaptic cell bodies and dendrites (*see* Fig. 4), suggesting that a dual action of neurotransmitter at a single synapse is a common synaptic mechanism. According to this idea, at many synapses,

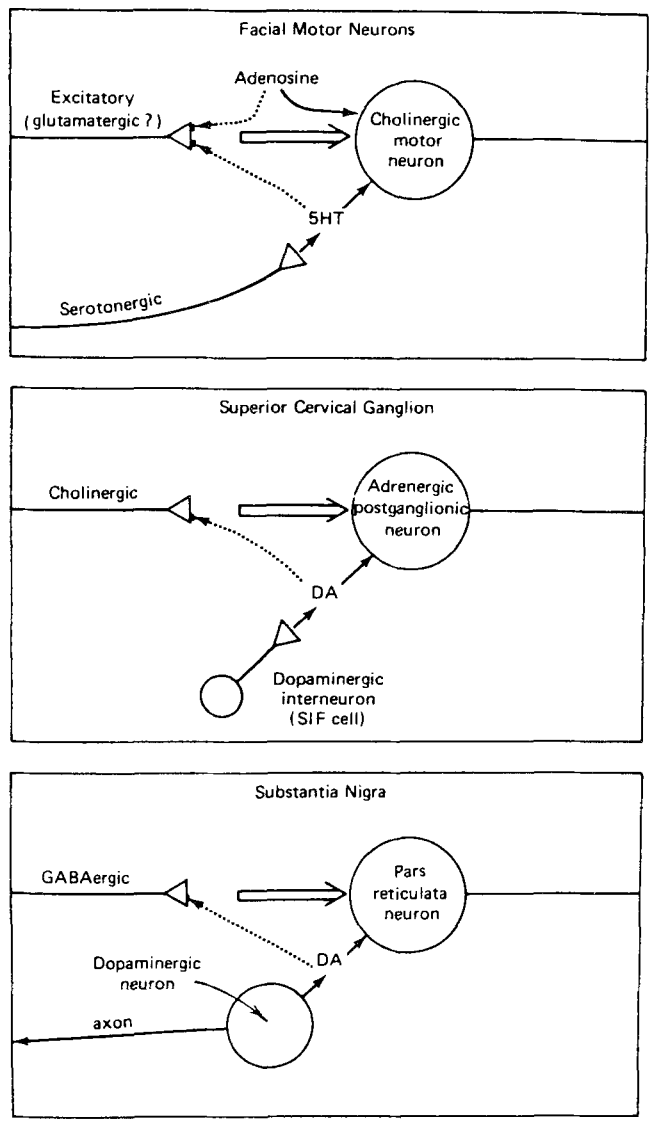

Fig. 4. Schematic diagram of the synaptic organization of rat facial motor nucleus (top panel); rabbit or cow superior cervical ganglion (middle panel); rat substantia nigra (bottom panel). The study of synapsin I phosphorylation in these three regions of the nervous system has revealed or confirmed the existence of types of neurotransmitter receptors on presynaptic nerve terminals that had previously been shown to exist on postsynaptic neurons. For simplicity, pathways representing the cholinergic innervation of dopaminergic interneurons in the superior cervical ganglion and the GABA-ergic innervation of dopaminergic neurons in the substantia nigra are omitted. It should be noted that, in the substantia nigra, dopamine is released from the dendrites, rather than from the axons, of dopaminergic neurons. Open arrows (⇨), main synaptic pathway; solid arrows (→), previously identified actions of neurotransmitter; dotted arrows (·····>), actions detected or verified by studies of synapsin I. (5HT) serotonin: (DA) dopamine; (SIF cell) small intensely fluorescent cell; (GABA) γ-aminobutyric acid. From Nestler and Greengard, 1984.

a given neurotransmitter acts both on presynaptic nerve terminals, where it regulates the amount of a second neurotransmitter released in response to nerve impulses, and on postsynaptic cell bodies and dendrites, where it regulates the responsiveness of the postsynaptic element to that second neurotransmitter (Nestler and Greengard, 1983, 1984).

Study of synapsin I phosphorylation can also be used to estimate the percentage of nerve terminals in a given region of the nervous system that possesses certain classes of presynaptic receptors. For example, norepinephrine, acting at β-adrenergic receptors, has been found to stimulate the phosphorylation of roughly one-third of the synapsin I present in the frontal cortex (Mobley and Greengard, 1985). These results indicate that at least one-third of all of the nerve terminals in this brain region possess presynaptic β-adrenergic receptors. Moreover, since it has been estimated that only a very small fraction of nerve terminals in the cortex is noradrenergic, these studies of synapsin I phosphorylation support the possibility that a substantial percentage of all nerve terminals in the cortex respond physiologically to norepinephrine released by distant noradrenergic nerve terminals. The results support the proposal (*see* Reader et al., 1979) that noradrenergic nerve terminals function in the cortex in a paracrine manner. Thus, study of synapsin I phosphorylation has provided a unique method for detecting and characterizing presynaptic receptors directly, as well as for elucidating their role in synaptic transmission.

Brief periods of impulse conduction, under physiological conditions, have been shown to increase the state of phosphorylation of synapsin I in nerve terminals of the rabbit superior cervical ganglion (Nestler and Greengard, 1982a,b) and rat posterior pituitary (Tsou and Greengard, 1982). In the ganglion it was found that as few as 20 nerve impulses significantly stimulated synapsin I phosphorylation. The amount of synapsin I phosphorylated in response to preganglionic nerve stimulation was calculated to be approximately 2% of the total presynaptic synapsin I per nerve impulse. Furthermore, the changes in the phosphorylation of site 1 of synapsin I, elicited by nerve impulse conduction, were found to be nearly stoichiometric: preganglionic nerve stimulation resulted in the conversion of roughly 80% of presynaptic synapsin I from the dephosphorylated to the phosphorylated form (Nestler and Greengard, 1982b). Figure 5 shows the change in dephosphosynapsin I, measured by "back phosphorylation," observed in response to 5 s of preganglionic nerve stimulation at 10 Hz, as a function of the time after initiation of nerve stimulation. Since the total amount of synapsin I, measured by radioimmunoassay, was not altered by nerve stimulation, the decreases observed in the amount of dephosphosynapsin I (Fig. 5) reflect increases in the state of phosphorylation of synapsin I in response to nerve impulse conduction. An increase in the state of phosphorylation of synapsin I was first observed within 20 s after initiation of nerve stimulation and appeared to be maximal at 30–60 s, after which time the state of phosphorylation returned to control levels (Nestler and Greengard, 1982a). The data shown in Fig. 5 reflect the state of phosphorylation of both the collagenase-insensitive and collagenase-sensitive domains of synapsin I. Other data (Nestler and Greengard, 1982a,b) indicate that the state of phosphorylation of the individual domains (i.e., site 1 and sites 2/3) changes with similar time courses.

Further studies on the rabbit superior cervical ganglion were performed to determine whether the changes observed in synapsin I phosphorylation in response to nerve impulse conduction or to dopamine occurred presynaptically or postsynaptically (Nestler and Greengard, 1982b). These studies, summarized in Fig. 6, revealed that the ganglion contains two "pools" of synapsin I. One pool, representing about 60% of total ganglion synapsin I, is located in presynaptic nerve terminals. This preganglionic pool disappears on surgical denervation of the ganglion, is unaffected by brief

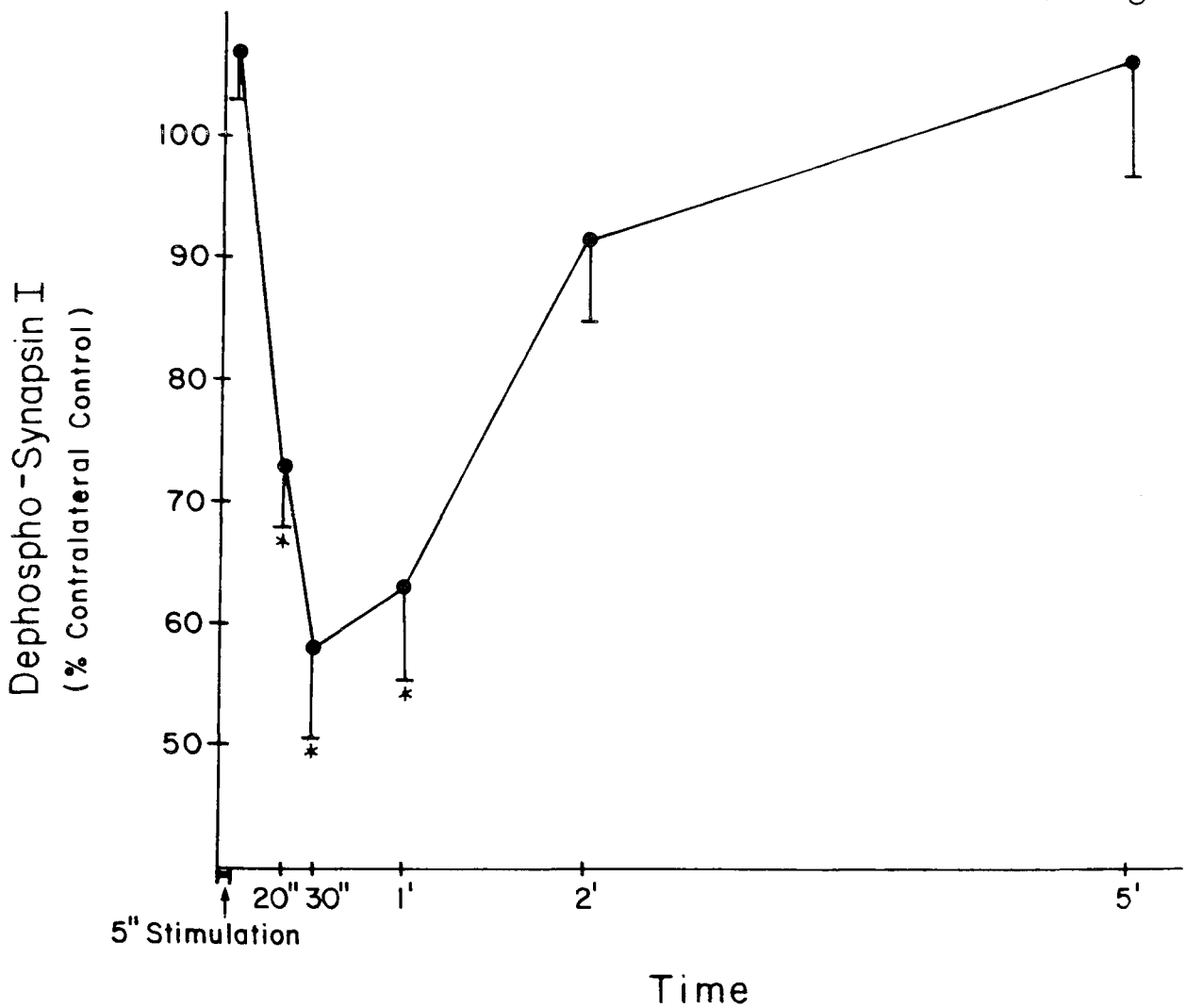

Fig. 5. Effect of a brief period (5 s) of impulse conduction on amount of dephosphosynapsin I in rabbit superior cervical ganglion, as a function of time after stimulation. The preganglionic nerve supplying one ganglion of each rabbit was stimulated via a suction electrode at 10 Hz for 5 s; stimulated and contralateral control ganglia were homogenized in 1% SDS at various times afterwards. Dephosphosynapsin I was determined by back phosphorylation, and the amount in the stimulated ganglion compared with that in the contralateral control ganglion. Values shown are means ± SEM. The number of pairs of ganglia used ranged from 3 to 7. *Significantly different from control ($p < 0.05$) by two-tailed test. From Nestler and Greengard, 1982a.

exposure to the protein synthesis inhibitor cycloheximide, and undergoes phosphorylation in response to nerve impulses, dopamine, or high potassium concentration. The other pool, representing about 40% of total ganglion synapsin I, is located in the cell bodies of ganglionic neurons. This postsynaptic pool is unaffected by denervation, is decreased by brief exposure to cycloheximide, and does not undergo phosphorylation in response to a variety of physiological and pharmacological stimuli. Rather, postsynaptic synapsin I appears to represent newly synthesized synapsin I, presumably en route to the nerve terminals arising from the ganglionic cell bodies, where it will serve its physiological function.

Study of the rabbit superior cervical ganglion and rat posterior pituitary has indicated that two first messengers regulate synapsin I phosphorylation in nerve terminals of these tissues: the nerve impulse itself and the neurotransmitter dopamine. Evidence indicates that these first messengers regulate synapsin I phosphorylation via different mechanisms: impulse conduction increases synapsin I phosphorylation through the activation of calcium-dependent protein kinases, and dopamine increases synapsin I phosphorylation through the activation of cyclic AMP-dependent protein kinase. First, the effects of nerve impulse conduction and high potassium concentration on synapsin I phosphorylation are dependent on extracellular calcium, whereas those of dopamine and cyclic AMP analogs are not (Nestler and Greengard, 1982a,b). Second, nerve impulse conduction and high potassium concentration stimulate the phosphorylation of synapsin I both in the collagenase-insensitive and the collagenase-sensitive domains of the molecule, observations consistent with the expectation, based on studies with purified synapsin I and protein kinases, that activation of calcium-dependent protein kinases would increase the state of phosphorylation of both regions of synapsin I. In contrast, dopamine, cyclic AMP analogs, and forskolin (which activates adenylate cyclase) stimulate the phosphorylation only of the collagenase-insensitive region of synapsin I, observations consistent with the expectation, based on studies with purified synapsin I and protein

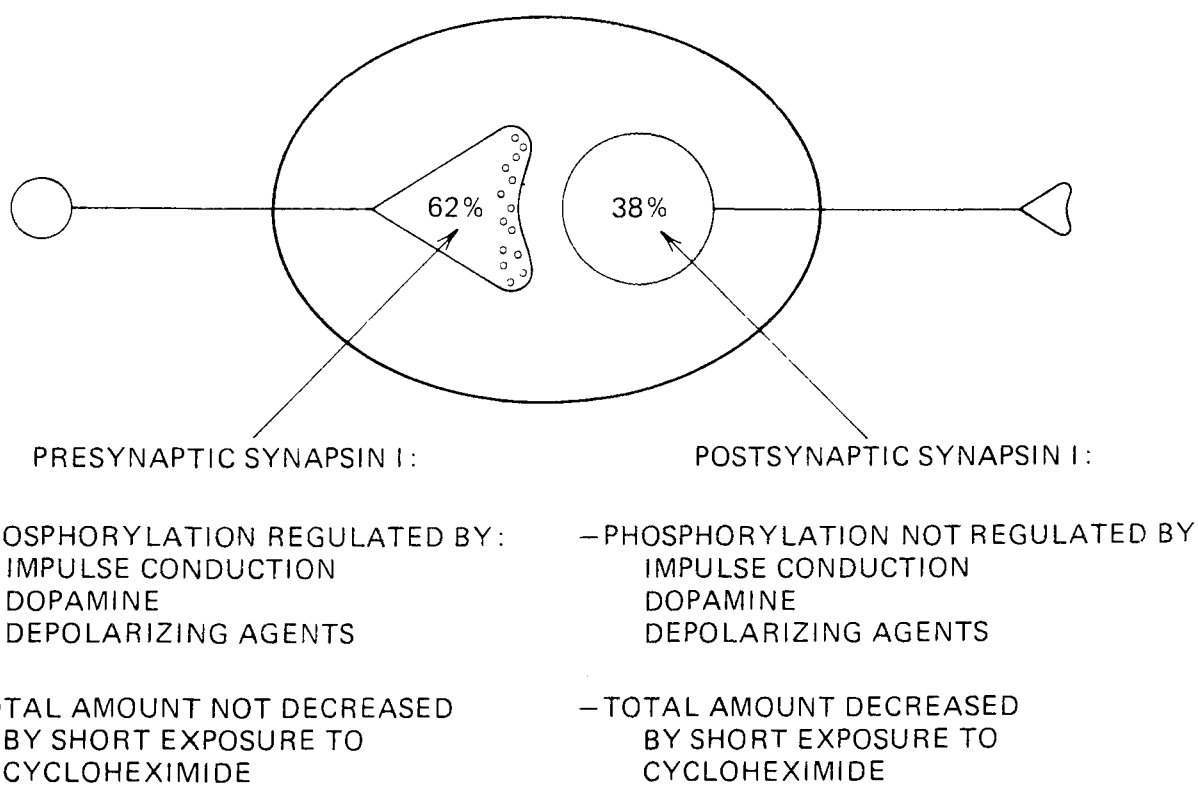

Fig. 6. Schematic diagram of the distribution and regulation of synapsin I in the rabbit superior cervical ganglion. From Nestler and Greengard, 1982b.

kinases, that activation of cyclic AMP-dependent protein kinase would increase the state of phosphorylation of synapsin I only in this region (Nestler and Greengard, 1982b; Tsou and Greengard, 1982). Based on studies with cell-free preparations of brain tissue, one can hypothesize that the calcium-dependent phosphorylation of the collagenase-insensitive domain of synapsin I in ganglionic and posterior pituitary nerve terminals is catalyzed by calcium/calmodulin-dependent protein kinase I, whereas that of the collagenase-sensitive domain of synapsin I is catalyzed by calcium/calmodulin-dependent protein kinase II.

Synapsin I phosphorylation is also regulated in whole animals (Strombom et al., 1979). Administration of convulsants to mice was found to increase synapsin I phosphorylation, whereas administration of central nervous system depressants was found to decrease such phosphorylation in whole cerebrum. Because convulsants increase and depressants decrease neuronal activity in brain, these results are consistent with the observations that nerve impulse conduction stimulates synapsin I phosphorylation in isolated intact nervous tissue.

Direct Evidence for a Role of Synapsin I in Neuronal Function

Direct evidence for a role of synapsin I in neurotransmitter release has recently been obtained in studies of the squid giant synapse (Llinas et al., 1985). In these studies neurotransmitter release was determined by measuring the amplitude, rate of rise, and latency of the postsynaptic potential generated in response to presynaptic depolarizing steps under voltage-clamp conditions. Injection of purified dephosphorylated synapsin I into nerve terminals at the synapse was found to decrease the amplitude and rate of rise of the postsynaptic potential. In contrast, injection of purified synapsin I that had been phosphorylated on its collagenase-sensitive domain (sites 2 and 3) by calcium/calmodulin-dependent protein kinase II, as well as injection of heat-denatured dephosphosynapsin I, had no effect on these parameters of neurotransmitter release. Injection of purified calcium/calmodulin-dependent protein kinase II into nerve terminals increased the amplitude and rate of rise, and decreased the latency, of the postsynaptic potential. The effects of dephosphosynapsin I and of calcium/calmodulin-dependent protein kinase II on the postsynaptic potential occurred in the absence of any detectable changes in presynaptic calcium current. These results provide support for the idea that neurotransmitter release can be modulated by biochemical mechanisms and that synapsin I phosphorylation is involved in such modulation.

Nicotinic Acetylcholine Receptor

I would now like to leave the presynaptic terminal, synapsin I, and the regulation of neurotransmitter release, move across the synaptic cleft to the postsynaptic membrane, and discuss a protein in the postsynaptic membrane, the phosphorylation of which appears to regulate neurotransmitter sensitivity. The protein is the nicotinic acetylcholine receptor, and the work I am going to describe was carried out in our laboratory primarily by Dr. Richard Huganir. It was found several years ago by Raftery, Changeux, Diamond, and their colleagues (Vandlen, 1979; Gordon et al., 1977; Saitoh and Changeux, 1981) that the acetycholine receptor is a phosphoprotein. During the past few years, Huganir has undertaken the biochemical characterization of the phosphorylation of the acetylcholine receptor and attempted to determine the physiological significance, if any, of this phosphorylation.

As is now well-known, the nicotinic acetylcholine receptor is composed of four types of subunits, known as alpha, beta, gamma, and delta, and these subunits can be separated from

one another on SDS gels and visualized by protein staining. Each of three distinct protein kinases is capable of catalyzing the phosphorylation of the acetylcholine receptor (Huganir et al., 1983, 1984; Huganir and Greengard 1983). cAMP-dependent protein kinase causes the rapid and stoichiometric phosphorylation of the gamma and delta subunits of the receptor. Protein kinase C causes the rapid and stoichiometric phosphorylation of the delta subunit of the receptor and a slow but very reproducible phosphorylation of the alpha receptor. Finally, there is an endogenous protein tyrosine kinase present in the plasma membranes that contains the nicotinic cholinergic receptor, and this endogenous protein tyrosine kinase catalyzes the rapid and stoichiometric phosphorylation of the receptor on the beta, gamma, and delta subunits. Thus, three distinct protein kinases phosphorylate the acetylcholine receptor on a total of seven distinct sites, with six of these sites being phosphorylated rapidly and stoichiometrically.

The acetylcholine receptor is believed to traverse the plasma membrane a total of five times and includes one relatively long cytoplasmic segment. It seems likely that all seven of the phosphorylation sites are located in this cytoplasmic region of the molecule (Huganir et al., 1984). In addition, for those subunits that undergo multisite phosphorylation, the phosphorylated amino acids appear to reside within a few residues of one another. Moreover, phosphorylation occurs in closely homologous regions of the different subunits.

What, if any, is the physiological significance of the phosphorylation of the acetylcholine receptor? To answer this question, Huganir phosphorylated the receptor on the gamma and delta subunits in the presence of cAMP-dependent protein kinase and then purified the receptor to homogeneity. For control purposes, he also purified the dephosphorylated form of the receptor. He then reconstituted the two forms of the purified receptor into lipid vesicles. In collaboration with Delcour and Hess at Cornell University, he then compared the rate of desensitization of the dephosphorylated and phosphorylated forms of the receptor in the presence of acetylcholine (Huganir et al., 1986). It was demonstrated that the phosphorylated form of the receptor desensitizes to acetycholine with a rate constant about seven-to-ten times that of the dephosphorylated form of the receptor. Interestingly, phosphorylation of the receptor had no effect on its sensitivity in the absence of acetylcholine.

The effect of partial phosphorylation of the acetylcholine receptor on its desensitization rate was also examined. The results of these studies indicated that phosphorylation of either the gamma or delta subunit of the receptor, or both, was sufficient to convert it from the slowly desensitizing form to the rapidly densensitizing form.

The physiological significance of the phosphorylation of the acetylcholine receptor can be interpreted as follows. It seems likely that there is a neurotransmitter, the identity of which remains to be determined, that activates adenylate cyclase in the plasma membrane of cells that contain the nicotinic cholinergic receptor, that the activation of the adenylate cyclase increases cAMP, which activates cAMP-dependent protein kinase, which then, by phosphorylation of the gamma or delta subunit of the receptor, causes its desensitization in the presence of acetylcholine. This is an example of receptor–receptor interaction within a single plasma membrane. Moreover, the fact that two additional classes of protein kinases also phosphorylate the acetylcholine receptor in the same region of the molecule makes it seem likely that there are at least two additional neurotransmitters that act through these two other protein kinase systems to bring about the desensitization of the acetylcholine receptor. Thus, there may be four neurotransmitters that act on four distinct receptors in this plasma membrane, three of them acting through three distinct protein kinases to alter the sensitivity of the acetyl-

choline receptor to the fourth neurotransmitter, namely acetylcholine. This is a rather complex example of receptor–receptor interactions within a plasma membrane, in this case, one involving four neurotransmitters and their receptors. However, it seems possible that such complex interactions are quite common within the nervous system.

DARPP-32, A Dopamine- and cAMP-Regulated Phosphoprotein

The third neuronal phosphoprotein that I shall describe, DARPP-32, is associated with neurons that contain dopamine receptors of the D-1 subclass. DARPP-32 is a phosphoprotein for which a biochemical mechanism of action has been found.

Identification

DARPP-32 was originally observed during the examination of the regional distribution of cAMP-and calcium-regulated protein phosphorylation systems in rat brain (Walaas et al., 1983a,b). Some of the cAMP-regulated phosphoproteins, one of which was DARPP-32, were found to have restricted regional distributions in the brain that paralleled the gross anatomical distribution of dopaminergic innervation. A detailed biochemical analysis of the distribution of DARPP-32 in rat brain revealed that it was specifically enriched in the basal ganglia (Walaas and Greengard, 1984). High concentrations were found in the caudatoputamen, nucleus accumbens, and olfactory tubercle (the main dopamine-innervated regions of the forebrain basal ganglia), and in the main targets of the output neurons of these three regions, the globus pallidus and substantia nigra. DARPP-32 was shown to be contained within intrinsic neurons of the caudatoputamen by use of the neurotoxin kainic acid. Injection into the caudatoputamen of kainic acid, which destroys intrinsic striatal neurons while sparing nerve terminals and glia (Schwartz and Coyle, 1977), produced a considerable decrease in the amount of striatal DARPP-32 present (Walaas and Greengard, 1984). Further studies, employing lesioning techniques that eliminated specific neuronal populations in these regions, demonstrated that DARPP-32 was absent from the nigrostriatal dopaminergic neurons themselves, but was present throughout the striatonigral dopaminoceptive neurons, specifically those dopaminoceptive neurons possessing D_1-dopamine receptors (Walaas and Greengard, 1984).

Regulation of Phosphorylation

The mammalian brain appears to contain at least two types of dopamine receptors designated D_1- and D_2-dopamine receptors (Kebabian and Calne, 1979). The D_1-dopamine receptor is linked to the activation of adenylate cyclase, whereas at least some D_2-dopamine receptors appear to be linked to inhibition of adenylate cyclase (Kebabian and Calne, 1979; Kebabian and Cote, 1981; Stoof and Kebabian, 1981). The striking correlation between dopaminergic innervation and DARPP-32 localization, and the localization of DARPP-32 to the subclass of dopaminoceptive neurons possessing D_1-dopamine receptors, suggested that dopamine, acting through D_1-dopamine receptors, might regulate the phosphorylation of DARPP-32 by cAMP-dependent protein kinase in these dopaminoceptive cells. Using techniques that allowed analysis of the state of phosphorylation of DARPP-32 in brain slice preparations containing intact cells (Walaas et al., 1983c), it was found that either dopamine or the 8-bromo analog of cAMP could convert DARPP-32 from the dephosphorylated to the phosphorylated form (Walaas and Greengard, 1984). Phosphorylation of DARPP-32 was observed at concen-

trations of dopamine that had previously been found to activate specific dopamine receptors linked to the activation of adenylate cyclase (Forn et al., 1974). The effect of dopamine could be inhibited by the dopamine receptor blocker fluphenazine (Walaas et al., 1983c) and was specific for dopamine insofar as other neurotransmitter candidates (norepinephrine, serotonin, and adenosine) were without effect. These studies, which established a physiological link between DARPP-32 phosphorylation and dopaminergic neurotransmission, provided the impetus for further anatomical and biochemical studies of this phosphoprotein.

Regional, Cellular, and Subcellular Distribution in Brain

The tissue, cellular, and subcellular distribution of DARPP-32 was investigated by immunochemical techniques involving the use of both monoclonal and polyclonal antibodies prepared against purified bovine DARPP-32. The regional and cellular distribution of DARPP-32 in the central nervous system of the rat was determined by a detailed immunocytochemical study (Ouimet et al., 1984). In general, the results of this study were supported by results obtained in a similar study of the distribution of DARPP-32 in the central nervous system of the rhesus monkey (Ouimet et al., manuscript in preparation).

The distribution of DARPP-32 exhibited large regional variations, with particularly strong labeling occurring in most of the brain regions that are heavily innervated by dopamine fibers, including the basal ganglia. Strong DARPP-32 immunoreactivity in neuronal cell bodies and dendrites was found within the caudatoputamen, nucleus accumbens, and olfactory tubercle (the forebrain basal ganglia), all of which receive dense dopamine inputs. Immunoreactive puncta, which represent nerve terminals as determined by electron microscopy (*see* below), were observed in the globus pallidus and pars reticulata of the substantia nigra, brain regions known to receive projections from the forebrain basal ganglia. Strong neuronal immunoreactivity was also found in the central, lateral, and cortical amygdaloid nuclei, whereas strong glial immunoreactivity was found in tanycytes in the arcuate nucleus and median eminence of the mediobasal hypothalamus. All of these regions receive dense dopaminergic innervation (Lindvall and Bjorklund, 1978).

Studies employing high-resolution light and electron microscopy have shown that DARPP-32 is present in the cytosol of the somata, dendrites, dendritic spines, axons, and axon terminals of the medium-sized spiny neurons of the caudatoputamen and nucleus accumbens, but not in the giant cholinergic striatal interneurons (Ouimet et al., manuscript in preparation). The medium-sized spiny neurons receive most of the dopamine input to the neostriatum (Groves, 1983) and represent more than 90% of the Golgi-impregnated neurons in this brain region (Pasik et al., 1979; Dimova et al., 1980). DARPP-32 immunoreactivity was absent from corticostriatal fibers and the dopaminergic nigrostriatal neurons.

Weak immunoreactivity was observed in cells of the deep layers of the medial prefrontal cortex and in cell clusters in the entorhinal cortex, regions with well-defined dopamine inputs (Lindvall and Bjorklund, 1978). Weak immunoreactivity was also found in several regions in which dopamine innervation is believed to be less important. Thus, some neurons were weakly labeled in the medial habenula, the cerebellum, and layers II, III, and VI of the neocortex, whereas the thalamus and hippocampus showed a weak nerve terminal staining pattern. A restricted number of astrocytes, located throughout the neuroaxis, were also found to be weakly immunoreactive.

The results obtained by immunocytochemical methods were supported by quantitative analysis, employing both a biochemical assay (Walaas and Greengard, 1984) and a specific

radioimmunoassay (Hemmings and Greengard, 1986) of the distribution of DARPP-32 in the rat central nervous system. The highest concentrations of DARPP-32 were found in the caudatoputamen, the globus pallidus, and the substantia nigra. Intermediate concentrations were found in the nucleus accumbens and the olfactory tubercle, and low concentrations in the frontal cortex, neocortex, hippocampus, amygdala, thalamus, cerebellum, and retina.

The levels of DARPP-32 in subcellular fractions prepared from the rat caudatoputamen were also analyzed with the biochemical phosphorylation assay and the radioimmunoassay. These studies demonstrated that the soluble fraction of this brain region is highly enriched in DARPP-32, consistent with its localization within cell bodies and dendrites. A significant amount of DARPP-32 was also found in the crude synaptosomal fraction that could be released by hypotonic lysis, suggesting that a fraction of DARPP-32 is also present in nerve terminals. This observation is supported by the finding that, in the substantia nigra, DARPP-32 is enriched in the particulate fraction and can be released by hypotonic lysis (Walaas and Greengard, 1984). In this brain region, evidence obtained by immunocytochemistry at both the light and electron microscope levels indicated that DARPP-32 is contained within nerve terminals. Thus, the results of studies of the subcellular localization of DARPP-32 determined by the analysis of subcellular fractions are consistent with those of ultrastructural studies employing immunocytochemical techniques in which cell bodies, dendrites, axons, and nerve terminals were all immunoreactive for DARPP-32. Furthermore, DARPP-32 immunoreactivity was evenly distributed throughout the cytoplasm, suggesting that DARPP-32 is not primarily associated with any subcellular organelle (Ouimet et al., 1984).

The nature of the neurotransmitter(s) in DARPP-32-containing neurons has not been firmly established. The vast majority of the medium-sized neurons in the rat caudatoputamen and nucleus accumbens contain γ-aminobutyric acid (GABA), as revealed by immunocytochemical staining for glutamic acid decarboxylase (Oertel and Mugnaini, 1984). It is likely that most, if not all, of these neurons also contain DARPP-32 (Ouimet et al., 1984). However, GABA-ergic neurons are much more widely distributed throughout the brain than are neurons immunoreactive for DARPP-32, so not all GABA-ergic neurons in the brain contain DARPP-32 (*see* Ouimet et al., 1984). Furthermore, since DARPP-32 is present in a variety of other nerve cell types that do not appear to be GABA-ergic, the general distribution of neurons immunoreactive for DARPP-32 is not identical to that of GABA-ergic neurons. Many striatal medium-sized spiny neurons are also immunoreactive for substance P (Ljungdahl et al., 1978) and enkephalin (Pickel et al., 1980). Thus, it is possible that DARPP-32 is also present in neurons that contain substance P and/or enkephalin.

Species and Tissue Distribution

Immunochemical methods have also been used to identify DARPP-32 in various vertebrate species and to study its distribution in peripheral nervous and non-nervous tissues. DARPP-32 has been detected in brain tissue from the mouse, rat, guinea pig, rabbit, cat, cow, rhesus monkey, human, canary, and turtle by phosphorylation, radioimmunoprecipitation, radioimmunoassay, and antibody labeling of nitrocellulose blots of SDS-polyacrylamide gels (Hemmings et al., unpublished observations). Studies of peripheral nervous tissues employing the same techniques have shown that DARPP-32 is present in the posterior pituitary, which has both D_1-and D_2-dopamine receptors, but is absent from the anterior pituitary, which has only D_2-dopamine receptors (Kebabian and Calne, 1979). DARPP-32 has also been detected in bovine and rabbit superior cervical sympathetic ganglia, which contain dopaminergic neurons and dopamine receptors coupled to

adenylate cyclase (Kebabian and Greengard, 1971); in bovine parathyroid cells, which contain D_1-dopamine receptors (Brown et al., 1977); and in the chromaffin cells of the bovine adrenal medulla (Hemmings and Greengard, 1986). DARPP-32 has not been detected in any of the peripheral nonnervous tissues tested other than the parathyroid gland.

Recently, immunochemical methods have been used to carry out a phylogenetic survey of DARPP-32 localization. In the basal forebrain of the turtle, strong immunoreactivity was detected by radioimmunoprecipitation, radioimmunoassay, and antibody labeling of nitrocellulose blots of SDS-polyacrylamide gels (Hemmings et al., unpublished observations) and in the paleostriatum of the canary (Hemmings et al., unpublished observations). In the canary, these findings have been confirmed by immunocytochemical studies (Burd et al., unpublished observations). In the turtle and in the canary (as in the rat), the brain regions that contain DARPP-32 are the regions that receive major dopaminergic inputs and that are thought to be homologous to the neostriatum of mammals (Reiner et al., 1984). Further studies of the immunocytochemical localization of DARPP-32 in these species should yield valuable information concerning the phylogenetic development of dopaminoceptive neurons, particularly within the basal ganglia. From these studies, it appears that the regional distribution of DARPP-32 in the brains of several mammalian and nonmammalian vertebrates corresponds to that of dopamine innervation and probably reflects the different locations of dopaminoceptive neurons possessing D_1-dopamine receptors in these species. Immunoreactive DARPP-32 could not be detected in the brains of two other vertebrate classes, the fish and the frog (Hemmings et al., unpublished observations.

DARPP-32 as a Marker for Dopaminoceptive Neurons

Results obtained by a variety of techniques indicate a close association between DARPP-32 localization and the subclass of dopaminoceptive neurons containing D_1-dopamine receptors. However, several cell types that appear to be weakly immunoreactive for DARPP-32 are not known to receive dopaminergic innervation (e.g., neurons in layers II, III, and VI of the cerebral cortex outside the prefrontal cortex, the cerebellar Purkinje cells, various astrocytes, and tanycytes). There are several possible explanations for these apparent discrepancies. First, the distribution of D_1-dopamine receptors may be wider than that of dopaminergic nerve terminals. Second, a minor dopaminergic input may have escaped detection by the currently available methods in some brain regions. Third, DARPP-32 may be able to act as an effector for neurotransmitters that elevate cAMP levels other than dopamine.

In some instances of apparent discrepancy, the presence of DARPP-32 appears to be correlated with the presence of D_1-dopamine receptors. Thus, dopamine-sensitive adenylate cyclase (which indicates the presence of D_1-dopamine receptors) has been found in the cat cerebellum (Dolphin et al., 1979) and in certain glial cells (Schubert et al., 1976; Henn et al., 1977). In other cases, the presence of DARPP-32 may indicate the existence of a previously unknown dopamine input, as for the tanycytes of the arcuate nucleus and median eminence (Calas, 1985). Furthermore, neurotransmitters other than dopamine that elevate cAMP may be able to regulate the phosphorylation of DARPP-32 in certain DARPP-32 containing neurons that possess receptors for these neurotransmitters. For example, in Purkinje cells, cAMP levels can be increased by norepinephrine (Kakiuchi and Rall, 1968), which could result in the phosphorylation of DARPP-32. In the rat caudatoput-

amen, however, the regulation of the state of phosphorylation of DARPP-32 appears to be specific for dopamine (Walaas and Greengard, 1984), although peptide neurotransmitters have not yet been tested.

Most brain regions found to contain high levels of DARPP-32 (Walaas and Greengard, 1984; Hemmings and Greengard, 1986) have been shown to contain D_1-dopamine receptors. Conversely, DARPP-32 has been found to be absent from cells containing D_2-dopamine receptors, but not containing D_1-dopamine receptors (e.g., nigrostriatal dopaminergic neurons, corticostriatal nerve terminals, and anterior pituicytes). Despite this strong correlation, it is not yet possible to conclude whether DARPP-32 is present in all dopaminoceptive neurons containing D_1-dopamine receptors or whether it is absent from all nondopaminoceptive neurons and from dopaminoceptive neurons containing only D_2-dopamine receptors.

Purification and Biochemical Characterization

DARPP-32 was identified in bovine caudate nucleus cytosol and purified 435-fold to apparent homogeneity from this source (Hemmings et al., 1984a). Purified DARPP-32 has been extensively characterized, and some of its biochemical properties are summarized in Table 7. It is an acidic, highly elongated monomer that is both heat stable and acid soluble. It has a relative molecular mass of 32 kdalton, as determined by SDS-polyacrylamide gel electrophoresis (Hemmings et al., 1984a), and a molecular mass of 24 kdalton, as determined by high-speed sedimentation equilibrium centrifugation (Hemmings et al., 1984b). Amino acid sequencing indicates that the actual molecular mass is ~22 kdalton (Williams et al., 1986).

DARPP-32 is phosphorylated at a single threonine residue by cAMP-dependent protein kinase (Hemmings et al., 1984a,c). The amino acid sequence surrounding the phosphorylated threonine of DARPP-32 (Hemmings et al., 1984d) was found to include two proline residues flanking the phosphothreonine residue and four consecutive arginine residues amino-terminal to the phosphothreonine residue. Many of the biochemical properties of DARPP-32 were found to be remarkably similar to those of protein phosphatase inhibitor-1, in addition to its phosphorylation site sequence. Phosphatase inhibitor-1, in its phosphorylated form, is a potent and specific inhibitor of the enzyme protein phosphatase-1 (Huang and Glinsmann, 1976; Nimmo and Cohen, 1978). In order to determine whether the biochemical similarities between DARPP-32 and phosphatase inhibitor-1 were indicative of similar biochemical mechanisms of action, the effect of purified DARPP-32 on protein phosphatase activity was studied and compared to that of phosphatase inhibitor-1.

Interactions with Protein Phosphatases

The protein phosphatase activities involved in the dephosphorylation of most of the known proteins phosphorylated on serine or threonine residues can be accounted for by four distinct enzymes (Cohen, 1982; Ingebritsen and Cohen, 1983a,b). These enzymes are grouped into two classes: type 1 protein phosphatase (protein phosphatase-1) and type 2 protein phosphatases (protein phosphatases-2A, -2B, and -2C). Type 1 protein phosphatase selectively dephosphorylates the β-subunit of phosphorylase kinase and is inhibited by nanomolar concentrations of phosphatase inhibitor-1 or phosphatase inhibitor-2 (another protein phosphatase inhibitor). Type 2 protein phosphatases selectively dephosphorylate the α-subunit of phosphorylase kinase and are insensitive to these inhibitors.

Analysis of the effect of DARPP-32 on purified preparations of these four protein phosphatases showed that the phosphorylated form

TABLE 7

Summary of Biochemical Properties of DARPP-32[a]

Property	Method of determination	Value
Molecular weight	SDS-polyacrylamide gel electrophoresis Sedimentation equilibrium centrifugation	32 kdalton 24 kdalton
Stokes radius	Gel filtration	34 Å
Sedimentation coefficient	Sucrose density gradient centrifugation	2.05 *S*
Frictional ratio (f/f_0)	Stokes radius and sedimentation coefficient	1.7
Axial ratio	Strokes radius and sedimentation coefficient	13.5
Isoelectric point	Isoelectric focusing Phospho form Dephospho form	 4.6 4.7
Amino acid composition		High Glu/Gln and Pro; low hydrophobic residues
Phosphorylatable residue	Thin layer electrophoresis and chromatography	Threonine
K_m for cyclic AMP-dependent protein kinase	Kinetic analysis	2.4 μ*M*
K_{cat} for cyclic AMP-dependent protein kinase	Kinetic analysis	2.7/s
K_m for cyclic GMP-dependent protein kinase	Kinetic analysis	5.4 μ*M*
K_{cat} for cyclic GMP-dependent protein kinase	Kinetic analysis	2.3/s

[a]Data from Hemmings et al. (1984a,b,c).

of DARPP-32 inhibited protein phosphatase-1 noncompetitively, with an IC_{50} of approximately $10^{-9}M$ under the experimental conditions employed (Fig. 7). It showed no inhibitory activity toward protein phosphatase-2A, -2B, or -2C (Hemmings et al., 1984b). The dephosphorylated form of DARPP-32 was inactive as an inhibitor of protein phosphatase-1. Phosphorylated DARPP-32 itself was not a substrate for protein phosphatase-1. Rather, it was dephosphorylated most efficiently by the calcium/calmodulin-regulated enzyme protein phosphatase-2B (Hemmings et al., 1984b; King et al., 1984). This observation is of interest, since the substrate specificity of protein phosphatase-2B (also known as calcineurin) has been reported to be quite limited (Stewart et al., 1982; Ingebritsen and Cohen, 1983b). The potency and specificity of DARPP-32 as a protein phosphatase inhibitor and a substrate for the various protein phosphatases were very similar to those of phosphatase inhibitor-1. Thus, the basal ganglia of mammalian brain contain a region-specific neuronal phosphoprotein, namely DARPP-32, that is a potent inhibitor of protein phosphatase-1 in its phosphorylated form and is regulated by dopamine. Although DARPP-32 is very similar in its biochemical and functional properties to phosphatase inhibitor-1, DARPP-32 and phosphatase inhibitor-1 are clearly distinct proteins, as determined by amino acid sequencing, peptide mapping, and sensitivity to cyanogen bromide (Williams et al., 1986).

Physiological Role

Dopamine, acting at D_1-dopamine receptors, increases the state of phosphorylation of DARPP-32 in intact nerve cells by elevating cAMP levels and thereby activating cAMP-dependent protein kinase (Walaas et al., 1983c; Walaas and Greengard, 1984). These observations suggest that DARPP-32, as an intracellular "third messenger" for dopamine, may be involved in mediating certain of the actions of dopamine acting at D1-dopamine receptors. A molecular mechanism by which DARPP-32 may mediate some of the effects of dopamine on dopaminoceptive neurons has been discovered by studying the effects of DARPP-32 on the activity of purified preparations of protein phosphatases. According to this scheme, dopamine would produce some of its physiological effects by increasing the phosphorylation of DARPP-32, thereby inhibiting protein phosphatase-1.

Figure 8 illustrates two possible mechanisms by which the inhibition of protein phosphatase-1 could mediate some of the physiological effects produced by dopamine acting through cAMP. Regions of the brain containing DARPP-32, most notably the basal ganglia, also contain many other substrates for cAMP-dependent protein kinase (Walaas et al., 1983a,b). It is likely that many of these substrates can be dephosphorylated by protein phosphatase-1, as has been determined for other substrates of cAMP-dependent protein kinase (Ingebritsen and Cohen, 1983b). The phosphorylation and activation of DARPP-32 in these brain regions would therefore inhibit the dephosphorylation of these other substrates for cAMP-dependent protein kinase, thereby amplifying the effects of cAMP by a positive feedback mechanism (Hemmings et al., 1984b). Furthermore, phosphoproteins that are phosphorylated by protein kinases other than cAMP-dependent protein kinase are also dephosphorylated by protein phosphatase-1 (Ingebritsen and Cohen, 1983b). Thus, phosphorylation and activation of DARPP-32 may also allow dopamine, acting through cAMP, to modulate the phosphorylation state of substrates for other second-messenger regulated protein kinases. By this mechanism, dopamine and cAMP would be able to interact with other first- and second-messenger systems by regulating the state of phosphorylation of some of the same substrate proteins. The inhibition by dopamine, acting through the cAMP-dependent phosphorylation of DARPP-32, of the dephosphorylation by protein phosphatase-1 of substrate protein(s) regulated by another neurotransmitter, acting through another second

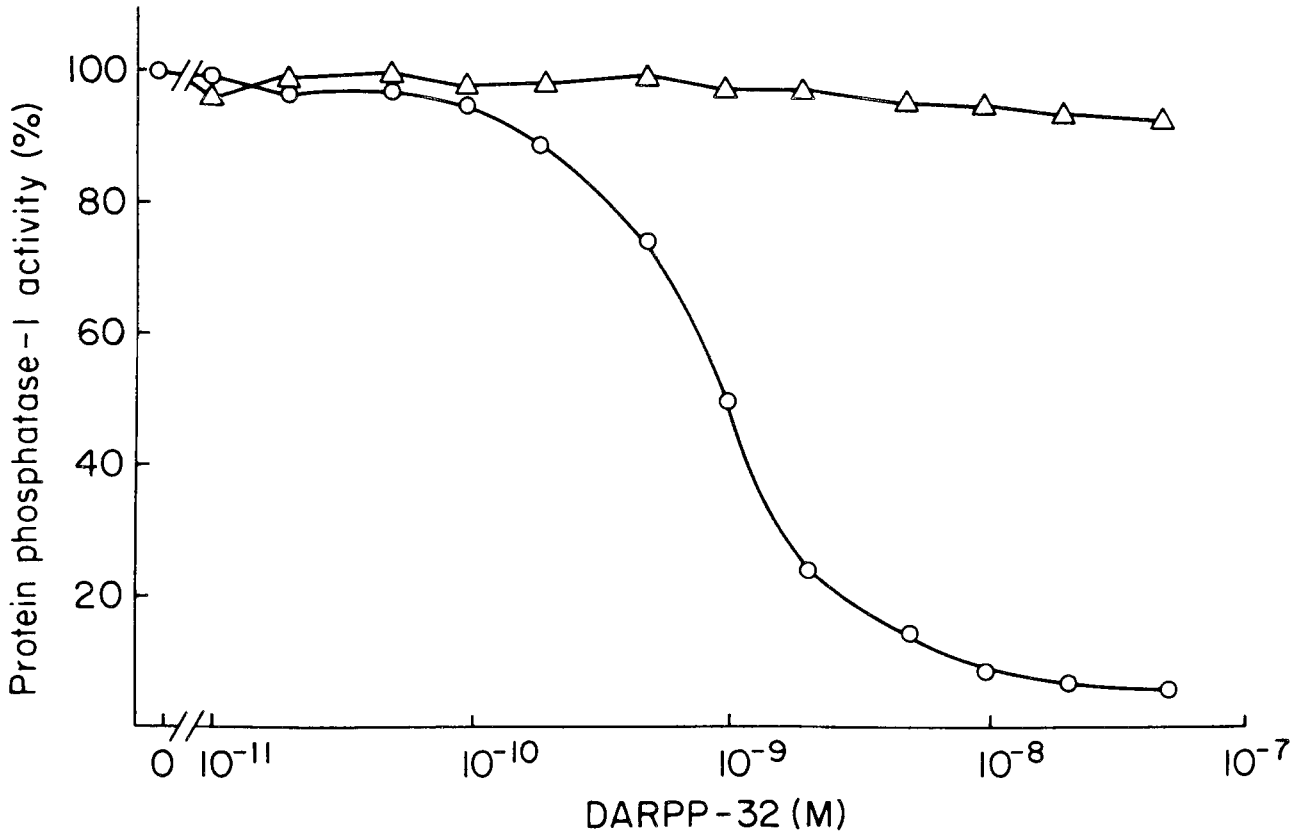

Fig. 7. Inhibiton of purified rabbit muscle protein phosphatase-1 by various concentrations of phosphorylated (circles) or dephosphorylated (triangles) DARPP-32. The activity of protein phosphatase-1 was determined by measuring the release of [^{32}P]-phosphate from [^{32}P]-phosphorylase a. Modified from Hemmings et al., 1984b.

messenger and protein kinase, would provide a molecular mechanism for the synergistic interaction between two neurotransmitters.

The interaction of dopamine and cAMP with other first- and second-messenger systems is also possible through another mechanism. DARPP-32 and protein phosphatase-2B are both highly concentrated in the basal ganglia within the medium-sized spiny neurons (Wallace et al., 1980; Wood et al., 1980; Ouimet et al., 1984; Walaas and Greengard, 1984; DeCamilli, unpublished observations). A role for protein phosphatase-2B in the regulation of the state of phosphorylation of DARPP-32 in vivo is likely, since DARPP-32 is a particularly effective substrate for this protein phosphatase in vitro (Hemmings et al., 1984b; King et al., 1984). The dephosphorylation of DARPP-32 by protein phosphatase-2B, a calcium/calmodulin-regulated enzyme (Fig. 9), provides a mechanism by which calcium, acting as a second messenger, could antagonize some of the effects of the dopamine-induced cAMP signal in dopaminoceptive neurons (Hemmings et al., 1984b).

Two potential interactions between the cAMP and calcium second-messenger systems, mediated through the regulation of the state of phosphorylation of DARPP-32 and the concomitant control of protein phosphatase-1 activity, are illustrated schematically in Fig. 9. These

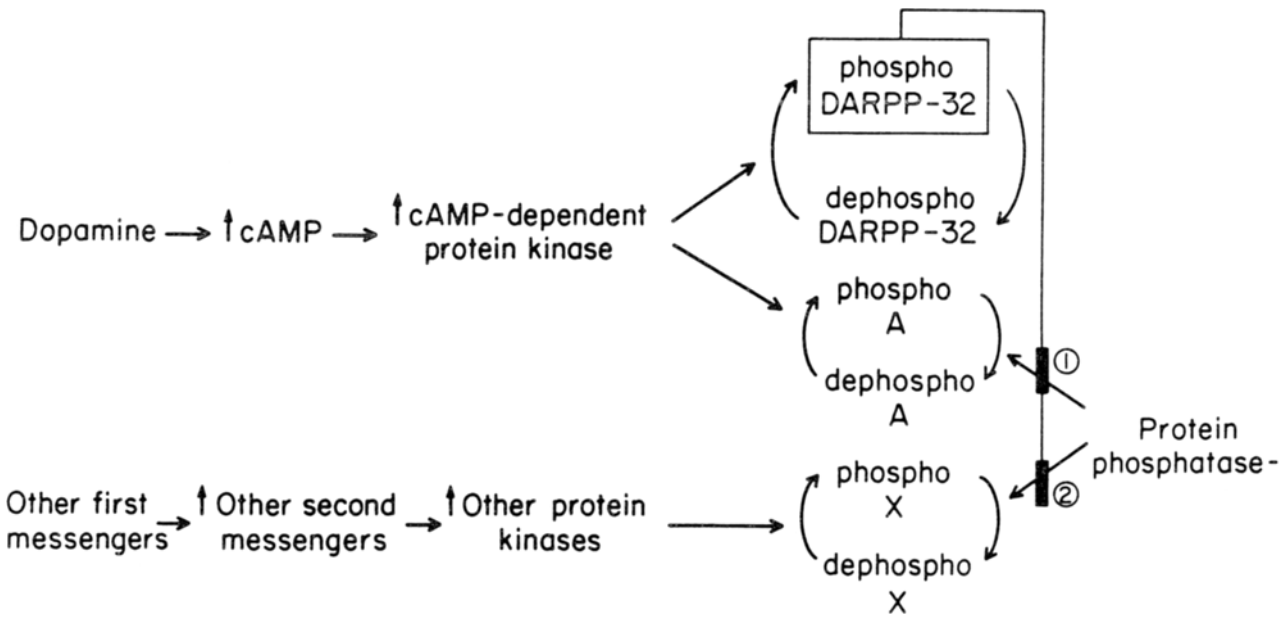

Fig. 8. Schematic diagram illustrating two possible mechanisms by which the inhibition of protein phosphatase-1 by DARPP-32 may be involved in mediating the physiological effects of dopamine on dopaminoceptive neurons posssessing D_1-dopamine receptors. The first messenger dopamine, acting through cyclic AMP and cyclic AMP-dependent protein kinase, stimulates the phosphorylation of DARPP-32 and of various other substrate proteins in target neurons. The phosphorylation of DARPP-32 converts it to an active inhibitor of protein phosphatase-1. Activated DARPP-32 then decreases the dephosphorylation of some of the proteins (represented by A), which are substrates for cyclic AMP-dependent protein kinase (arrow 1) and of other proteins (represented by X), which are substrates for other protein kinases (arrow 2). By increasing the phosphorylation of A, phosphorylated DARPP-32 represents a positive feedback signal through which the actions of dopamine are amplified. By increasing the phosphorylation of X, phosphorylated DARPP-32 represents a mediator through which dopamine modulates the actions of other first messengers.

interactions provide molecular mechanisms by which a protein phosphatase inhibitor can mediate either synergistic or antagonistic effects of one first messenger on another through the second-messengers cAMP and calcium. In this scheme, dopamine is shown to regulate the phosphorylation of DARPP-32 (Walaas et al., 1983c; Walaas and Greengard, 1984), and glutamate is shown as an example of a neurotransmitter that produces some of its effects through the elevation of intracellular calcium levels. Calcium may antagonize the effects of cAMP by activating a calcium/calmodulin-dependent protein phosphatase (protein phosphatase-2B), thereby leading to the dephosphorylation of DARPP-32 and, possibly, other substrate proteins for cAMP-dependent protein kinase. A synergistic effect of cAMP on the effects of calcium could occur by the cAMP-dependent phosphorylation of DARPP-32, leading to the inhibition of the dephosphorylation by protein phosphatase-1 of substrate proteins for calcium-dependent protein kinases. The synergistic effects produced by this latter mechanism would be reversed if the substrate protein being affected inhibited the response to glutamate by a negative feedback mechanism. This may occur in the medium-sized spiny neurons of the stria-

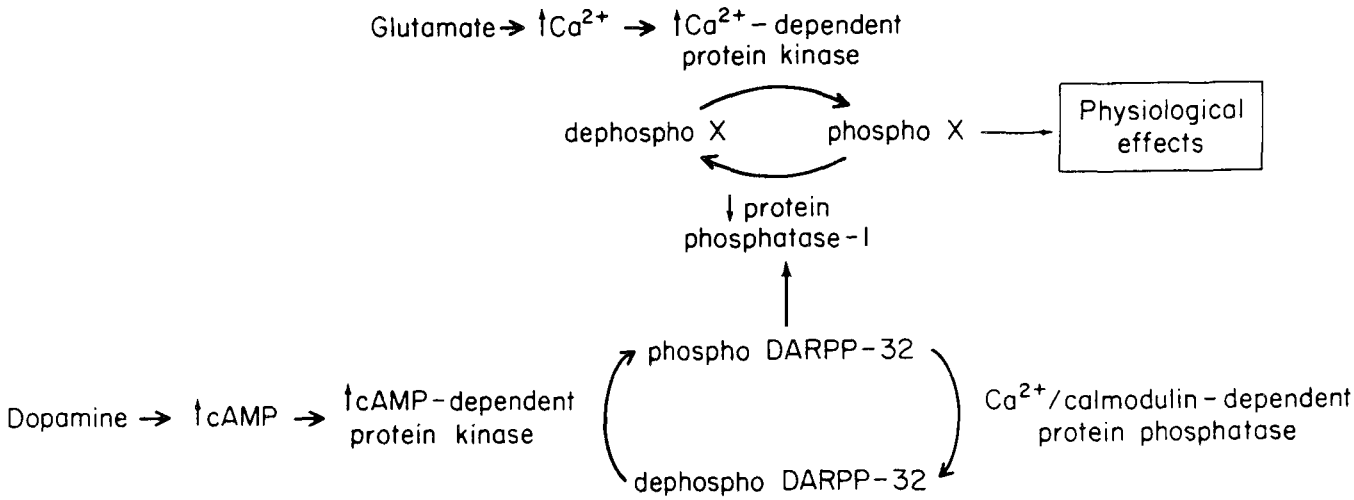

Fig. 9. Schematic diagram illustrating a possible role for DARPP-32 in mediating the interaction of dopamine with glutamate. The first messenger glutamate, which increases intracellular calcium levels, causes the activation of a calcium-dependent protein kinase and, subsequently, the phosphorylation of specific substrate proteins (represented by X), which are involved in mediating the physiological effects of glutamate. The first messenger dopamine, acting through cyclic AMP and cyclic AMP-dependent protein kinase, stimulates the phosphorylation of DARPP-32, which converts it to an active inhibitor of protein phosphatase-1 and thereby decreases the dephosphorylation of these substrate proteins for the calcium-dependent protein kinase. In this way, dopamine may modulate the response to glutamate. Another possible interaction between the cyclic AMP and calcium second messenger systems is through the calcium/calmodulin-dependent protein phosphatase (protein phosphatase-2B), which can dephosphorylate and inactivate phosphorylated DARPP-32.

tum, where dopamine, apparently acting through cAMP, antagonizes the ability of glutamate to depolarize certain dopaminoceptive neurons (Moore and Bloom, 1978; Bunney, 1979), possibly by inhibiting the dephosphorylation by protein phosphatase-1 of an "inhibitory modulator protein," which is phosphorylated and activated by glutamate acting through a calcium-dependent protein kinase and thereby inhibits glutamate-induced depolarization by a negative feedback mechanism (*see* Nestler and Greengard, 1984). From this example and the previous discussion, it is clear that many variations in the types of interactions between neurotransmitters are possible at the level of protein phosphatase inhibitors. It is likely that these interactions occur in many different types of neurons and between many pairs of neurotransmitters.

The regulation of protein phosphatase activity by the reversible phosphorylation of specific protein phosphatase inhibitors appears to be a particularly important mechanism of cellular regulation in the brain. In addition to phosphatase inhibitor-1, which is present in many tissues, including brain (Hemmings and Greengard, unpublished observations), two cell type-specific protein phosphatase inhibitors have been identified in brain. One of these, DARPP-32, is present in dopaminoceptive neurons possessing D_1-dopamine receptors and may function as a protein phosphatase inhibitor specific

for the dopamine system. The other, G-substrate, is a specific substrate protein for cGMP-dependent protein kinase that resembles phosphatase inhibitor-1 and DARPP-32 in many of its biochemical properties (Aitken et al., 1981; Aswad and Greengard, 1981; King et al., 1984). It is specifically localized within the Purkinje cells of the cerebellum (Schlichter et al., 1980; Detre et al., 1984) and has been found to inhibit a protein phosphatase isolated from cerebellum (Simonelli et al., unpublished observations). G-substrate thus appears to function as a Purkinje cell-specific protein phosphatase inhibitor. The study of these two neuronal phosphoproteins has revealed that the regulation of protein phosphatase activity by the phosphorylation and activation of specific inhibitor proteins appears to be a prominent regulatory mechanism in brain and suggests that this mechanism may be common to the action of several neurotransmitters. Further studies on the role of DARPP-32 in the control of protein phosphorylation in dopaminoceptive neurons should provide additional insights into the molecular mechanisms of the transsynaptic actions of dopamine.

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