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EXPERIMENTAL APPROACHES TO UNDERSTANDING THE ROLE OF PROTEIN PHOSPHORYLATION IN THE REGULATION OF NEURONAL FUNCTION

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INTRODUCTION

Studies by Earl Sutherland and his colleagues on hormonal regulation of the breakdown of glycogen in liver resulted in the discovery that the first step in the action of many hormones is to increase the synthesis of cAMP by activating adenylate cyclase (Rall et al 1957, Sutherland & Rall 1958, Robison et al 1968). It was later established that cAMP exerts its effects by stimulating protein kinases that catalyze the phosphorylation of specific functional proteins and thereby regulate their activity (Walsh et al 1968, Kuo & Greengard 1969, Krebs & Beavo 1979). The discovery that the brain contains a high concentration of cAMP-dependent protein kinase led to the proposal that protein phosphorylation might play an important role in regulation of neuronal properties by neurotransmitters and neurohormones (Miyamoto et al 1969). In particular, it seemed that protein phosphorylation, which usually takes place on a time scale of hundreds of milliseconds or longer, might be a mechanism underlying relatively long-lasting changes in neuronal properties such as "slow" changes in post-synaptic potentials (McAfee & Greengard 1972), changes in the rate of transmitter synthesis (Morgenroth et al 1975), or changes in gene expression (Klein & Berg 1970). The biochemists and neurobiologists who took up the study of brain protein phosphorylation hoped to gain insight into some of the mechanisms

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underlying changes in neuronal excitability and synaptic efficacy and also, perhaps, into processes that govern the development of various neuronal types during the formation of the nervous system. This line of research was bolstered by the findings that the brain contains not only high concentrations of protein kinases, but also protein phosphatases, adenylate cyclase, and phosphodiesterase (Greengard 1976), and also by the discovery that several neurotransmitters stimulate the synthesis of second messengers such as cyclic AMP and cyclic GMP by binding to specific receptors on the surfaces of neurons (for reviews see Nathanson 1977, Greengard 1981).

Further progress in understanding the role of protein phosphorylation in the regulation of neuronal function has until recently been slow. This problem has often been as difficult as the general problem of identifying and understanding proteins that mediate complex neuronal functions such as transmitter release, or the gating of ion channels. However, increasingly sophisticated methods for examining neuronal structure and function at cellular and molecular levels have begun to yield clear answers.

Three strategies have resulted in new insights in the past few years and are discussed in this review. The first involves the study of the role of protein phosphorylation in regulating electrical properties of membranes of invertebrate neurons. The large size and ease of identification of these neurons make them useful for such studies for two reasons:

1. They are easy to impale with microelectrodes, consequently many of their membrane ion channels have been well characterized physiologically.
2. Both small molecules and proteins can be injected into them for the purpose of studying the resulting functional changes.

Recent experiments in which protein kinase or its inhibitor were injected into identified invertebrate neurons have shown that cAMP-dependent protein phosphorylation can lead to changes in the properties of membrane ion channels. Such changes probably underlie the modulatory actions of many agents that stimulate adenylate cyclase.

Another, more open-ended, strategy has been to identify substrate proteins for various protein kinases in mammalian brain homogenates, characterize them, then attempt to learn their functions by examining their subcellular locations and the physiological changes that alter their phosphorylation. This is a rather risky and long-term approach, but it has resulted in the discovery of two neuronal proteins with specific distributions in the brain and intriguing regulatory properties that point toward certain functions. In addition, this approach has led to the discovery of calcium-regulated protein phosphorylation systems in neural tissue.

A third strategy has been to look for phosphorylation in vitro of specific proteins whose functional properties appear to be modulated by second

messengers. The rate-limiting enzymes in the synthetic pathways for the catecholamines and serotonin are more active following neuronal activity. They have now been shown by several groups to be regulated by phosphorylation. Tyrosine hydroxylase is activated by both cAMP-dependent and calcium-dependent phosphorylation, while tryptophan hydroxylase is regulated by calcium-dependent phosphorylation.

Due to space limitations, several topics of interest are not covered here. Two of these, the use of hippocampal slices to examine the role of protein phosphorylation in long-term potentiation, and the possible involvement of protein phosphorylation in photoreceptor function, were discussed in recent articles in the *Annual Review of Neuroscience* (Lynch & Schubert 1980, Hubbell & Bownds 1979).

REGULATION OF ELECTRICAL PROPERTIES OF INVERTEBRATE NEURONS

Several distinct membrane ion channels have been identified in the somas of invertebrate neurons (for a review see Adams et al 1980). Pharmacological and physiological techniques have been developed by which properties of many of the channels can be studied individually and under well-controlled conditions. Such studies have resulted in a better understanding of the ion channels that underlie the complex electrical properties of several specialized neuronal cell types. In three types of neurons in ganglia of the marine mollusk *Aplysia californica,* agents that stimulate adenylate cyclase have been found to modify the cell's electrical properties. A combination of physiological and biochemical experiments indicate that changes in K^+-conductances in each of the cells underlie the electrical modifications and that cAMP-dependent protein phosphorylation can initiate these K^+-conductance changes. Initial biochemical experiments have identified phosphoproteins that are candidates for mediating the physiological effects.

The Bursting Pacemaker, R_{15}

Levitan and co-workers have studied the regulation of ion channels in the giant Aplysia neuron, R_{15}. This cell is one of a class of cells called bursting pacemakers. Such cells produce rhythmic bursts of action potentials, interspersed by periods of transient hyperpolarization. This pattern of activity is produced by the interaction of several ionic currents (see Adams et al 1980). A characteristic component of bursting pacemakers is a small voltage-dependent inward current (I_B) carried by Na^+ and/or Ca^{2+} (Eckert & Lux 1975). This current is activated very slowly by depolarizing pulses and tends to excite the cell, leading to bursts of action potentials. It can be offset

by several voltage-dependent K^+-currents. Two neurotransmitters, serotonin (Drummond et al 1980a) and dopamine (Ascher 1972, Wilson & Wachtel 1978), alter pacemaker activity. Serotonin lengthens the interburst hyperpolarization and at sufficiently high concentrations (10 μM) suppresses bursting altogether. Serotonin also activates adenylate cyclase in R_{15} and causes a rise in intracellular cAMP concentrations (Levitan 1978, Drummond et al 1980a). The serotonin receptor that activates adenylate cyclase is the same one that mediates the effect of serotonin on the electrical properties of the R_{15} membrane (Drummond et al 1980a,b). Both extracellular serotonin and intracellular cAMP increase an anomalously rectifying K^+ conductance (J. A. Benson and I. B. Levitan, personal communication). The resulting K^+-current presumably offsets the slow inward current that triggers bursting. This provides a possible mechanism for the lengthened interburst interval at intermediate doses of serotonin and the suppression of bursting at high doses.

The role of cAMP-dependent protein phosphorylation in control of this K^+-conductance was examined by injecting into R_{15} a specific protein inhibitor (PKI) of cAMP-dependent protein kinase (Levitan & Adams 1981, Adams & Levitan 1982). This small inhibitory protein, purified from mammalian skeletal muscle (Demaille et al 1977), is a potent and specific inhibitor of cAMP-dependent protein kinase (Ashby & Walsh 1972). It has little effect on any other known kinases. Injection of the inhibitor into R_{15} blocks the effect of serotonin on bursting pacemaker activity. In an interesting control experiment, Adams & Levitan (1982) showed that the inhibitor does not block the effect of dopamine, which slows pacemaker activity by a different mechanism that involves a different ionic current and does not act through cAMP (Drummond et al 1980a). These experiments provide strong evidence that cAMP-stimulated protein phosphorylation is directly involved in the serotonin response. Further work by the Levitan laboratory on the nature of the proteins that are phosphorylated in R_{15} in response to serotonin is discussed below.

Peptidergic Bag Cells

L. Kaczmarek and others working in the laboratory of F. Strumwasser have analyzed the regulation of electrical activity of the Aplysia bag cells. These neurons occur in clusters around the base of the two anterior connectives that connect the abdominal ganglion with the head ganglia. The cells secrete a peptide hormone that triggers the release of eggs and associated egg-laying behavior. The cells can be induced to fire long discharges of sodium-calcium action potentials in response to brief stimulation of an anterior connective or to application of peptides from the atrial gland, which is part of the reproductive tract. Several lines of evidence suggest that the afterdischarge

is triggered by a rise in intracellular cAMP (Kaczmarek et al 1978). For example, cAMP concentration in the cells rises during afterdischarge, and injection of 8-benzylthio-cAMP into the cells can produce an afterdischarge (Kaczmarek & Strumwasser 1981a). The electrical correlates of the onset of afterdischarge are complex. Prior to discharges that are induced by extracellular 8-benzylthio-cAMP there is an increase in membrane resistance, a corresponding decrease in the action potential threshold, and a broadening of the action potentials. Large subthreshold voltage oscillations are observed, which eventually trigger the long-lasting afterdischarge (Kaczmarek & Strumwasser 1981a). Preliminary voltage clamp analyses on isolated bag cells in culture indicate that the primary effect of cAMP is to decrease the net outward currents in the bag cells. Cyclic AMP has no effect on the inward currents, the transient early K^+-current, or the voltage activated late K^+-current. These results suggest that the decrease may be in either the Ca^{2+}-activated K^+-current or in an as yet undescribed K^+-current (Kaczmarek & Strumwasser 1981b).

In a collaborative study between the Strumwasser and Greengard laboratories, Kaczmarek et al (1980) made use of the wealth of information about mammalian cAMP-dependent protein kinase and of its availability in purified form to test whether protein phosphorylation by cAMP-dependent protein kinase might be involved in the onset of afterdischarge. There are two types of cAMP-dependent protein kinases (Hofmann et al 1975, Corbin et al 1975). Both are tetrameric enzymes containing two cAMP-binding regulatory subunits and two catalytic subunits. The two types of enzyme have different regulatory subunits, but the same type of catalytic subunit. Cyclic AMP activates the kinases by binding to the regulatory subunits, causing them to release the catalytic subunits in an active form. The free catalytic subunits can be purified (Beavo et al 1974) and will phosphorylate appropriate proteins even in the absence of cAMP. Injection of purified free catalytic subunits into a cell would thus be expected to mimic activation of endogenous cAMP-dependent protein kinase. Microinjection of purified bovine heart catalytic subunits into bag cells produced an increase in membrane resistance and a broadening of action potentials in more than half of the cells injected, whereas control injections of the microelectrode solution or heat inactivated enzyme did not (Kaczmarek et al 1980). These results support the hypothesis that cAMP-dependent protein phosphorylation results in a decrease in K^+-conductance in the cells. However, only three of the 16 injected cells showed subthreshold voltage oscillations, and only one showed brief repetitive firing. This could mean either that additional factors unrelated to protein phosphorylation are also involved in initiation of afterdischarge, or that the amount of kinase injected into most of the cells was insufficient to induce afterdischarge. These studies provide clear evidence

that cAMP-dependent protein phosphorylation is involved in regulation of K^+-currents in *Aplysia*. Future experiments should clarify which of the K^+-channels is primarily affected. Further experiments of the Strumwasser group have shown that the bovine heart cAMP-dependent kinase phosphorylates proteins in bag cell homogenates that are also phosphorylated by an endogenous *Aplysia* cAMP-dependent protein kinase (Jennings et al 1982), thus confirming that the specificity of the kinase is highly conserved in different species. The nature of bag cell proteins that are phosphorylated by the cAMP-dependent protein kinase is discussed below.

Sensory Neurons of the Aplysia Gill-Withdrawal Reflex Pathway

E. Kandel and his colleagues have carried out an extensive analysis of the neuronal mechanisms underlying habituation and sensitization of the gill-withdrawal reflex in *Aplysia*. When either the mantle edge or siphon of an *Aplysia* is tapped, it will withdraw its gill, which normally extends beyond the mantle and siphon. If the mantle is tapped at low frequencies (once per 3 min), the reflex disappears, or habituates. If the animal is then tapped on the head, the reflex returns, or is sensitized. The neural circuits underlying the reflex, and the relationship of the habituation and sensitization to classical learning paradigms in higher animals have been thoroughly reviewed (Kandel 1976, 1981). It is sufficient for the present discussion to say that the behavior of the reflex seems to be correlated with changes in synapses between the mantle and siphon sensory neurons and the interneurons and motor neurons that innervate the gill and siphon. Sensitization is correlated with a sudden increase at these synapses in the number of quanta of transmitter released per impulse. This synaptic facilitation can be triggered in vitro by stimulation of the nerve leading into the abdominal ganglion from the head, by bath application of serotonin, or by injection of cAMP into the sensory neuron soma (Brunelli et al 1976). Thus, facilitation by serotonin apparently results from its stimulation of adenylate cyclase. Klein & Kandel (1978, 1980) found that the increased transmitter release is due, at least in part, to an increased calcium current in the synapses during depolarization. This effect is not due to a direct effect on the voltage-sensitive calcium channels, but is caused by an inhibition of the net K^+-current that normally repolarizes the membrane following an action potential.

In a collaborative study between the Kandel and Greengard laboratories, Castelluci et al (1980) microinjected catalytic subunits of bovine heart cAMP-dependent protein kinase into sensory neurons in order to test whether protein phosphorylation plays a role in synaptic facilitation. The neurons were pretreated with tetraethyl ammonium (TEA) to enhance calcium current during the action potential. In 29 of 35 injected cells, they

observed an additional enhancement of calcium influx which resulted in a further prolongation of the action potentials. In five of ten injected cells, the conductance of the membrane decreased, as it would if K^+ channels were closed. Finally, in three of three injected sensory cells, the amplitude of the post-synaptic potential in the motor neuron increased, mimicking facilitation caused by stimulation of the nerve from the head, by bath application of serotonin, or by injection of cAMP. These effects were not observed following control injections of inactive catalytic subunits. Castelucci et al (1981) also showed that injection of the protein kinase inhibitor blocked facilitation of the synapse by serotonin and reversed facilitation after it had been produced by application of serotonin. These results indicate that under the experimental conditions studied, activation of the kinase is a limiting factor in determining the time course of the facilitation. Taken together, these experiments provide strong evidence that cAMP-dependent protein phosphorylation is involved in modulation of a K^+-current in the sensory cells, and thus in sensitization of the gill-withdrawal reflex.

Camardo et al (1981) have studied the specific K^+-current that is modulated by serotonin using voltage clamp analysis. The current does not have the characteristics of any of three well-known K^+-currents in invertebrate somas, the early I_K, the delayed I_K, or the Ca^{2+}-activated I_K. Thus, it is possible that serotonin regulates a K^+-current that has not yet been described. Further work by Bernier & Schwartz on the nature of the proteins that are phosphorylated in the sensory neurons in response to serotonin is discussed in the following section.

Substrate Proteins for cAMP-dependent Protein Kinase in Invertebrates

All three of the groups whose work is discussed above have attempted to identify proteins that are phosphorylated in Aplysia neurons by cAMP-dependent protein kinase, and thus may be mediating the changes in electrical properties induced by cyclic AMP.

Lemos et al (1981, and personal communication) have developed a technique for measuring protein phosphorylation within R_{15} after intracellular injection of γ-^{32}P-ATP. They showed that the injected ATP remained confined to the cell for the period immediately following the injection; thus only proteins inside R_{15} were labeled with ^{32}P during this time. Following the labeling period and application of test solutions, the entire ganglion was homogenized and the labeled proteins were separated by SDS polyacrylamide gel electrophoresis. This technique has two primary advantages.

1. It circumvents the problem of glial contamination when protein phosphorylation is measured in cell bodies that have been dissected from

Table 1 Summary of effects of cyclic AMP-dependent protein phosphorylation on K^+-conductances in *Aplysia* neurons

Cell type	Direction of regulation	Functional consequence	Evidence
R_{15} (bursting pacemaker)	Increased conductance	Increase in interburst interval	Effect of serotonin on K^+-conductance is blocked by protein kinase inhibitor
Peptidergic bag cells	Decreased conductance	Triggering of afterdischarge leading to release of egg-laying hormone	Decrease in K^+-conductance is induced by injection of protein kinase catalytic subunit
Sensory neurons	Decreased conductance	Increased influx of calcium following action potential leads to increased transmitter release, e.g. sensitization	Decrease in K^+-conductance and sensitization are induced by injection of protein kinase catalytic subunit, and blocked by injection of protein kinase inhibitor

ganglia following conventional labeling with bath-applied inorganic ^{32}P-phosphate.

2. The cell can be voltage clamped during and following the injection, so that changes in protein phosphorylation can be related to changes in conductance.

With this method, Lemos et al showed an increase in the phosphorylation of four particulate proteins in conjunction with serotonin induced conductance changes. Three high molecular weight proteins, 230,000, 205,000, and 135,000 daltons, and one 26,000 dalton protein were more highly phosphorylated in the presence of serotonin than in its absence.

Jennings et al (1982) have examined protein phosphorylation in bag cell clusters. They used two methods to measure changes in phosphorylation that occur during afterdischarge. In one method, abdominal ganglia were incubated with ^{32}P-labeled inorganic phosphate to pre-label the intracellular ATP pools. Some of the ganglia were then stimulated to initiate afterdischarge. At appropriate times, a bag cell cluster was removed from the ganglion and homogenized. Labeled proteins were separated by SDS gel electrophoresis. Incorporation of labeled phosphate into proteins was compared to that in control ganglia that had not been stimulated. In the second method, a "back-phosphorylation" technique was employed to measure changes in cAMP-dependent protein phosphorylation during afterdischarge. Bag cells in intact ganglia were stimulated to afterdischarge. At appropriate times, bag cells were dissected from the ganglia and homogenized in a medium containing phosphodiesterase and phosphatase inhibitors. The catalytic subunit of cAMP-dependent protein kinase was added

to the homogenates, and incorporation of ^{32}P from γ-^{32}P-ATP into protein, catalyzed by the kinase, was measured. Proteins that had been substantially phosphorylated in the intact cells would be expected to incorporate less ^{32}P-labeled phosphate in this assay because the phosphorylation sites would already be occupied by cold phosphate. Both techniques revealed an approximate doubling in phosphorylation of a 21,000 dalton protein 20 min after initiation of afterdischarge. No change in this protein was detectable 2 min after the beginning of afterdischarge. The pre-labeling technique, but not back-phosphorylation, revealed a 33,000 dalton protein with increased labeling both at 2 (82%) and 20 (69%) min after initiation of afterdischarge. Both of these proteins could be phosphorylated by endogenous cAMP-dependent protein kinase in bag cell homogenates and by added bovine catalytic subunit, although the 21,000 dalton protein was more heavily phosphorylated in both instances. The 33,000 dalton protein was found in several parts of the nervous system, whereas the 21,000 dalton protein was relatively specific to the isolated bag cell cluster. Goy et al (1981) have also reported an effect of serotonin and cAMP on phosphorylation of a 28,000 dalton protein in a lobster neuromuscular preparation. It may be that phosphorylation of a protein in this molecular weight range is a response to cAMP that is common to many invertebrate neurons.

L. Bernier and J. Schwartz have begun to examine phosphorylated proteins in the sensory cells of Aplysia (personal communication). They have concentrated on examining high molecular weight proteins in wedges of sensory cells removed from ganglia that have been pre-labeled with bath applied inorganic ^{32}P-phosphate. They have preliminary evidence that several proteins that are present in the sensory neurons are labeled under these conditions and they are beginning to examine whether their labeling is stimulated by serotonin.

The description of these phosphoproteins in identified neural systems in which it may be possible to study their functions is an important development. However, because the Aplysia nervous system is so small, it will be more difficult than in mammals to obtain the proteins in sufficient quantities to study their structure and subcellular localization, and to develop reagents for use in determining their functions. This illustrates a limitation of the invertebrate neural systems for biochemical studies. It would be helpful to know whether related proteins exist in the mammalian nervous system from which one can obtain large quantities of tissue easily and cheaply.

BIOCHEMICAL STUDIES OF PROTEIN KINASES AND THEIR SUBSTRATES IN MAMMALIAN BRAIN

There are three major second messengers, cAMP, cGMP, and calcium, that are known to be involved in the responses of neurons to regulatory agents

(Greengard 1978). It is widely accepted that the first two, cAMP and cGMP, act primarily, if not exclusively, by stimulating specific protein kinases. The respective kinases have been purified from brain and are relatively well characterized (for review see Walter & Greengard 1981). This discussion focuses on more recent work on some of their neuronal substrates. Two of these, Synapsin I and tyrosine hydroxylase, are substrates for cAMP-dependent protein kinase. Another, the G-substrate, is a specific substrate for cGMP-dependent protein kinase.

Calcium ion, in contrast to the cyclic nucleotides, apparently has several molecular effects in brain tissue. The full extent of the functional interactions of calcium ion is not known, but many of its effects are mediated through the calcium-binding regulatory protein, calmodulin. Studies on the phosphorylation of Synapsin I led to the discovery of a protein kinase activity in brain that is activated by calcium and calmodulin. More recently, several brain calcium-dependent protein kinases and their substrate proteins have been described. Some of these will be reviewed here.

Synapsin I

The physiological effects of increased levels of cAMP in mammalian neurons are numerous and well-documented (see Nathanson 1977). They range from short-term changes in transmitter release (Miyamoto & Breckenridge 1974) to induction of enzyme synthesis (Mackay & Iversen 1972). In order to learn more about the biochemical pathways underlying these effects, Greengard and colleagues have studied neuronal protein substrates for cAMP-dependent protein kinase (for review see Greengard 1981). One of the most intriguing is Synapsin I, until recently called Protein I, a doublet of closely related proteins found only in nervous tissue (Ueda et al 1973). It has been purified to homogeneity from bovine and rat brain (Ueda & Greengard 1977, L. De Gennaro and P. Greengard, unpublished), where it comprises about 0.4% of the total brain protein (Goelz et al 1981). Its molecular properties have been summarized in other reviews (Greengard 1979, 1981). Here, I discuss recent experiments describing its cellular and subcellular localization and also its phosphorylation by calcium-dependent in addition to cAMP-dependent protein kinases. These studies have given clues concerning possible functions of this abundant brain protein.

CELLULAR LOCALIZATION De Camilli et al (1979) and Bloom et al (1979) have used antisera raised against bovine Synapsin I to localize it in sections of rat tissues. Specific staining was confined to synaptic regions in the central and peripheral nervous systems. These studies thus confirmed biochemical work (Ueda & Greengard 1977) that had indicated that Synapsin I was present only in neural tissue; they extended the finding to indicate

that it was present in high concentrations only in synaptic regions, hence the name Synapsin I.

In these early studies, it seemed that Synapsin I was present in only some of the synaptic regions that were examined and not in others. For example, no specific staining was seen at the neuromuscular junction (De Camilli et al 1979). As techniques for fixation, sectioning, and staining of tissue have improved, however, terminals that at first could not be labeled have been shown to contain Synapsin I. These terminals include the neuromuscular junctions of the rat diaphragm and ocular muscles (P. De Camilli, R. Cameron, and P. Greengard, personal communication). It now appears that Synapsin I is contained in many different types of synapses, both excitatory and inhibitory, cholinergic and adrenergic. It seems increasingly likely that Synapsin I is a component of most, and possibly all, mammalian nerve terminals (P. De Camilli, S. M. Harris, W. Huttner, and P. Greengard, personal communication).

In order to determine the function of Synapsin I, it is important to know where it is located within the synaptic terminals. It is a particulate protein, having the characteristics of a non-integral membrane protein, and therefore one would expect it to be associated with subcellular structures. Its location was initially studied in two ways. Both immunocytochemistry at the electron microscope level (Bloom et al 1979) and measurement of the levels of Synapsin I in subcellular fractions of brain (Ueda et al 1979) gave the same surprising result. The protein appeared to be associated both with synaptic vesicles and with postsynaptic densities. Both types of experiments indicated that the largest proportion of Synapsin I was present on presynaptic vesicles; nevertheless, a significant portion was also associated with postsynaptic densities. It now seems likely that in both cases the apparent association with postsynaptic densities was artifactual for different, but related reasons. The tendency of charged soluble proteins to bind nonspecifically to postsynaptic densities is by now well documented. This has led to misidentification of its peptide components in the past, as pointed out by Matus et al (1980). Synapsin I is a highly charged "sticky" protein that could easily redistribute during the detergent extraction used to prepare isolated densities. The same tendency of the postsynaptic density to bind charged molecules could also cause an artifactual precipitation of the electron dense horseradish peroxidase reaction product on the density during the staining procedure used for immunocytochemical localization. These problems, together with the relatively low specific activity of Synapsin I in the post-synaptic density fraction (Ueda et al 1979), left some doubt about whether its presence there was a reflection of its location in vivo.

In order to examine the location of Synapsin I in a way less subject to artifact, P. De Camilli and colleagues developed a method for staining

synaptic regions with a ferritin-labeled antibody under conditions that do not require detergent treatment for antibody penetration. A purified synaptosomal preparation was made from brain, fixed under conditions of varying hypotonicity, and embedded in agarose. Following such fixation, the ferritin-labeled antibody can penetrate into synaptosomes that have been broken by the hypotonic shock. With this procedure, synaptic vesicles were heavily labeled, but the label over postsynaptic densities was not greater than the background in preparations stained with control sera (Figure 1; P. De Camilli, S. M. Harris, W. Huttner, and P. Greengard, manuscript in preparation). These experiments indicate that Synapsin I is associated, at least in part, with synaptic vesicles and that it is probably not present in postsynaptic densities.

CALCIUM-DEPENDENT PHOSPHORYLATION OF SYNAPSIN I The first indication that Synapsin I might be phosphorylated, and thus regulated, in response to an increase in calcium ion concentration came from an experiment of Krueger et al (1977). It is known that a number of synaptic processes are regulated by the influx of calcium ion that occurs when a nerve terminal is depolarized by an impulse. The most clearly established of these is, of course, transmitter release (Katz & Miledi 1967, Douglas 1968). The biosynthesis of certain neurotransmitters is also regulated by calcium ion (Patrick & Barchas 1974), as is the number of quanta of transmitter released per nerve impulse (Rosenthal 1969, Klein & Kandel 1980).

In order to evaluate whether protein phosphorylation might be one of the mechanisms by which calcium exerts its regulatory actions, Krueger et al (1977) studied the effect of depolarization-induced calcium influx on the rate of phosphorylation of synaptic proteins. Brain synaptosomes were prepared and incubated with ^{32}P-labeled inorganic phosphate to prelabel the intrasynaptosomal ATP pool. The synaptosomes were then transferred to solutions containing either veratridine or high concentrations of potassium, in the presence or absence of calcium. Both of these test media induce artificial depolarization of the synaptosomes and therefore permit the influx of calcium through voltage-dependent channels. After a brief incubation, the synaptosomes were dissolved in sodium-dodecyl sulfate (SDS) and applied to SDS polyacrylamide gels. Incorporation of phosphate into proteins was examined by autoradiography. The rate of phosphorylation of several proteins was specifically increased by depolarization in the presence of calcium. The most prominent of these was the Synapsin I doublet (Krueger et al 1977, Sieghart et al 1979).

These results raised a question about the mechanism by which calcium influx into synaptosomes increased the phosphorylation of Synapsin I. Did the influx of calcium raise the level of cAMP inside the synaptosomes and

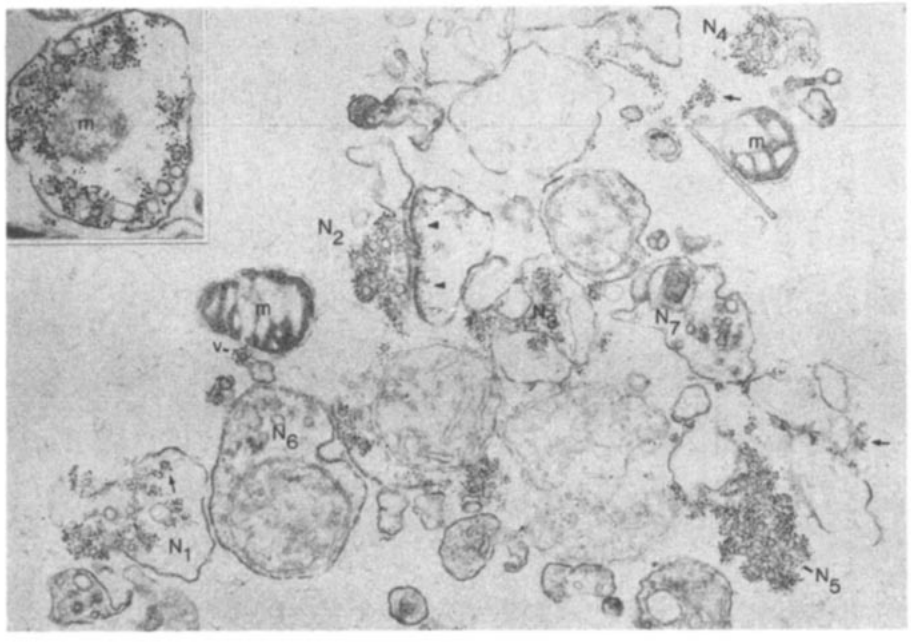

Figure 1 Immunoferritin labeling of Synapsin I in a crude synaptosomal fraction embedded in agarose. A crude synaptosomal suspension was immunostained for Synapsin I by an indirect immunoferritin procedure after a mildly hypotonic fixation and embedding in an agarose matrix. The state of preservation of individual nerve endings appears highly variable. Severely disrupted nerve endings (N_1–N_5) as well as nerve endings with an apparently sealed plasma membrane (N_6 and N_7) are visible in the figure. Isolated synaptic vesicles (v), which have probably leaked out of disrupted nerve endings, can also be observed. Prominent labeling by ferritin particles is seen on all synaptic vesicles visible in the picture except when they appear to be inaccessible to marker proteins due to the presence of a sealed plasma membrane, as in the endings labeled N_6 and N_7. Structures other than synaptic vesicles are not labeled by ferritin. Note in particular the absence of ferritin labeling on nerve ending plasma membranes. A postsynaptic density is indicated by *arrowheads*. A few clusters of ferritin particles apparently not in direct proximity to synaptic vesicles (*arrows*) might be associated with synaptic vesicles out of the plane of the section. *Inset:* higher magnification of a labeled nerve ending, showing the specific association of ferritin particles with synaptic vesicles. m = mitochondria. (X 53,200; inset, X 76,500) (Courtesy of P. De Camilli and S. M. Harris.)

thereby stimulate phosphorylation by cAMP-dependent protein kinase, or was calcium ion stimulating a separate protein kinase, or even inhibiting a phosphatase? Krueger et al (1977) showed in their study that the conditions that produced the calcium influx into synaptosomes did not cause a measurable rise in cAMP in the synaptosomes. This suggested that calcium was not acting through the cAMP system.

Additional evidence for a separate calcium pathway came from an examination of the sites on Synapsin I that were phosphorylated in response to calcium or cAMP. Huttner & Greengard (1979) were able to stimulate phosphorylation of endogenous Synapsin I in lysates of synaptosomes by the addition of calcium or cAMP. The Synapsin I phosphorylated after either cAMP stimulation or calcium stimulation was subjected to proteolysis by Staph aureus V8 protease according to the Cleveland-Laemmli procedure (Cleveland et al 1977). The resulting phosphopeptides were separated by SDS gel electrophoresis. Stimulation of phosphorylation by cAMP occurred at a site recovered in a 10,000 dalton phosphopeptide, while stimulation of phosphorylation by calcium occurred at sites recovered in two fragments: the same 10,000 dalton fragment and an additional 30,000 dalton fragment. Further studies (Huttner et al 1981, L. DeGennaro and P. Greengard, personal communication) showed that the 10,000 dalton fragment contains a single phosphorylation site that is phosphorylated in the presence of either cAMP or calcium and is located in a globular portion of the Synapsin I molecule that is resistant to digestion by collagenase ("the globular head portion"). The 30,000 dalton fragment contains two phosphorylation sites, both of which are phosphorylated only in the presence of calcium. They are located in an elongated portion of the molecule that is sensitive to digestion by collagenase ("the collagen-like tail"). These experiments provide firm evidence that a separate calcium-sensitive phosphorylation system is responsible for part of the calcium-stimulated phosphorylation of Synapsin I. Studies of the molecular components of this calcium regulated system are discussed below.

POSSIBLE FUNCTIONS OF SYNAPSIN I Purified Synapsin I has been tested for a variety of different enzymatic activities, and all of these tests have yielded negative results. Thus it seems possible that it is not a chemical catalyst but serves some other functional role. From its location and other characteristics, one can make some educated guesses about its possible functions. The specific association of Synapsin I with synaptic vesicles suggests that it is involved in the regulation of a vesicle function. In addition, its phosphorylation by both cAMP and calcium-regulated systems indicates that its function can be regulated by agents that affect either or both of these systems.

The most prominent role of synaptic vesicles is the release of transmitter into the synaptic cleft, triggered by an influx of calcium during depolarization of the terminal (Katz & Miledi 1967, Heuser et al 1979). There is evidence from a number of different systems that both calcium ion and cAMP can alter the level of transmitter released at various synapses. Calcium influx is necessary for the phenomenon of "post-tetanic potentiation" (Rosenthal 1969) in which a burst of impulses in the presynaptic terminal leads to an increase in the amount of transmitter released per impulse. Agents that increase the level of cAMP inside terminals have also been shown to potentiate release (Dudel 1965, Kravitz et al 1975, Goldberg & Singer 1969, Miyamoto & Breckenridge 1974). In the nerve terminals that have been well studied (for review see Kelly et al 1979), the final release process is probably too fast to be directly mediated by protein phosphorylation, unless the kinase involved has a much higher turnover number than other known protein kinases. The delay between calcium influx into the terminal and fusion of vesicles with the presynaptic membrane is estimated to be about 200 μsec (Llinas et al 1976), whereas the cAMP-dependent protein kinase catalyzes only two to ten phosphate transfers per second per active site under optimum conditions in a test tube (Sugden et al 1976, Glass & Krebs 1979). However, most models of exocytosis incorporate several partial reactions leading to the final calcium-triggered fusion. These postulated partial reactions include (*a*) movement of the vesicle toward the presynaptic membrane, (*b*) recognition between the vesicle and "active zones" in the presynaptic membrane, (*c*) close apposition of the vesicle membrane and the presynaptic membrane at the site at which fusion will occur, and finally (*d*) fusion itself (see Kelly et al 1979). Any one of these partial reactions could, in theory, be a limiting factor in determining how many quanta of transmitter are released during an impulse. Although there are certainly other possibilities, it may be that phosphorylation of Synapsin I by calcium or cAMP-regulated kinases regulates one or more of the "partial reactions" that precede the final calcium-dependent fusion event, and thus is involved in the control of the number of quanta of transmitter released per impulse.

Physiological studies are consistent with this possibility. Synapsin I can be phosphorylated and dephosphorylated within minutes in slices of rat cerebral cortex during cycles of depolarization by high K^+ and repolarization in normal K^+ (Forn & Greengard 1978). This indicates that its phosphorylation in intact tissue is highly dynamic and can change with a rapid time course. Phosphorylation of both the cAMP-regulated and calcium-regulated sites on Synapsin I can be increased by impulse conduction along the preganglionic nerve fibers of the rabbit superior cervical ganglion (Nestler & Greengard 1981, 1982). In addition, specific neurotransmitters that

raise the concentration of cAMP can stimulate phosphorylation of Synapsin I. This is true for serotonin in the rat facial nucleus (Dolphin & Greengard 1981), and for dopamine in bovine and rat superior cervical ganglia (Nestler & Greengard 1980, 1982).

A direct test of the hypothesis that Synapsin I regulates some aspect of transmitter release would require introduction into a nerve cell of reagents that specifically activate or inhibit phosphorylation of Synapsin I, such as antibodies that block its phosphorylation (see Naito & Ueda 1981a,b). It would be helpful to know if an homologous protein or set of proteins exists in invertebrate species in which one has greater access to the interior of neurons of known function than in mammals. S. Goelz and P. Greengard (personal communication) have used a specific radioimmunoassay to determine the concentration of Synapsin I-like proteins in nonmammalian species. The results indicate that homologous proteins do exist in several such species, but their concentrations appear to be low and the proteins have divergent properties in the species that have been examined. Thus, Synapsin I does not seem to have been as highly conserved during evolution as the cAMP-dependent protein kinase. Nevertheless, it should be possible to characterize and localize homologous proteins in invertebrate species and thus make functional studies possible. Alternatively, it may be possible to test the function of Synapsin I by introducing appropriate reagents into mammalian neurons or synaptosomes using techniques involving fusion of liposomes with the plasma membrane (see Rahamimoff et al 1978).

Calcium-Dependent Protein Kinases and Their Substrates

Although it has been known for years that phosphorylase kinase, a central enzyme in the regulatory cascade that mediates hormonal control of the breakdown of glycogen, is stimulated by calcium (Brostrom et al 1971), the general significance of calcium-dependent protein kinases has only recently been appreciated. The characterization of calcium-dependent protein kinases other than phosphorylase kinase was aided by the discovery by Cheung (1970) and Kakiuchi & Yamazaki (1970) of a calcium-binding regulatory protein called calmodulin which activates the enzyme phosphodiesterase in the presence of micromolar concentrations of calcium ion. Calmodulin has since been found to regulate a variety of other enzymes (Cheung 1980), including protein kinases.

Calcium and calmodulin-dependent protein kinase activity was discovered in brain tissue by Schulman & Greengard, who were investigating the molecular basis of calcium-regulated phosphorylation of Synapsin I. They found that calcium stimulates the phosphorylation of several proteins in hypotonically lysed synaptosomes (Schulman & Greengard 1978a) and subsequently showed that this protein kinase activity depends on the pres-

ence of both calcium and calmodulin (Schulman & Greengard 1978a,b). The calmodulin-dependent protein kinase activity is not unique to synaptic membranes; it is present in membranes of a variety of tissues in which it phosphorylates a tissue-specific array of proteins (Schulman & Greengard 1978b).

At about the same time, Dabrowska et al (1978) and Yagi et al (1978) reported that calcium and calmodulin activate myosin light chain kinase, the enzyme that phosphorylates the P-light chains of myosin, rendering the myosin ATPase sensitive to activation by actin (Sherry et al 1978). Shortly thereafter, Cohen et al (1978) found that calmodulin is an integral part of phosphorylase kinase and mediates the activation of this enzyme by calcium.

CALMODULIN DEPENDENT SYNAPSIN I KINASE In the experiments of Schulman & Greengard, Synapsin I, which is present in total particulate fractions prepared from synaptosomes, was nevertheless a relatively minor substrate for the particulate calmodulin-dependent kinase. In order to determine whether this kinase could be responsible for the calcium-stimulated phosphorylation of Synapsin I in intact synaptosomes, and to more thoroughly characterize the calcium-dependent Synapsin I kinase, an assay was developed to measure calmodulin-dependent phosphorylation of purified Synapsin I (Kennedy & Greengard 1981). This made it possible to characterize the kinase activity under conditions in which the rate of phosphorylation was not limited by the concentration of substrate. Under optimal conditions in such an assay, the rate of phosphorylation of Synapsin I by a crude brain homogenate is stimulated as much as 40-fold by calcium ion to an initial rate of about 5 nmol/min/mg protein. Phosphorylation of both the globular head portion and the collagenase sensitive tail of Synapsin I occurs under these conditions. Phosphorylation of these two regions is catalyzed by two distinct calcium-stimulated protein kinases that can be resolved by DEAE-cellulose chromatography (Figure 2) (Kennedy & Greengard 1981). One of them is primarily cytosolic and phosphorylates only the globular head portion of Synapsin I. After partial purification, it is reversibly activated by calcium ion and inhibited by 50 μM trifluoperazine (a calmodulin antagonist), but it does not require the addition of exogenous calmodulin. Further characterization will be required to determine whether it is activated by calcium ion alone or whether it contains tightly bound calmodulin that is required for its activation.

The second Synapsin I kinase, which phosphorylates the collagenase-sensitive tail of Synapsin I, has been purified about 200-fold and characterized in greater detail than the first (Kennedy, McGuinness, and Greengard, 1982 and in preparation). It is completely dependent on calcium and exo-

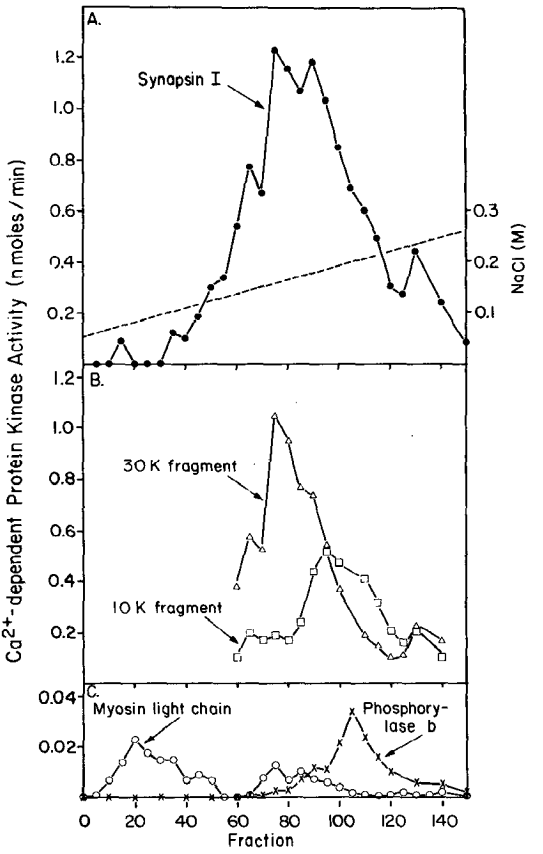

Figure 2 Resolution of two distinct calcium-dependent Synapsin I kinase activities in brain cytosol by DEAE-cellulose chromatography. Brain cytosol proteins were applied to a DEAE-cellulose column, which was then washed with 0.05 M NaCl. Various protein fractions were eluted with a salt gradient from 0.05 M NaCl to 0.3 M NaCl. A. Each fraction was assayed for total calcium-stimulated Synapsin I kinase activity. B. The fractions were further assayed for kinase activity that was specific for sites on the collagen-like tail of Synapsin I that are recovered in a 30K dalton fragment after proteolysis with Staph aureus V8 protease, or for the site in the globular head group that is recovered in a 10K dalton fragment. The figure shows that the curve in A is actually the sum of two curves representing enzmes that are specific for each of the two sites. C. The same fractions were also assayed for previously described calcium-dependent protein kinases, myosin light-chain kinase, and phosphorylase b kinase. Their peaks do not correspond to those of the two Synapsin I kinases. Pure phosphorylase b kinase phosphorylates only the 30K dalton region of Synapsin I, thus it does not contribute at all to the 10K dalton kinase peak. From Kennedy & Greengard (1981).

genous calmodulin. It is found in both the soluble and particulate fractions of brain homogenates. The partially purified enzymes from the two sources are indistinguishable by several criteria. An interesting finding was that the enzyme preparations from both sources contain three proteins that are phosphorylated in the presence of calcium and calmodulin, a 50K dalton protein, and two proteins in the 60K dalton region. When compared by phosphopeptide mapping and two-dimensional gel electrophoresis, these proteins were identical to three proteins of corresponding molecular weights that were shown by Schulman & Greengard (1978a,b) to be prominent substrates for calcium and calmodulin-dependent protein kinase in a crude particulate preparation from rat brain. In addition, the 50K dalton substrate was the major Coomassie blue staining protein in both partially purified enzyme preparations, and its presence coincided with enzyme activity during the purification steps. These findings suggested that the 50K dalton phosphoprotein may be an autophosphorylatable subunit of the Synapsin I kinase, or may exist in a complex with it. The association of the 50K dalton phosphoprotein with Synapsin I kinase activity has recently been confirmed in our laboratory. The enzyme has been purified to near homogeneity. A monoclonal antibody raised against the enzyme specifically precipitates both Synapsin I kinase activity and the 50K dalton protein (M. K. Bennett, N. E. Erondu, and M. B. Kennedy, unpublished observations). Thus the prominent brain particulate 50K dalton substrate protein for calcium and calmodulin-dependent protein kinase appears to be a subunit of the enzyme itself. The functional significance of this autophosphorylation is not yet known.

The two calcium-dependent Synapsin I kinases differ from both myosin light chain kinase and phosphorylase kinase. They have different mobilities on DEAE-cellulose (Figure 2), different substrate specificities, and they are more highly concentrated in brain than in other tissues. The apparent multiplicity of calcium-dependent kinases is of considerable general interest, since it contrasts with the uniformity of the cAMP-dependent protein kinases. As mentioned above, the two major types of cAMP-dependent kinases have virtually identical catalytic subunits and consequently have identical substrate specificities. The calcium-dependent protein kinases may be more diverse and have narrower substrate specificities. This could reflect a fundamental difference between cyclic nucleotide- and calcium-linked regulatory systems. Additional evidence for several calcium-dependent kinases in neural tissue is discussed below.

SUBCELLULAR LOCALIZATION OF BRAIN CALMODULIN-DEPENDENT PROTEIN KINASES Two other research groups have observed a

particulate calmodulin-dependent protein kinase activity in nervous tissue and have carried out studies on its subcellular localization. De Lorenzo and co-workers (De Lorenzo & Freedman 1977, 1978, Burke & De Lorenzo 1981, 1982) reported that calcium-stimulated phosphorylation of tubulin and other endogenous proteins occurs in a particulate preparation enriched in synaptic vesicles and that this phosphorylation system requires calmodulin (De Lorenzo et al 1979). Grab et al (1981) reported that isolated postsynaptic densities contain calmodulin and a calmodulin-dependent protein kinase that phosphorylates endogenous PSD proteins. Information about whether these organelles contain the same calmodulin-dependent kinase or distinct, highly specific kinases in vivo will await more thorough characterization of each of the enzymes and their substrate proteins.

De Lorenzo et al (1979) have postulated that calmodulin-dependent protein phosphorylation may be involved directly in the final stage of transmitter release. This hypothesis is based largely on the observation that the addition of calcium to an enriched vesicle fraction stimulates the release of transmitter from the vesicles and also the phosphorylation of certain proteins. Removal of calmodulin from the vesicles suppresses both the calcium-stimulated loss of bound transmitter and calcium-stimulated protein phosphorylation. Both can be restored by the addition of pure calmodulin. Although these results are intriguing, they show only that calmodulin is necessary for both processes. Major questions remain about the relationship between the loss of transmitter from purified vesicles and the release of transmitter from intact terminals. The physiological relevance of neurotransmitter release from isolated vesicles in the absence of plasma membrane is not clear. It will be important to test the possibility that the release of transmitter from isolated vesicles results simply from a nonspecific degradation of the vesicles by a contaminating calcium and calmodulin-dependent lipase or protease. Another difficulty with this hypothesis is the discrepancy in time course between calcium-stimulated release from intact synaptic terminals, which takes a few hundred microseconds, and calcium-stimulated vesicle phosphorylation, which takes several seconds. As discussed above, the final in vivo release process appears to be too fast to be mediated by a conventional phosphorylation cascade mechanism.

CALCIUM AND LIPID-ACTIVATED PROTEIN KINASE Nishizuka and colleagues have described a calcium-activated protein kinase in brain that does not require calmodulin. It can be activated either by limited proteolysis by a calcium-dependent protease (Inoue et al 1977) or by the simultaneous presence of calcium ion and lipid (Takai et al 1979). The first process is irreversible and requires about 500 μM calcium for half maximal activation (Takai et al 1979). Because of the high concentration of calcium required

for activation, this process may not be physiologically significant. However, the second process is reversible and occurs at lower calcium concentrations. The K_a for calcium is about 50 μM in the presence of mixed membrane lipids (Takai et al 1979), and as low as 5 μM in the presence of phosphatidyl serine and diacylglycerol (Kishimoto et al 1980). The latter lipid has very little stimulatory effect of its own, but it potentiates the effect of phospholipids by increasing the affinity of the phospholipid-enzyme complex for calcium. The effect of diacylglycerol is quite potent, occurring at concentrations less than 5% (w/w) of the concentration of phospholipid and reducing the K_a for calcium from about 100 μM to 5 μM. Consequently, Kishimoto et al (1980) have postulated that activation of the kinase may be linked to hydrolysis of phosphatidyl inositol, which produces diacylglycerol and inositol phosphate. Specific turnover of phosphatidyl inositol has been reported to occur in response to several extracellular messengers including α-adrenergic and muscarinic agonists (Hokin & Hokin 1955, Michell 1979).

Kuo and colleagues (1980) demonstrated that the calcium and lipid-activated protein kinase is present in many different phyla and tissues. The highest concentrations were found in mammalian spleen and neural tissue, where the enzyme was roughly evenly distributed between the soluble and particulate fractions. Wrenn et al (1980) and Katoh et al (1981) showed that substrate proteins for the calcium and lipid-activated kinase are distinct from those for calcium and calmodulin-dependent protein kinases in brain and heart, respectively.

It is clear that this enzyme can be activated by physiologically relevant concentrations of calcium ion when it interacts with the proper mixture of lipids and also that it has a unique specificity that distinguishes it from other known protein kinases. It remains to be demonstrated whether and under what circumstances the enzyme is activated in intact cells.

A Neuronal Substrate for cGMP-Dependent Protein Kinase

Cyclic GMP, as well as cAMP, acts as a second messenger in the nervous system. In vertebrate rod photoreceptors, photolyzed rhodopsin catalyzes a cascade of reactions that leads to a fall in the concentration of intracellular cGMP (Hubbell & Bownds 1979, Stryer et al 1981). The change in cGMP concentration has been postulated to be linked either directly to the decrease in sodium conductance that underlies the light response, or to adaptation of that response to background illumination.

There are several other reports of alterations of cGMP metabolism by neurally active agents. For example, activation of muscarinic receptors on neuroblastoma cells leads to an increase in intracellular cGMP that is secondary to an increased flux of calcium ion into the cells (Matsuzawa &

Nirenberg 1975). Activation of nicotinic receptors in muscle can also raise internal cGMP levels (Nestler et al 1978). Woody et al (1978) have presented evidence that iontophoresis of acetylcholine or cGMP onto cortical neurons produces a specific and relatively long-lasting increase in the input resistance of the cells. The physiological significance of these effects is as yet unknown.

As an approach to learning more about the role of cGMP in neuronal tissue, Schlicter and Greengard looked for specific substrate proteins for cGMP-dependent protein kinase in homogenates of nervous tissue. One such substrate, a soluble 23,000 dalton protein termed G-substrate, was discovered in cerebellar homogenates (Schlicter et al 1978) and has been purified to homogeneity (Aswad & Greengard 1981a). Studies with the purified G-substrate have shown that it has a 20-fold higher affinity for the cGMP-dependent protein kinase than for the cAMP-dependent protein kinase, thus confirming the specificity of its regulation by cGMP (Aswad & Greengard 1981b).

The cellular localization of the G-substrate was examined by measuring the concentrations of G-substrate in mutant mice that lack particular classes of cerebellar neurons. These studies showed that the substrate is highly concentrated in Purkinje cells (Schlicter et al 1980). Low levels of a protein that resembled G-substrate were detected in mice lacking Purkinje cells (Schlicter et al 1980) and in brain regions other than the cerebellum (Aswad & Greengard 1981a); however, examination of phosphopeptides of this protein has shown that it is not the G-substrate, but a related protein called inhibitor I (see below) (A. Nairn, D. Aswad, and P. Greengard, personal communication). The localization of the G-substrate in Purkinje cells is paralleled by a high concentration of the cGMP-dependent protein kinase measured in mutant cerebelli by a photo-affinity labeling technique (Schlicter et al 1980) and by immunofluorescent labeling with antibody raised to purified cGMP-dependent protein kinase (Lohmann et al 1981).

Some insights into the possible functions of the G-substrate have come from studies of the purified protein (Aitken et al 1981). Many of its properties suggest that it may be homologous to a protein isolated from rabbit skeletal muscle by Nimmo & Cohen (1978). This protein, termed inhibitor I, is a substrate for the cAMP-dependent protein kinases. In its phosphorylated form it is an inhibitor of phosphatase I, the enzyme responsible for removing the regulatory phosphates from several enzymes that participate in the cascade of enzymatic reactions that regulates glycogenolysis in muscle. Preliminary experiments have indicated that the G-substrate also possesses some phosphatase inhibitor activity (Aitken et al 1981); thus, it may play a role in a neuronal regulatory cascade that is analogous to the role of inhibitor I in the glycogenolytic cascade.

Specific immunohistochemical staining for cGMP (Ariano & Matus 1981), guanylate cyclase (Ariano et al 1982), and cGMP-dependent protein kinase (M. A. Ariano, personal communication) has been observed in neurons in brain regions other than the cerebellum; for example, the striatum. It will be interesting to see whether other specific substrates for cGMP-dependent protein kinase are present in these regions.

PHOSPHORYLATION OF NEURAL PROTEINS OF KNOWN FUNCTION

Regulation of Synthesis of the Biogenic Amines

Because most neurotransmitters have a unique biosynthetic pathway and the enzymes that catalyze each synthetic step can be assayed in a test tube, regulation of transmitter synthesis can be studied directly in tissue homogenates. There is now considerable evidence that the rate of synthesis of catecholamines and serotonin can be controlled by phosphorylation of the rate-limiting enzymes in their biosynthetic pathways.

Several years ago, evidence was presented that the activity of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines (Nagatsu et al 1964, Levitt et al 1965), could be enhanced in brain homogenates by added cAMP-dependent protein kinase (Morgenroth et al 1975). Recently, Joh et al (1978) have demonstrated that purified tyrosine hydroxylase is phosphorylated and thereby activated by the cAMP-dependent protein kinase. Earlier work with impure enzyme had suggested that this increase in activity resulted from an increased affinity of the enzyme for its pteridine cofactor (Lovenberg et al 1975, Goldstein et al 1975, Lloyd & Kaufman 1975); however, the experiments with highly purified enzyme indicate that phosphorylation results in an increased maximal velocity with no change in the affinity of the enzyme for cofactors or substrates. The physiological circumstances under which tyrosine hydroxylase might be activated by cAMP in vivo are not yet certain. One possible scenario was presented by Erny et al (1981), who showed that adenosine can activate tyrosine hydroxylase in pheochromocytoma cells in culture by increasing intracellular cAMP. These workers have suggested that adenosine, which is rapidly formed from vesicular ATP following its release along with neurotransmitter, may act in vivo to activate tyrosine hydroxylase via presynaptic adenosine receptors.

Both tyrosine hydroxylase, and tryptophan hydroxylase, the rate-limiting enzyme in serotonin biosynthesis (Jequier et al 1967), are activated in brain homogenates by a phosphorylation reaction that is independent of cAMP and dependent on calcium and calmodulin (Hamon et al 1978, Kuhn et al 1978, Yamauchi & Fujisawa 1979a,b, Yamauchi et al 1981). The activation

is unusual in that it requires both a calcium-dependent protein kinase and a distinct "activator protein." It appears to occur in two sequential steps, phosphorylation of the hydroxylase, followed by activation by the activator protein (Yamauchi et al 1981). The calcium and calmodulin-dependent protein kinase appears to be distinct from either phosphorylase kinase or myosin light chain kinase (Yamauchi & Fujisawa 1980). The activator protein is a dimer of two identical 35,000 dalton subunits that has been purified to homogeneity (Yamauchi et al 1981). It is both more widely distributed and more highly concentrated than either of the two hydroxylases or the calmodulin-dependent protein kinase. Thus, it may have a variety of different functions.

Stimulation of the synthesis of both serotonin and norepinephrine by increased electrical activity has been demonstrated in a number of different laboratories (e.g. Eccleston et al 1970, Morgenroth et al 1974). Because increased electrical activity results in an increased flux of calcium ion into the synaptic terminals, calcium-dependent phosphorylation resulting in activation of the transmitter synthesizing enzymes provides a plausible, direct mechanism for this stimulation.

Phosphorylation of the Acetylcholine Receptor

The electroplax, a specialized organ of electric fishes, is a rich source of relatively homogeneous cholinergic terminals (Gordon et al 1977a). For this reason, it has frequently been used as a source of tissue for biochemical studies of pre- and postsynaptic structures. In the course of such studies, Gordon et al (1977a,b) and Teichberg et al (1977) discovered that the acetylcholine receptor is phosphorylated by an endogenous protein kinase in receptor-enriched membranes. Two of the four receptor subunits, the δ (Gordon et al 1977a,b, Saitoh & Changeux 1981, Smilowitz et al 1981) and the γ (Saitoh & Changeux 1981, Smilowitz et al 1981) are phosphorylated.

Early studies indicated that the receptor kinase was stimulated by K^+ ion (Gordon et al 1977a, Saitoh & Changeux 1980), but was not sensitive to second messengers such as cyclic nucleotides or calcium. However, Smilowitz et al (1981) have recently presented evidence that a receptor kinase is stimulated by calcium in the presence of calmodulin. This suggests that receptor phosphorylation might be regulated in vivo by postsynaptic activity that raises the intracellular concentration of calcium.

The functional significance of the receptor phosphorylation is unclear. It seems unlikely that it is required either for agonist-induced conductance changes or for desensitization, since these two processes can occur in purified membranes in the absence of an energy source (Sugiyama et al 1976) and in artificial lipid bilayers (Nelson et al 1980, Schindler & Quast 1980). It has been suggested that phosphorylation may be required for stabilization and maintenance of receptor clusters at the synapse (Gordon et al 1977a,

Saitoh & Changeux 1980, 1981). Prior to innervation of developing muscle and following denervation of mature muscle, acetylcholine receptors are present over the whole surface of the muscle as well as at the immature or denervated endplate (for review see Fambrough 1979). Although the subunit structure of the two populations of receptors is the same, the "extrajunctional" receptors differ from the junctional receptors in several ways:

1. They turn over more rapidly [17 hr (Merlie et al 1976) vs 2 wk or longer (Frank et al 1975, Linden & Fambrough 1979)].
2. Their mean channel open time is longer (Michler & Sakmann 1980, Fischbach & Schuetze 1980).
3. Their isoelectric point is 0.1 pH units more basic (Brockes & Hall 1975).

Saitoh & Changeux (1981) have presented evidence that these differences may be accounted for in part by receptor phosphorylation. They found that neonatal electric fish have a population of acetylcholine receptors that have an isoelectric point 0.1 pH units more basic than that of adult receptors. Treatment of the adult receptors with alkaline phosphatase has two effects:

1. It shifts their isoelectric point toward the more basic pH.
2. It results in an increased incorporation of phosphate during subsequent phosphorylation by the endogenous kinase.

The neonatal receptors are affected to a smaller extent by this treatment. These results suggest that the adult receptors are more phosphorylated than the neonatal receptors, and that this phosphorylation may contribute to their stabilization at mature endplates.

FUTURE DIRECTIONS

The goal of studies of neuronal protein phosphorylation is to better understand, at the molecular level, both short and long-term regulation of neuronal properties. Considerable progress has been made in the past several years. There is now evidence that phosphorylation plays a role in the regulation of ion channels, transmitter synthesis, synaptic vesicle function, and possibly, in the stabilization of clusters of receptors at synapses. In most of these cases, the detailed biochemistry underlying the functional changes is not yet understood. More precise descriptions of these and other, as yet unrecognized, regulatory mechanisms will come both from continued application of the techniques and approaches that have been discussed, and from the use of new techniques that have just begun to be exploited to their fullest. The production of monoclonal antibodies (Köhler & Milstein 1975) that recognize protein kinases and their substrates should facilitate their purification and localization, and may also provide highly specific inhibitors for use in the dissection of regulatory pathways. Internal perfusion of single

cells (Lee et al 1980) and the study of single channels by "patch-clamping" (Hamill et al 1981) will provide the technical means for obtaining a complete description of the regulation of individual channels by purified components. Characterization of proteins involved in specialized neuronal functions will make it possible to identify and isolate their messenger RNAs. This will, in turn, allow the preparation of probes of complementary DNA for use in studying the control of gene expression during neuronal development.

It seems likely from the studies I have discussed that different types of neurons will contain distinct regulatory pathways that participate in specialized functions. For example, it appears that invertebrate neurons contain different mechanisms for modulating K^+-channels. In one cell type (R_{15}), cAMP increases K^+-conductance, and in two others (bag cells and sensory neurons), it decreases K^+-conductance. In addition, the G-substrate appears highly localized in cerebellar Purkinje cells. Consequently, descriptions of neuronal regulatory pathways, and immunocytochemical localization of the proteins involved, may begin to uncover subpopulations of functionally related neurons within complex neural structures. Thus, biochemical studies of regulatory mechanisms involving protein phosphorylation may be helpful not only in analyzing neuronal function at the cellular level, but also in understanding the organization and function of the nervous system as a whole.

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