Neuronal Phosphoproteins: Physiological and Clinical Implications

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Summary. The presence of a great variety of neuron-specific phosphoproteins in nervous tissue supports the view that protein phosphorylation plays many roles in neuronal function. The physiological significance of several of these phosphoproteins has already been established. Some neuronal phosphoproteins have been detected throughout the entire nervous system, whereas the distribution of others is limited to one or a few neuronal cell types. These various neuron-specific phosphoproteins are proving of value in the study of the physiology, anatomy, developmental biology, and pathophysiology of the nervous system.

Many types of physiological stimuli produce diverse synaptic responses in the nervous system by regulating the state of phosphorylation of specific phosphoproteins in target neurons (*1, 2*). The detection, purification, and characterization of neuronal phosphoproteins, and the elucidation of their physiological roles, is leading to an understanding of the molecular mechanisms by which neurons react with specific physiological responses to various stimuli. In this article, we summarize recent evidence for a vital role of phosphoproteins in neuronal function.

Regulation of Neuronal Protein Phosphorylation

A large number of extracellular signals have been shown to regulate protein phosphorylation in the nervous system. Those extracellular signals, or first messengers, include several types of neurotransmitters and hormones, as well as the nerve impulse itself (Table 1).

A first messenger can alter protein phosphorylation in target tissues by either directly or indirectly regulating the activity of a protein kinase, the enzyme responsible for the phosphorylation of substrate proteins, or a protein phosphatase, the enzyme responsible for the dephosphorylation of substrate proteins. Activation of specific protein kinases appears to be the most prominent mechanism in nervous tissue by which first messengers regulate protein phosphorylation. Numerous first messengers activate protein kinases indirectly by increasing the intracellular level of a second messenger in target neurons. Prominent second messengers in the nervous system include cyclic adenosine monophosphate (AMP), cyclic guanosine monophosphate (GMP), and calcium (Table 1). Some first messengers have been shown to activate protein kinases directly. For example, insulin activates a protein kinase activity associated with its plasma membrane receptors in diverse tissues, including brain (*1, 3*).

Brain contains virtually one type of cyclic AMP–dependent protein kinase and one type of cyclic GMP–dependent protein kinase, but several types of calcium-dependent protein kinases (*1, 4*). Two subclasses of calcium-dependent protein kinases are known. One is activated by calcium in conjunction with the calcium-binding protein calmodulin and is referred to as calcium-calmodulin–dependent protein kinase. The other is activated by calcium in conjunction with phosphatidylserine and diacylglycerol and is referred to as calcium-phosphatidylserine–dependent protein kinase or protein kinase C (*5*).

Direct evidence for a role of these second messenger–dependent protein kinases in neuronal function has been obtained by a number of investigators (*1, 2, 6*). Injection of purified protein kinases into individual neurons or other excitable cells has been shown to mimic the ability of first messengers to elicit specific physiological responses in those cells. Conversely, injection of inhibitors of the kinases into the cells has been shown to block the ability of the first messengers to elicit those responses. These results indicate that activation of protein kinases is an obligatory step in the sequence of events by which first messengers produce some physiological responses in the nervous system.

A number of other protein kinases, which are reported to phosphorylate specific substrate proteins and/or to be associated with specific subcellular fractions, have been found in brain. These include rhodopsin kinase, myelin kinase, neurofilament kinase, and coated vesicle kinase (*1*). Further work is needed to clarify whether these kinases are related to the second messenger–dependent protein kinases mentioned above. In addition, several other protein kinases that appear to play roles in the regulation of generalized cellular processes, such as intermediary metabolism, protein synthesis, and cell growth, are present in diverse tissues, including brain.

Several distinct types of protein phosphatases have been found in brain. These include phosphatases 1, 2A, 2B, and 2C (*7*). Some protein phosphatases, like the protein kinases, are regulated by the second messengers—cyclic AMP, cyclic GMP, and calcium (*1, 7, 8*). In some cases, second messengers regulate protein phosphatase activity directly. For example, phosphatase 2B, also referred to as calcineurin, is activated directly upon binding calcium and calmodulin. In other cases, second messengers regulate protein phosphatases indirectly via actions on phosphatase inhibitors, as discussed below. These findings indicate that certain first messengers in the nervous system produce physiological responses in part through the regulation of protein phosphatases (see below).

Classes of Neuronal Proteins Regulated by Phosphorylation

Analysis of patterns of protein phosphorylation in different brain regions, by one-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, has revealed that more than 70 proteins are substrates for cyclic AMP–dependent, cyclic GMP–dependent, or calcium-dependent protein kinases and appear to be specific to nervous tissue (*9*). These 70 proteins exhibit a great

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diversity in their characteristics. Analysis of brain protein phosphorylation by two-dimensional electrophoresis has revealed the existence of an even larger number of neuron-specific phosphoproteins in the nervous system. The large number and diversity of neuronal proteins that undergo phosphorylation supports the view that protein phosphorylation has numerous and varied roles in the nervous system. Those neuronal phosphoproteins that have been most intensively studied fall into certain categories (Table 2) and include enzymes involved in neurotransmitter biosynthesis, enzymes involved in cyclic nucleotide metabolism, autophosphorylated protein kinases, protein phosphatase inhibitors, proteins involved in the regulation of transcription and translation, cytoskeletal proteins, synaptic vesicle–associated proteins, neurotransmitter receptors, and ion channels. Regulation of these neuronal proteins by phosphorylation has been reviewed recently (*1*, *2*). In several cases, the functional roles of the phosphorylation reactions have already been established.

Some general points should be made about certain of the classes of phosphoproteins listed in Table 2. Autophosphorylated protein kinases are among the most prominent substrates observed for those kinases in endogenous phosphorylation assays in nervous and non nervous tissue. Thus, prominent substrates for protein kinases activated by cyclic AMP, cyclic GMP, calcium, epidermal growth factor, and insulin have been shown to be autophosphorylated subunits of the respective protein kinases in certain target cells (*1*). These findings indicate that protein kinases are present in cells at higher concentrations than are many of their substrate proteins. This might be expected for certain substrate proteins, such as neurotransmitter receptors and ion channels, that are present in cells at very low concentrations. In such cases, high levels of protein kinase may be necessary in order to ensure rapid phosphorylation of the substrates. This is in contrast to the classical concept that the concentration of enzyme is much less than that of its substrate.

It now appears that autophosphorylation of protein kinases is the rule rather than the exception, although the physiological role of autophosphorylation is not yet clear. The autophosphorylation of a number of protein kinases is associated with an increase in kinase activity. In some instances, autophosphorylation may represent an obligatory step in the sequence of molecular events through which those kinases are activated, and in others a positive feedback mechanism through which the kinases are activated to a greater extent. Inhibitors of protein phosphatases, like autophosphorylated protein kinases, are among the most prominent phosphoproteins found in brain. If, as seems likely, the phosphorylated moieties of the protein kinases are involved in the regulation of kinase activity, then those phosphorylated moieties and the phosphatase inhibitors subserve analogous functional roles as regulatory components of the protein kinases and protein phosphatases, respectively.

Several neurotransmitter receptors have been reported to be associated with protein phosphorylation systems (see Table 2), although the exact nature of the association may differ in each case. For example, the nicotinic acetylcholine receptor from the electroplax tissue of *Torpedo* is rapidly and specifically phosphorylated on the γ and δ subunits by cyclic AMP–dependent protein kinase, on the δ subunit by calcium-phosphatidylserine–dependent protein kinase, and on the β, γ, and δ subunits by a tyrosine-specific protein kinase endogenous to *Torpedo* electroplax (*10*). As another example, the β-adrenergic receptor is phos-

Table 1. Signals in the nervous system (*1*, *33*).

Extracellular signals	Intracellular signals		
First messenger*	Second messenger	Protein kinase†	Third messenger (phosphoprotein)
Nerve impulse	Calcium	Calcium-calmodulin	Synapsin I; protein III
	Calcium	Calcium-phosphatidylserine	87K
Neurotransmitters			
Dopamine	Cyclic AMP	Cyclic AMP	Synapsin I; protein III; DARPP-32
Serotonin	Cyclic AMP	Cyclic AMP	Synapsin I; protein III; substrates in *Aplysia* neuron R15, pineal gland
Norepinephrine	Cyclic AMP	Cyclic AMP	Synapsin I; protein III
Acetylcholine	Cyclic AMP	Cyclic AMP	Tyrosine hydroxylase
	Calcium	Calcium-calmodulin	Tyrosine hydroxylase
	Calcium	Calcium-phosphatidylserine	Tyrosine hydroxylase
	Calcium		Substrates in *Torpedo* synaptosomes
Opiates			Substrates in striatum
Hormones			
Corticosterone		Cyclic AMP	Cyclic AMP-dependent protein kinase (type 2 regulatory subunit)
Estradiol			Substrates in hypothalamus, cerebral cortex
Testosterone			Substrates in hypothalamus, amygdala
Other			
Light	None	Rhodopsin	Rhodopsin
	Cyclic GMP	Cyclic GMP	Substrates in rod outer segments
Nerve growth factor			Ribosomal protein S6; tyrosine hydroxylase; histones H1 and H3; substrates in sympathetic ganglia, pheochromocytoma cells
Repetitive neuronal activity			Synapsin I; substrates in hippocampus, cerebral cortex
		Pyruvate dehydrogenase	Pyruvate dehydrogenase

*Only those first messengers that have been shown to regulate protein phosphorylation in intact nervous tissue are included. Where the second messenger and protein kinase are not indicated, it is not known whether the first messenger alters substrate protein phosphorylation by regulating the activity of a protein kinase or a protein phosphatase. †Protein kinase abbreviations: calcium-calmodulin, calcium-calmodulin–dependent protein kinase; calcium-phosphatidylserine, calcium-phosphatidylserine–dependent protein kinase (protein kinase C); cyclic AMP, cyclic AMP–dependent protein kinase; rhodopsin, rhodopsin kinase; cyclic GMP, cyclic GMP–dependent protein kinase; pyruvate dehydrogenase, pyruvate dehydrogenase kinase.

phorylated in intact turkey erythrocytes, where it appears to be a substrate for cyclic AMP–dependent protein kinase. This phosphorylation is stimulated by β-adrenergic agonists and may mediate desensitization of the receptor (*11*).

In addition to neurotransmitter receptors, several other classes of receptors are associated with protein phosphorylation systems (*1*). Membrane receptors for several types of growth factors contain tyrosine-specific protein kinase activity. Binding of the growth factors to their respective receptors results in the activation of this receptor-associated kinase activity and in the phosphorylation of the receptor molecule. Cytosolic receptors for several types of steroid hormones, including estradiol, progesterone, and glucocorticoids, have been shown to be phosphorylated by various protein kinases in vitro or in vivo. Phosphorylation of the estradiol receptor by a calcium-calmodulin–dependent protein kinase in vitro alters the affinity of the receptor for estradiol. Membrane receptors for immunoglobulin E, plasma glycoproteins, and transferrin (*12*) are also phosphoproteins. The large number and diversity of receptors that undergo phosphorylation suggests that regulation of receptor function by phosphorylation is a common and physiologically important property of a wide variety of receptors.

It appears that diverse molecular mechanisms underlie the regulation of receptor function by protein phosphorylation (*1*).

1) The nature of the physical association between the receptor molecule and the protein kinase involved in the regulation of its function varies among receptors. In some cases, the protein kinase and the ligand binding domain of the receptor reside in the same polypeptide chain, whereas, in other cases, they reside in different subunits of the same receptor molecule. In still other cases, the protein kinase and the receptor molecule are only loosely associated.

2) The nature of the physical association between the phosphorylated protein and the receptor molecule also varies among receptors. For example, the phosphorylated protein and the ligand binding subunit in some cases represent the same polypeptide chain, and in other cases distinct subunits of the receptor molecule. In still other cases, the phosphorylated protein appears to be a "regulator" protein and not a constituent of the receptor.

3) Some receptor-associated protein kinases may be activated by first messengers other than ligands for the receptors, whereas other such kinases are activated by the receptor ligands. In each case, such activation could be achieved either directly through some allosteric interaction with the receptor, as is the situation with growth factor-associated protein kinases, or indirectly through some second messenger.

4) Although little is known about the role of receptor-associated phosphorylation reactions in receptor function, several possibilities exist. Phosphorylation of the receptor or of receptor-associated proteins may be an obligatory step in the sequence of events through which ligand

Table 2. Neuronal proteins regulated by phosphorylation. Some of the proteins included are specific to neurons. The others are present in many cell types in addition to being present in neurons and are included because among their multiple functions in the nervous system is the regulation of neuron-specific phenomena. Not included are the many phosphoproteins present in diverse tissues (including brain) that play roles in generalized cellular processes, such as intermediary metabolism, and that do not appear to play roles in neuron-specific phenomena (*1, 2, 8, 10, 11, 34*).

Enzymes involved in neurotransmitter biosynthesis
Tyrosine hydroxylase
Tryptophan hydroxylase
Enzymes involved in cyclic nucleotide metabolism
Adenylate cyclase
Guanylate cyclase
Phosphodiesterase
Autophosphorylated protein kinases
Cyclic AMP–dependent protein kinase
Cyclic GMP–dependent protein kinase
Calcium-calmodulin–dependent protein kinases
Calcium-phosphatidylserine–dependent protein kinase (protein kinase C)
Casein kinases
Tyrosine-specific protein kinases
Double-stranded RNA-dependent protein kinase
Rhodopsin kinase
Phosphatase inhibitors
Inhibitor 1
Inhibitor 2
DARPP-32
G substrate
Proteins involved in transcription and translation regulation
RNA polymerase
Histones
Nonhistone nuclear proteins
Ribosomal protein S6
Other ribosomal proteins
Cytoskeletal proteins
MAP-2
Tau
Other microtubule-associated proteins
Neurofilaments
Calspectin
Myosin light chain
Actin
Synaptic vesicle-associated proteins
Synapsin I
Protein III
Clathrin
Neurotransmitter receptors
Nicotinic acetylcholine receptor
Muscarinic acetylcholine receptor
β-Adrenergic receptor
GABA receptor (GABA-modulin)
Ion channels*
Voltage-dependent:
Sodium channel
Potassium channel
Calcium channel
Calcium-dependent:
Potassium channel
Neurotransmitter-dependent:
Nicotinic acetylcholine receptor
Serotonin-regulated potassium channel in *Aplysia* sensory neurons
Serotonin-regulated (anomalously rectifying) potassium channel in *Aplysia* neuron R15
Other:
Sodium channel in rod outer segments

*Several of the ion channels listed have been shown to be physiologically regulated by protein phosphorylation reactions, although it is not yet known whether such regulation is achieved directly through the phosphorylation of the ion channel, or indirectly through the phosphorylation of a modulatory protein that is not a constituent of the ion channel molecule.

binding leads to a physiological response. Alternatively, such phosphorylation could modulate receptor activation either by altering the affinity of the receptor for its ligand or by altering the total number of receptors available to the ligand. In either situation, such phosphorylation would alter the ability of the ligand to produce a physiological response.

Many types of ion channels are also regulated by phosphorylation (Table 2). Some neurotransmitter-dependent ion channels, like the nicotinic acetylcholine receptor, are "receptor–ion channels" in that the neurotransmitter receptor and the ion channel involved reside in the same physical complex. In this situation, the neurotransmitter regulates ion channel function by directly inducing some allosteric change in the receptor–ion channel complex. With other neurotransmitter-dependent ion channels, such as the serotonin-regulated potassium channels in *Aplysia*, the neurotransmitter receptor and the ion channel are distinct entities and the neurotransmitter regulates ion channel function indirectly through some second messenger. The diversity of ion channels altered by phosphorylation suggests that regulation of ion channel function by phosphorylation is a common and physiologically important property of many ion channels. Diverse molecular mechanisms, analogous to those described above for receptors, presumably underlie the regulation of ion channel function by protein phosphorylation (*1*).

Among the most prominent neuronal phosphoproteins is the synaptic vesicle–associated protein synapsin I (*1, 2*). Study of this protein has suggested a new mechanism by which synaptic vesicle traffic is regulated. Calcium-calmodulin–dependent protein kinase II phosphorylates two sites in the "tail region" of synapsin I and their phosphorylation appears to control the attachment of synapsin I to neurotransmitter vesicles (*13*). Recent studies, with the squid giant synapse, have provided direct evidence for a role of synapsin I and of calcium-calmodulin–dependent protein kinase II in regulating neurotransmitter release (*14*). In these studies these proteins were injected into the presynaptic terminal digit of the giant synapse, and the effect on presynaptic calcium influx (as measured under presynaptic voltage-clamp conditions), and on neurotransmitter release (as measured by the postsynaptic potential), were determined. Injection of these substances modified neurotransmitter release, without detectable alteration of the presynaptic calcium current. The results obtained support the hypothesis that the dephospho form of synapsin I limits the availability of synaptic vesicles for neurotransmitter release, and that its phosphorylation by calcium-calmodulin–dependent protein kinase II relieves this inhibition (*14*). This mechanism may act synergistically with other protein phosphorylation mechanisms (*1, 2, 6*) that regulate calcium currents in presynaptic nerve terminals.

Regional Distribution of Neuron-Specific Phosphoproteins

Distinct patterns of phosphoproteins are observed when discrete regions of the brain are analyzed (Fig. 1). These distinct patterns of phosphoproteins can be understood, given the microhomogeneity of the brain, that is, the limited number of cell types present within any brain region. Such studies on the regional distribution of neuron-specific phosphoproteins have indicated that, of the large number of such proteins so far detected, some are present throughout the entire nervous system, whereas others have more limited distributions.

Neuron-specific phosphoproteins can be conveniently divided into three categories, according to their patterns of regional distribution (*9*). Category A phosphoproteins are widely and fairly evenly distributed throughout the nervous system, and may be present in virtually all nerve cells. Category B phosphoproteins are relatively widely, but unevenly, distributed throughout the nervous system; these phosphoproteins may be enriched in certain classes of nerve cells, and present in low levels or absent from other nerve cells. Category C phosphoproteins are restricted to one brain region only, where they are presumably localized to a single neuronal cell type.

The distribution patterns for certain neuron-specific phosphoproteins have been studied in considerable detail. Category A phosphoproteins include the synaptic vesicle–associated proteins synapsin Ia, synapsin Ib, protein IIIa, and protein IIIb (*1*). They appear to be present in virtually all axon terminals throughout the nervous system, and their expression coincides with synaptogenesis. These synaptic vesicle–associated phosphoproteins have proved useful as general markers for the study of axon terminals in both developing and adult brain. For instance, measurement of the total amount of synaptic vesicle–associated phosphoproteins by radioimmunoassay has provided a quantitative

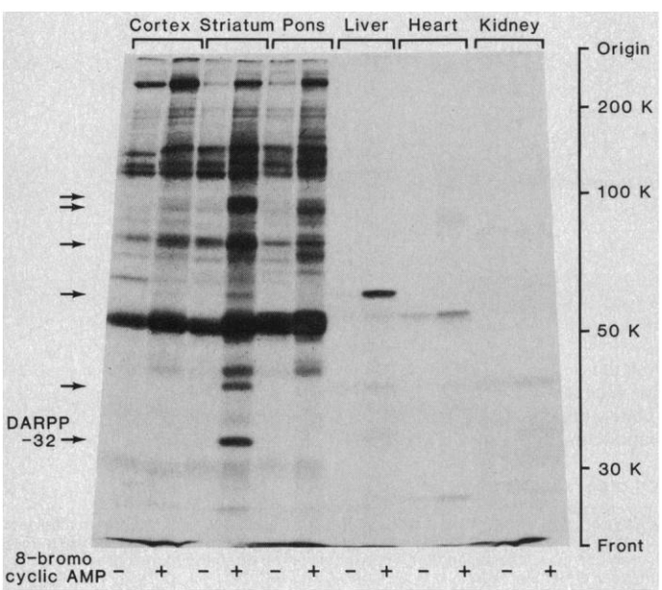

Fig. 1. Protein phosphorylation in cytosol from three brain regions and various peripheral organs of the rat. Portions of cytosol from rat cerebral cortex, striatum and brainstem (pons), and from liver, heart, and kidney, containing equal amounts of protein, were incubated with [γ-^{32}P]ATP in the absence or presence of 8-bromo cyclic AMP (2 μM) for 60 seconds. The reactions were stopped by the addition of SDS, and the phosphoproteins were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. The autoradiogram was overexposed in order to visualize the phosphoproteins from the peripheral organs. Brain tissue showed much more extensive cyclic AMP–dependent protein phosphorylation than did the peripheral organs, as evidenced by total ^{32}P incorporation and the number of individual phosphoproteins. Different brain regions contained some similar and some distinct phosphoproteins. Arrows point to phosphoproteins prominant in the striatum, including DARPP-32, a dopamine- and cyclic AMP–regulated phosphoprotein discussed in the text. Similar analysis of Ca^{2+}-dependent protein phosphorylation systems also showed much greater protein phosphorylation in brain than in peripheral organs.

method by which to determine the density of nerve terminals in various regions of the central and peripheral nervous systems under normal and pathological conditions (*1, 15*). Moreover, measurement of the state of phosphorylation of these proteins has been used to characterize presynaptic neurotransmitter receptors in various brain regions. Each of the four phosphoproteins is an endogenous substrate in nervous tissue for both cyclic AMP–dependent and calcium-calmodulin–dependent protein kinase, and their phosphorylation is regulated in intact nervous tissue by neurotransmitters that act through either cyclic AMP or calcium (*1*). Thus, by measuring changes in the phosphorylation state of synapsin I or protein III in response to a given neurotransmitter, it has been possible to reveal previously undetected presynaptic receptors, to study the physiological regulation of these receptors, and to quantify the fraction of nerve terminals in a particular region of the nervous system that contains a certain class of receptor. As an example of this latter application, quantitation of norepinephrine-induced phosphorylation of synapsin I has disclosed that at least 30 percent of nerve terminals in the rat frontal cortex have β-adrenergic receptors (*16*). Similarly, quantitation of dopamine-induced phosphorylation of synapsin I within the basal ganglia has revealed that the proportion of axon terminals with D_1-dopamine receptors is much greater in the substantia nigra than in the neostriatum (*17*).

Category B phosphoproteins include DARPP-32, a dopamine- and cyclic AMP–regulated phosphoprotein with a molecular weight of 32,000 (32K) (Fig. 1), which is enriched in brain regions that are densely innervated by dopaminergic neurons (*18*). Within these regions, DARPP-32 is localized to dopaminoceptive neurons that possess the D_1 receptor. Anatomical studies of DARPP-32 have revealed an intricate and specific distribution pattern for dopaminoceptive neurons throughout the brain (see cover). Another category B phosphoprotein is tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines. This phosphoprotein is restricted to nerve cells that utilize catecholamines as neurotransmitters, and has been used to map the localization of catecholaminergic neurons in the nervous system (*19*). Other examples of category B phosphoproteins are the 50K and 60K autophosphorylated subunits of calcium-calmodulin–dependent protein kinase II (*9, 20*). This protein kinase appears to be present in all nerve cells, but its concentration varies markedly among different brain regions. Its distribution in brain is strikingly similar to that of sodium-independent glutamate binding sites, which probably represent glutamate receptors (*21*). Since activation of glutamate receptors leads to calcium flux into neurons (*22*), this protein kinase may be involved in mediating certain of the transsynaptic effects of glutamate in the brain. Calcium-calmodulin–dependent protein kinase II may prove useful in the study of the anatomical and functional properties of those neurons that are innervated by glutamatergic nerve terminals. Presumably, other category B phosphoproteins in the nervous system are similarly associated with specific neurotransmitters or their receptors.

Two substrate proteins specific to the cerebellar Purkinje cell are examples of category C phosphoproteins. These are a cytosolic substrate for cyclic GMP–dependent protein kinase (G substrate), and a particulate substrate for cyclic AMP–dependent protein kinase (*23*). In addition, within the brain, cyclic GMP–dependent protein kinase is also highly enriched in Purkinje cells (*1, 23*). These three proteins represent ideal tools with which to analyze Purkinje cell anatomy and development (*24*). Furthermore, identification of the functions of these category C phosphoproteins should reveal functional characteristics specific to this type of neuron.

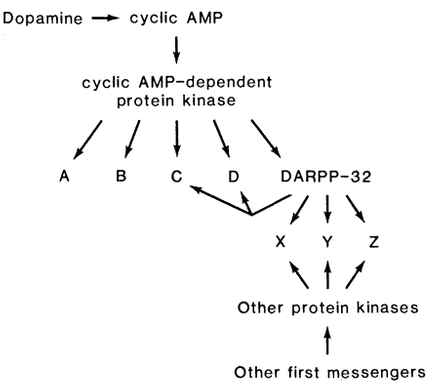

Fig. 2. Diagram of proposed roles that protein phosphatase inhibitors may play in the regulation of neuronal function. The first messenger dopamine, acting through cyclic AMP and cyclic AMP–dependent protein kinase, stimulates the phosphorylation of several proteins (A–D, DARPP-32) in an individual target neuron. The phosphorylation of A, B, C, and D leads directly to certain of the physiological effects of dopamine in this neuron. The phosphorylation of DARPP-32 converts it to an active phosphatase inhibitor. Activated DARPP-32 then decreases the dephosphorylation of some proteins (C and D) that are substrates for cyclic AMP-dependent protein kinase, and of other proteins (X, Y, and Z) that are substrates for other protein kinases. By increasing the phosphorylation of C and D, DARPP-32 represents a positive feedback signal through which dopamine modulates its own actions. By increasing the phosphorylation of X, Y, and Z, DARPP-32 represents a mediator through which dopamine modulates the actions of other first messengers.

Phosphoproteins of categories A, B, and C should also prove of value in clinical neurology. Recent studies have shown that protein III, a category A phosphoprotein, is present in small amounts in the cerebrospinal fluid of healthy human subjects (*25*). This raises the possibility that neuronal damage in the brain can be detected and quantitated in clinical settings by measurement of protein III levels in cerebrospinal fluid. The presence of category B or C phosphoproteins in such samples may indicate the region of the brain sustaining damage. Such proteins could thereby represent highly specific and sensitive tools to diagnose the progression or regression of a variety of neurological diseases (*1*), and to evaluate potential neurotoxic substances (*26*).

Phosphoproteins of the Basal Ganglia

Study of the regional distribution of brain phosphoproteins has revealed several that are specifically enriched in the basal ganglia (*9*). The basal ganglia, which in the mammalian brain include the caudatoputamen, the nucleus accumbens, the olfactory tubercle (together comprising the neostriatum), the globus pallidus, the subthalamic nucleus, and the substantia nigra, are of considerable interest to both the basic and clinical neuroscientist (*27*). The anatomy and neurotransmitters of these regions have been relatively well characterized, and several well-defined disease entities have been shown to involve them. For example, Parkinson's disease is associated with degeneration of nigrostriatal dopaminergic neurons, while Huntington's chorea is associated with degeneration of striatonigral dopaminoceptive neurons. Furthermore, several classes of antipsychotic drugs are believed to induce extrapyramidal side effects by virtue of their blockade of dopamine receptors in the basal ganglia. Studies of basal ganglion phosphoproteins should help elucidate the pathophysiology of these disorders.

The transsynaptic effects of dopamine are believed to be mediated by at least two dopamine receptors, one of which, the D_1 receptor, is linked to stimulation of adenylate cyclase. It is, therefore, of considerable interest that the basal ganglia, which are rich in dopamine, contain

several substrates for cyclic AMP–dependent protein kinase (*9*). These substrates, which include at least one particulate and six soluble phosphoproteins, among which is DARPP-32, may be involved in physiological or pharmacological effects of dopamine mediated through the D_1 receptor.

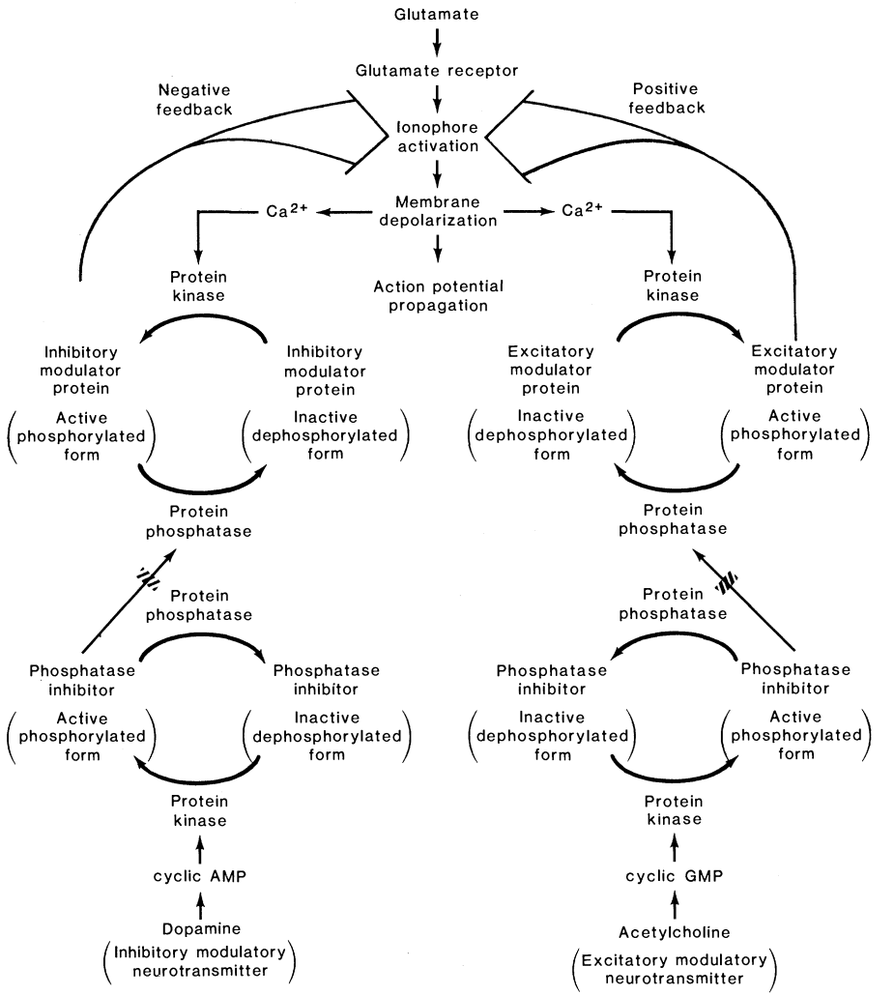

Fig. 3. Mechanism by which some modulatory neurotransmitters may affect synaptic transmission through the regulation of phosphatase inhibitors. A mediatory neurotransmitter (glutamate) elicits a biological response by binding to its membrane receptor, which leads successively to the activation of specific ion channels, the depolarization of the neuronal membrane, and the propagation of action potentials. In addition to eliciting this direct biological response, glutamate may also stimulate (negative or positive) feedback pathways that modulate the direct response. Such modulation might occur through an increase in intracellular calcium (achieved through an increase in the conductance either of ion channels associated with the glutamate receptor or of voltage-sensitive calcium channels activated as a result of membrane depolarization). The increase in intracellular calcium causes the activation of a calcium-dependent protein kinase and changes in the phosphorylation and activity of an inhibitory or excitatory modulator protein. Modulatory neurotransmitters (dopamine in the striatum and acetylcholine in the cerebellum, respectively) inhibit or enhance the response to glutamate through increases in cyclic AMP or cyclic GMP, which lead successively to the activation of cyclic AMP–dependent or cyclic GMP–dependent protein kinase, the phosphorylation and activation of a specific phosphatase inhibitor (DARPP-32 in the case of dopamine in the striatum; G substrate in the case of acetylcholine in the cerebellum), and the decreased dephosphorylation of a modulator protein, which, in its phosphorylated form, opposes (dopamine–cyclic AMP system) or facilitates (acetylcholine–cyclic GMP system) the responsiveness of the neurons to glutamate. Modulation of this responsiveness could occur at any of several levels from glutamate-receptor interaction to the propagation of action potentials, as indicated by the broad arrow. Variations in the types of interactions between neurotransmitters at the level of phosphatase inhibitors probably occur in various types of neurons. As examples of such variations, a phosphatase inhibitor or a modulator protein could be active in the dephosphorylated, rather than the phosphorylated, form. Inhibitor 2, a known phosphatase inhibitor, loses its inhibitory activity upon phosphorylation (*32*). Also, a given neurotransmitter may produce both inhibitory and excitatory effects by indirectly stimulating, through the regulation of a phosphatase inhibitor, the phosphorylation of both inhibitory and excitatory modulator proteins. The glutamate-dopamine and glutamate-acetylcholine interactions proposed are examples of the interactions that we believe occur among many pairs of neurotransmitters at the level of phosphatase inhibitors [modified from (*1*)].

DARPP-32 is the best characterized of these substrates and has served as a prototype for the study of basal ganglion phosphoproteins (*18*). A variety of techniques, including microdissection, lesion studies, and immunocytochemistry, have shown that the protein is enriched in dopaminoceptive cells, and that it is restricted to the subclass of those cells that possess D_1 receptors. It is present in the dendrites, cell bodies, axons, and axon terminals of all medium spiny neurons in the neostriatum, which have D_1 receptors, but not in the giant cholinergic interneurons, the corticostriatal fibers, or the dopaminergic neurons themselves, all of which have D_2 receptors. DARPP-32 is also present in the posterior pituitary, which has both D_1 and D_2 receptors, but is absent from the anterior pituitary, which has only D_2 receptors. Furthermore, the protein is phosphorylated in intact slices of neostriatum by dopamine or 8-bromo cyclic AMP, and it is a good substrate for cyclic AMP–dependent protein kinase in broken cell and purified preparations. These studies have, therefore, established both an anatomical and a physiological link between DARPP-32 and dopaminergic neurotransmission.

DARPP-32 represents a unique tool for the identification and ultrastructural study of the D_1 subclass of dopaminoceptive neurons throughout the nervous system, and for the hodological analysis of the dendritic and axonal projections of these cells (*18*). High resolution electron microscopic immunocytochemical studies of DARPP-32 have enabled the visualization of medium spiny neurons in the neostriatum (which represent about 96 percent of all neurons in this region), and the projections of these neurons to the globus pallidus and substantia nigra. The part of the ventral pallidum that receives fibers from the ventral striatum can be displayed with unequaled clarity and definition by DARPP-32 staining. DARPP-32 has also yielded valuable information about the phylogenetic development of dopaminoceptive neurons, particularly in the basal ganglia. Thus, the regional distribution of DARPP-32 in the brains of several mammalian and nonmammalian vertebrates corresponds to that of dopamine, and probably reflects the different locations of dopaminoceptive neurons in these various species (*28*).

Studies of region-specific phosphoproteins should contribute to the construction of a biochemical anatomy of the brain [see also (*29*)]. A special advantage of this approach, in contrast to standard

neuroanatomical techniques, is that for a given type of neuron the localization of a specific phosphoprotein enables the histological identification of all of those neurons and of all of their projections. Thus, immunocytochemical studies of region-specific phosphoproteins should allow a global and, at the same time, detailed view of individual neuronal cell types.

Cellular Messenger Interactions at the Level of Protein Phosphorylation

Cyclic AMP, cyclic GMP, and calcium appear to regulate many of the same neuronal processes. In such cases, the actions of these second messengers are often synergistic or antagonistic. An understanding of such interactions at the molecular level is now possible. For example, if two second messengers regulate membrane potential in a given neuron, the interactions between them could occur at any of several levels. At one extreme, interactions could occur at the initial steps in these pathways, whereby one second messenger regulates membrane potential by altering the intracellular concentration of the other second messenger. At the other extreme, interactions could occur at the last steps in these pathways: one second messenger might regulate membrane potential by altering the conductance of potassium channels and the other by altering the conductance of sodium channels. Indeed, interactions between a wide variety of cellular messengers have been found to occur at every conceivable level of protein phosphorylation systems: at the level of the intracellular concentration of second messengers, at the level of protein kinases, at the level of protein phosphatases, at the level of the same substrate protein, and at the level of different substrate proteins. These interactions have been reviewed (*1*) and only one example is discussed here.

One apparent function of DARPP-32 has recently been established through biochemical studies (*30*). The phosphorylated form of the protein inhibits phosphatase 1, with an IC_{50} (inhibition dose) of approximately $10^{-9}M$, while it shows no inhibitory activity toward phosphatases 2A, 2B, and 2C. The dephosphorylated form of DARPP-32 is inactive as an inhibitor of phosphatase 1. DARPP-32 shares a number of properties with two proteins that are also active as phosphatase inhibitors in their phosphorylated forms, namely the Purkinje cell-specific G substrate and the widely distributed phosphatase inhibitor 1 (*1*). These similarities include low molecular weight, heat and acid stability, amino acid composition, phosphorylation on threonine residues, and very similar amino acid sequences around their phosphorylation sites. Two types of mechanisms by which phosphatase inhibitors can regulate neuronal function are illustrated in Fig. 2, with DARPP-32 as an example. First, a phosphatase inhibitor, phosphorylated and activated by some first and second messenger system, can act as a positive feedback signal for that system by enhancing the phosphorylation of other substrates for the same protein kinase. Second, the same phosphatase inhibitor can also enhance the phosphorylation of substrate proteins for another protein kinase and, in so doing, can mediate the effects of one first and second messenger system on another.

A mechanism by which a phosphatase inhibitor can mediate the effects of one first messenger on another is illustrated in Fig. 3. As we have already mentioned, DARPP-32, an endogenous substrate for cyclic AMP–dependent protein kinase, is predominantly localized to the D_1 subclass of dopaminoceptive neurons, and phospho-DARPP-32 has been shown to inhibit protein phosphatase activity in vitro. Dopamine, apparently acting through cyclic AMP, inhibits the ability of glutamate to depolarize certain dopaminoceptive neurons, such as the medium spiny neurons in the striatum (*31*). We suggest that this effect of dopamine may be achieved in part through the phosphorylation and activation of DARPP-32, and through subsequent increases in the phosphorylation and activity of some inhibitory modulator protein. This process would represent negative feedback. Such a mechanism of action of dopamine is consistent with its physiological effects in striatal medium spiny neurons (*31*). Dopamine decreases the firing rate of these neurons relatively slowly, that is, over a period of seconds to minutes. In addition, the inhibitory actions of dopamine cannot be explained by a direct effect of the neurotransmitter on the conductance of a specific ion channel. Interestingly, medium spiny neurons exhibit an unusually low spontaneous firing rate in vivo. The high concentration of DARPP-32 in these neurons (*18*), through the type of mechanism illustrated in Fig. 3, may contribute to this low firing rate.

G substrate, an endogenous substrate protein for cyclic GMP–dependent protein kinase, is localized to cerebellar Purkinje cells, and phospho–G substrate has been shown to inhibit cerebellar protein phosphatase activity in vitro (*23*). Acetylcholine, apparently acting through cyclic GMP, and glutamate have each been shown to depolarize and increase the firing rate of cerebellar Purkinje cells (*32*). The excitatory effect of acetylcholine, which is slower in onset and longer in duration than that of glutamate, may be achieved in part through the phosphorylation and activation of G substrate, and through subsequent increases in the phosphorylation and activity of some excitatory modulator protein. Although the combined effect of acetylcholine and glutamate has not yet been studied, it seems possible that acetylcholine may potentiate the excitatory action of glutamate on these cells through such a mechanism. This process would represent positive feedback. Purkinje cells display an unusually high spontaneous firing rate in vivo. It is possible that the high concentration of G substrate in these neurons, through the type of mechanism illustrated in Fig. 3, contributes to this high firing rate.

While the phosphorylated form of DARPP-32 inhibits protein phosphatase 1, this phosphatase is unable to dephosphorylate and inactivate this inhibitor protein in vitro. Rather, the inhibitor is dephosphorylated and inactivated most effectively by the calcium-calmodulin–dependent protein phosphatase 2B (*1*, *30*). These in vitro observations suggest the existence of a type of interaction between calcium and phosphatase inhibitors in addition to those shown in Fig. 3. Thus, a first messenger, such as glutamate, which acts through calcium, may terminate those actions of another first messenger, such as dopamine, which are achieved through the activation of phosphatase inhibitors.

Although the specific details of the schemes shown in Figs. 2 and 3 are clearly conjectural, they do represent our current thinking about certain aspects of the physiological significance of phosphatase inhibitors in the nervous system.

Conclusions

Many individual molecular pathways involving protein phosphorylation have been demonstrated in nervous tissue over the past several years and appear to mediate diverse physiological responses to a wide variety of first and second messengers in the nervous system. In addition, numerous interactions have been found among these various protein phosphorylation pathways, indicating that the molecular mechanisms through which individual regulatory agents pro-

duce specific responses frequently converge at the level of protein phosphorylation. The existence of numerous pathways and of numerous interactions between these pathways attests to the extraordinary complexity of physiological processes at the molecular level and supports the view that protein phosphorylation is a final common pathway of fundamental importance in neurons. Because of the central role of protein phosphorylation as a regulatory mechanism in neurons, it provides an experimental framework within which to unravel the layers of molecular steps involved in the regulation of neuronal function.

Identification of the specific phosphoprotein involved in a particular biological response is crucial to the elucidation of the molecular pathway through which that response is achieved. Already, a large number of phosphoproteins have been found in nervous tissue, and in several instances their functional role is known. These studies support the view that through the detection of neuronal phosphoproteins, and through the characterization of their biochemical, anatomical, and physiological properties, much will be learned about the molecular basis of nervous system function. The vital role of protein phosphorylation in neuronal function indicates that many of the highly differentiated characteristics of distinct adult neuronal cell types, as well as many of the developmental mechanisms used by neurons to attain this diversity in characteristics, involve specific phosphoproteins. Indeed, many phosphoproteins have been found in specific association with certain neuronal cell types. This specific association has enabled the use of these proteins to investigate several areas of neuroscience that are not related primarily to the study of the biological functions of those proteins. Neuronal phosphoproteins, and cell type–specific phosphoproteins in particular, have already proved to be powerful tools with which to study various anatomical and developmental aspects of the nervous system and to characterize presynaptic neurotransmitter receptors. They also offer novel approaches to elucidating the pathophysiological basis for a variety of neurological and psychiatric disorders.

References and Notes

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