Cyclic nucleotides, phosphorylated proteins, and the nervous system[1,2]

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ABSTRACT

Some postsynaptic effects of several classes of neurotransmitters appear to be mediated or modulated through the cyclic nucleotides, cyclic AMP and cyclic GMP. Available evidence suggests that the molecular mechanism by which the cyclic nucleotides carry out this second messenger role in nerve cells involves regulation of the state of phosphorylation of specific neuronal proteins. Phosphorylated proteins also appear to be involved in mediating certain of the actions of several other classes of regulatory agents, including calcium and the steroid hormones.—**Greengard, P.** Cyclic nucleotides, phosphorylated proteins, and the nervous system. *Federation Proc.* 38: 2208–2217, 1979.

CYCLIC NUCLEOTIDES

The possibility that communication between cells in the nervous system might share characteristics in common with communication between cells in the endocrine system led us, a number of years ago, to begin an investigation of possible roles for cyclic AMP in neuronal tissue. As in the case with hormones acting on nonneuronal tissues (41), there is now evidence that cyclic AMP may act as an intracellular mediator for the actions of certain neurotransmitters on nerve cells (for recent reviews, see refs 2, 3, 9, 15–17, 38). There is also evidence that certain of the effects of some other neurotransmitters on their target cells may be mediated by cyclic GMP.

Neurotransmitter molecules, when released from the terminals of a presynaptic neuron, induce a change in the permeability properties of the membrane of the postsynaptic neuron by interacting with specific receptors located on the cell surface. The molecular nature of the events by which the binding of the neurotransmitter to its receptor is coupled with the alteration in the ionic properties of the postsynaptic cell membrane is not yet well understood. However, there appear to be two general classes of mechanism by which this process may occur (22).

The "receptor–ionophore" model: In one class the neurotransmitter receptor is thought to be coupled directly to an ionophore in such a manner that binding of the neurotransmitter to the receptor induces a conformational change in the ionophore, leading to a change in the permeability of the membrane to specific ions. One receptor that appears to work in this manner, and that has been intensively studied by Changeux, Heilbronn, Karlin, Raftery, Reich, and their colleagues, is the nicotinic cholinergic receptor.

The "receptor–second messenger" model: A second general class of mechanisms by which a neurotransmitter could affect the electrical properties of postsynaptic neurons is through a process in which the binding of the neurotransmitter to its receptor on the outside of the cell membrane leads to the production of another molecule, a second messenger, such as cyclic AMP, on the inside of the cell. This second messenger could then initiate a sequence of biochemical reactions that would ultimately result in a change in the electrophysiological properties of the neuronal membrane. Such electrophysiological changes might be caused by a biochemical modification of a membrane protein involved in regulating the permeability of the membrane to specific ions.

One might anticipate that the physiological responses caused by activation of the two different classes of receptors would have different characteristics. In the first case, where the binding of the neurotransmitter to the receptor induces a conformational change in an ionophore, the permeability change resulting from this one-step, nonenzymatic process might be expected to have a very short latency and duration, on the order of several milliseconds. In contrast, it seems likely that the effects of cyclic nucleotides on the permeability properties of neuronal membranes are mediated by means of a sequence of biochemical reactions; therefore, the permeability changes generated in response to a cyclic nucleotide might be expected to have a much longer latency and duration.

There is now evidence for two types of synaptic transmission involving the receptor–second messenger system (Fig. 1). Thus, the postsynaptic cell of the receptor–second messenger modulatory synapse can be either the presynaptic or the postsynaptic cell of the synapse being modulated. In other words, the receptor–second messenger system can be utilized for either presynaptic (Fig. 1*A*) or postsynaptic (Fig. 1*B*) modulation of synaptic transmission. In addition to mediating the effects of modulator neurons on the permeability properties of input and output neurons, cyclic nucleotides appear to have many other roles in neuronal function (15, 16).

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[1] From the American Society for Pharmacology and Experimental Therapeutics Symposium on *Cyclic Nucleotides and Synaptic Function* presented at the 62nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, NJ, April 10, 1978.

[2] Supported by Public Health Service grants DA-01627, MH-17387 and NS-08440.

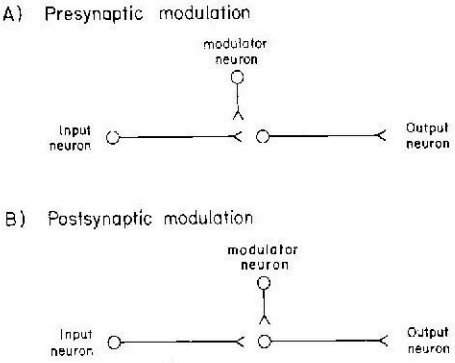


Figure 1. Two types of modulation of synaptic transmission. In both types, neurotransmitter released from the modulator neuron uses cyclic AMP as a second messenger in the target cell. *A*) presynaptic modulation. In this type of modulation, the postsynaptic cell of the receptor–second messenger synapse is in turn a presynaptic terminal, and is labeled "input neuron." *B*) postsynaptic modulation. In this type of modulation, the postsynaptic cell of the receptor–second messenger synapse is also the postsynaptic cell for another synapse, and is labeled "output neuron." (Modified from 17.)

A variety of experimental criterions useful in evaluating whether a cyclic nucleotide mediates a postsynaptic permeability change have been presented elsewhere (2). Failure to satisfy one or more of these criterions does not exclude the possibility that a cyclic nucleotide mediates a particular postsynaptic response. Some of the difficulties associated with testing certain of the criterions are discussed in detail elsewhere (2).

The demonstration of the existence of neurotransmitter-sensitive adenylate cyclases in nervous tissue, their regional and subcellular distribution, and the properties of these enzymes suggests a role for cyclic AMP in the physiology of synaptic transmission. For each of the neurotransmitters listed in Table 1, an adenylate cyclase activated by low concentrations of the neurotransmitter in question has been found in nervous tissue. Moreover, compounds that act as physiological agonists (i.e., mimic the physiological actions) of each of these neurotransmitters stimulate the appropriate adenylate cyclase. Conversely, those substances that act as antagonists of the physiological effects of these neurotransmitters in general block the activation by the neurotransmitter of the particular adenylate cyclase. Representative antagonists for these neurotransmitters are also included in Table 1. For the group of neurotransmitters listed in Table 1, the close similarity between the properties of the neurotransmitter receptor, as characterized in physiological experiments, and the properties of the neurotransmitter-sensitive adenylate cyclase, using a variety of physiological and pharmacological agonists and antagonists, suggests an intimate association between neurotransmitter receptors and adenylate cyclase molecules.

TABLE 1. Neurotransmitters associated with cyclic AMP system

Neurotransmitter	Antagonist
Dopamine	Chlorpromazine
Serotonin	LSD
Norepinephrine (β)	Propranolol
Histamine (H_2)	Metiamide
Octopamine	Phentolamine

(Taken from 17.)

Table 2 lists those neurotransmitters that cause increases in cyclic GMP in certain target cells. Each of these neurotransmitters is capable of causing increases in cyclic GMP in postsynaptic cells under conditions in which they produce a physiological response. In addition, substances that mimic the physiological effects of these neurotransmitters also mimic the neurotransmitter in causing increases in cyclic GMP in postsynaptic cells. Conversely, substances that act as antagonists of the physiological effects of these neurotransmitters block the increase in cyclic GMP brought about by the appropriate neurotransmitter in the postsynaptic cells. Representative antagonists for these neurotransmitters are included in Table 2. The available data suggest that cyclic GMP mediates some effects, and modulates other effects, of neurotransmitters on postsynaptic cells. The possible relationship of the neurotransmitter-induced increase in cyclic GMP to the physiological response of the postsynaptic cells is discussed in detail elsewhere (17).

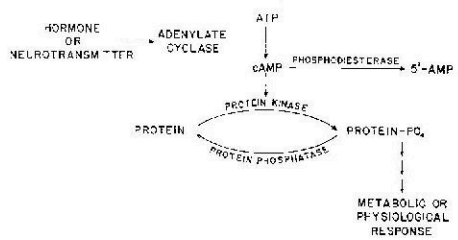
Studies of cyclic nucleotides in several neuronal preparations, including the superior cervical ganglion (17) and the mammalian cerebellum (3), leave little doubt that synaptic transmission under physiological conditions can result in an increase in the cyclic nucleotide content of certain postsynaptic neurons. The precise role or roles of the cyclic nucleotides formed in the postsynaptic cells, during physiological activity, are less well established. We have proposed that cyclic nucleotides formed in neurons in response to neurotransmitter action may have multiple roles including, but by no means limited to, the generation of certain slow postsynaptic potentials (15, 17, 22). Two other possible roles for cyclic AMP in nervous tissue include regulation of microtubule function and of neurotransmitter biosynthesis. The increase in cyclic AMP during increased impulse flow may also serve to mobilize carbohydrate (12) and lipid reserves, and thus help to meet the additional energy requirements associated with elevated functional activity in nervous tissue. In certain invertebrate nerve networks, an increase in cyclic AMP mediated by serotonin has been shown to be responsible for the increased neurotransmitter release that accompanies behavorial sensitization (5). It is also of interest that, in various model systems, cyclic AMP may act at a tran-

TABLE 2. Neurotransmitters associated with cyclic GMP system

Neurotransmitter	Antagonist
Acetylcholine (muscarinic)	Atropine
Histamine (H_1)	Diphenhydramine
Norepinephrine (α)	Phentolamine
Glutamate	?

(Taken from 17.)

Figure 2. Schematic diagram of apparent role played by protein phosphorylation in mediating the biological effects of those hormones and neurotransmitters that act through cyclic AMP. The appropriate hormone or neurotransmitter stimulates a specific adenylate cyclase present in the target tissue, causing an increased conversion of ATP to cyclic AMP; the newly formed cyclic AMP is either hydrolyzed by a phosphodiesterase to 5'-AMP or else it activates a protein kinase, leading to the phosphorylation of a substrate protein; the substrate protein, through one or more steps, controls the rate of some metabolic or physiologic response; the phosphorylation of this substrate protein leads to an increase or decrease in the rate of this response. (Taken from 16. Copyright 1978 by the American Association for the Advancement of Science.)



scriptional level to regulate the de novo synthesis of a number of proteins with specific functional roles (see ref 17 for review). It is possible that, in the nervous system, elevation of the cyclic AMP content of presynaptic or postsynaptic cells in response to increased presynaptic impulse flow may also result in altered synthesis of certain proteins having specific functional roles in synaptic physiology. This might constitute one component of the mechanism by which long-term information storage (i.e., learning) and retrieval (i.e., memory) can occur.

It is probably important to view the synaptic and nonsynaptic actions of cyclic AMP not so much in isolation but rather as part of an integrating system. It may be that enough synaptic stimulation not only raises cyclic AMP levels sufficiently to alter the permeability properties of neurons, but also initiates a logical sequence of events mediated by the cyclic nucleotide. This sequence might include an increase or decrease in the synthesis or release of neurotransmitter in response to synaptic stimulation, an initiation of intracellular movements in order to transport newly synthesized products, the activation of carbohydrate metabolism to supply the necessary cellular energy requirements, and direct effects on the genetic material in the cell nucleus that may lead to long-term alterations of behavior.

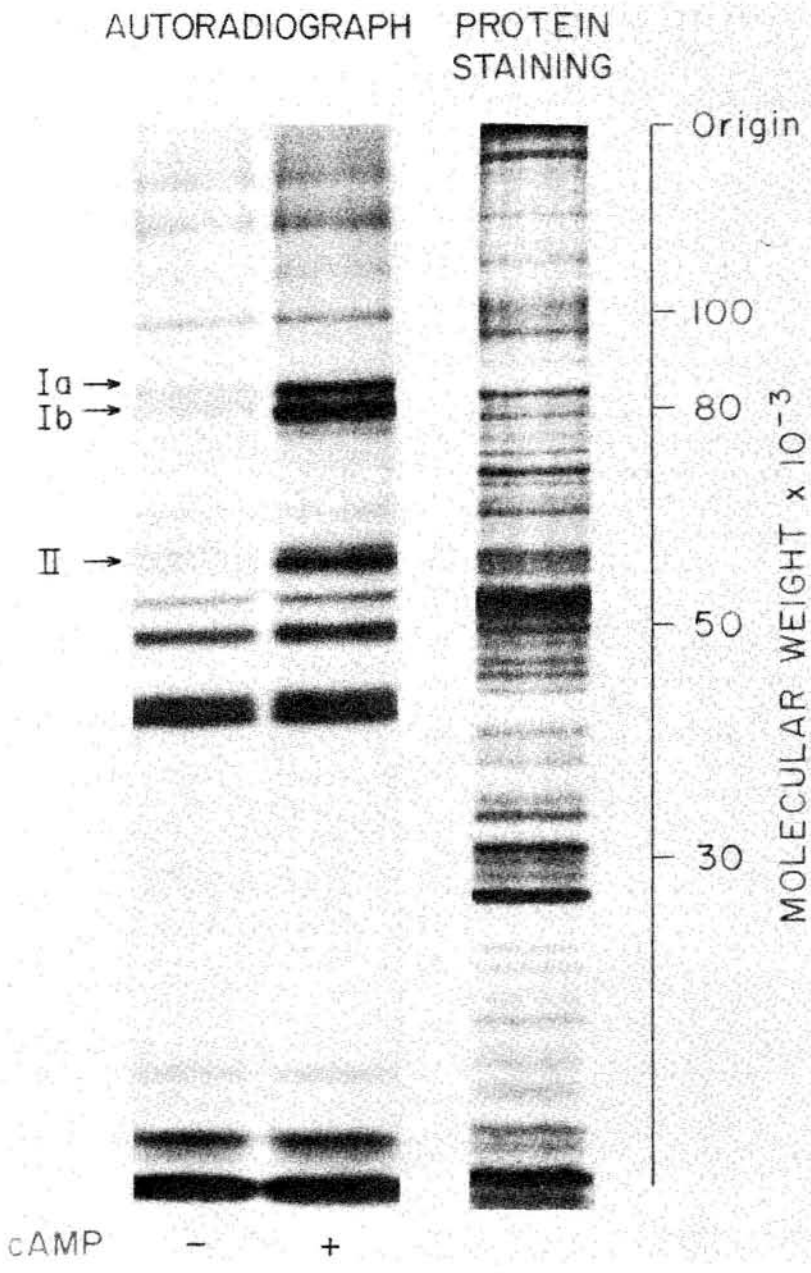
CYCLIC AMP-DEPENDENT PROTEIN PHOSPHORYLATION

I would like now to discuss a mechanism by which cyclic AMP may mediate the effects of neurotransmitters on postsynaptic cells. The protein kinase hypothesis, illustrated schematically in Fig. 2, provides a theoretical mechanism by which one simple molecule, cyclic AMP, could achieve a diversity of biological responses (28). According to this concept, cyclic AMP directly regulates the activity of a single class of enzymes, namely protein kinases. The specificity of the response to the cyclic AMP then resides in the nature of the protein kinases, and especially in the nature of the substrate proteins for the protein kinases in the various tissues.

An increasing amount of evidence supports the protein kinase hypothesis. Thus, numerous examples have now been found (for reviews, see refs 15–17, 25, 31, 39, 44) of systems in which the effects of cyclic AMP on various metabolic and physiological responses appear to be mediated through protein phosphorylation. At the present time, it seems likely that in eukaryotic organisms most, and conceivably all, of the metabolic and physiological effects of cyclic AMP will prove to be mediated through regulation of protein phosphorylation.

The literature on the subject of protein phosphorylation, even when restricted to the nervous system, is now vast. In this presentation, I shall review one study of this subject from our own laboratory (49, 53–55, and S. M. Loh-

Figure 3. Effect of cyclic AMP on endogenous protein phosphorylation in a synaptic membrane fraction from rat caudate nucleus. The synaptic membrane fraction was incubated with 7 μM [γ-^{32}P]ATP for 15 sec at 30 C, in the absence (−) or presence (+) of 10 μM cyclic AMP. The reaction was terminated by the addition of the detergent sodium dodecyl sulfate, which also solubilized the membrane proteins. An aliquot of the reaction product was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in order to separate the membrane proteins from one another. The separated proteins were located in the gel by standard procedures of protein staining. Autoradiography was then carried out to determine those protein bands into which radioactive phosphate had become incorporated. Left, phosphorylation of endogenous protein in the absence or presence of cyclic AMP; right, protein staining. (Modified from 49.)



mann, U. Walter, W. Sieghart and P. Greengard, ms in preparation). In view of the apparent importance of cyclic AMP-dependent protein phosphorylation in the nervous system, my colleagues and I have searched for substrate proteins for cyclic AMP-dependent protein kinase in nervous tissue. The ability of endogenous protein kinase in a synaptic membrane fraction from the rat caudate nucleus to phosphorylate endogenous substrate proteins in this fraction is demonstrated by the experiment shown in Fig. 3. As shown by the protein staining pattern in this figure, a large number of distinct bands were found upon gel electrophoresis of the solubilized proteins from this synaptic membrane fraction. Of these many bands, the phosphorylation of three was markedly stimulated by cyclic AMP. We refer to these three bands as Proteins Ia, Ib, and II. Recently, it has been possible to resolve Protein II into two bands (Lohmann et al., ms in preparation), one of which has been identified as the regulatory subunit of a protein kinase present in synaptic membranes (55); this regulatory subunit is phosphorylated by the catalytic subunit in an autophosphorylation reaction (55). Proteins Ia plus Ib, collectively designated Protein I, are present only in nervous tissue (53). In view of the possibility that Protein I might be involved in the physiology of synaptic transmission, we have investigated its biological and biochemical properties. The results of those studies indicate that Protein I has a number of unique properties.

Some of the biological properties of Protein I are summarized in Table 3. Not only does Protein I appear to be confined to the nervous system (53, 54), but within the nervous system Protein I appears to be present only in neurons (49). This latter conclusion is based on observations that injection,

TABLE 3. Some biological properties of Protein I

Present only in nervous system
Within nervous system, present only in neurons
Within neurons, present only in synaptic membranes and synaptic vesicles
Appears simultaneously with synapse formation during development
Substrate for membrane-bound protein kinase and protein phosphatase

(Taken from 17.)

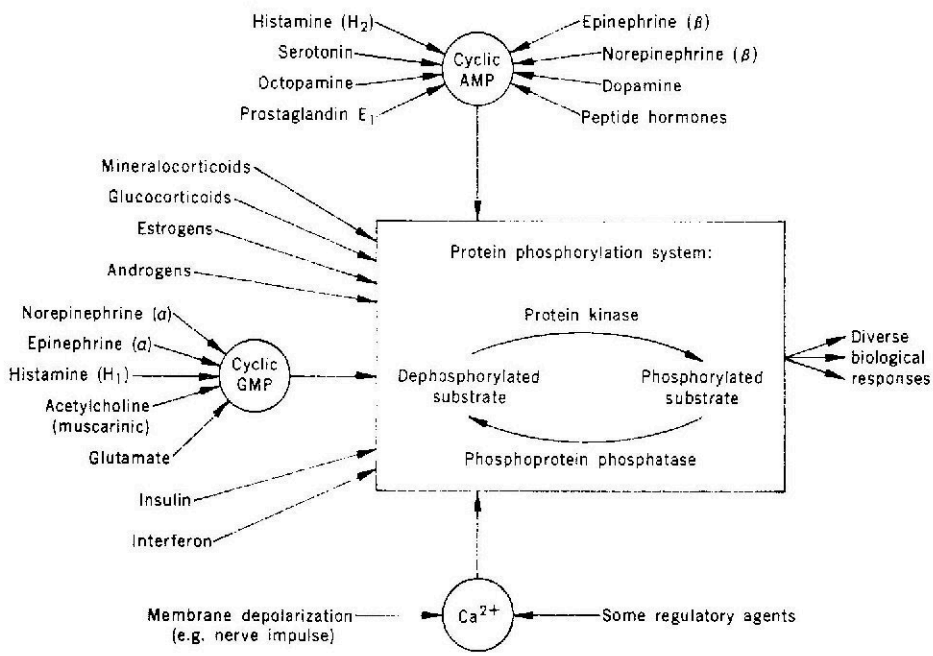
TABLE 4. Some chemical properties of Protein I purified from bovine brain

	Protein IA	Protein IB
Molar proportion	1	2
Molecular weight	86,000	80,000
Isoelectric point	10.3	10.2
Stokes radius	59 Å	59 Å
Shape	Highly elongated	Highly elongated
Amino acid composition	Rich in glycine and proline	Rich in glycine and proline
Other structural features	A globular region (phosphorylated) and an elongated collagenous tail (not phosphorylated)	

(Taken from 17.)

into brain, of kainic acid, a substance that selectively destroys neurons (8, 19, 35), causes an extensive depletion of several brain phosphoproteins, including Proteins Ia and Ib. Subcellular fractionation studies carried out by Tetsufumi Ueda, in collaboration with the research group of Philip Siekevitz at Rockefeller University, indicate that, within neurons, Proteins Ia and Ib are present in high concentration in synaptic membrane fractions, and in particularly high concentration in fractions enriched in synaptic vesicles and postsynaptic densities (T. Ueda, P. Greengard, K. Berzins, R. S. Cohen, F. Blomberg, D. J. Grab and P. Siekevitz, ms in preparation). The concentration of Proteins Ia and Ib in postsynaptic densities is much higher than that in the synaptic membrane fractions from which the postsynaptic densities are prepared; therefore, it is possible that the content of Proteins Ia and Ib in synaptic membrane fractions may be

Figure 4. Schematic diagram of postulated role played by protein phosphorylation in mediating some of the biological effects of a variety of regulatory agents. The diagram gives examples of regulatory agents some of whose effects may be mediated through regulation of the phosphorylation of specific proteins, and is not intended to be complete. In addition to cyclic AMP and a variety of neurotransmitters and hormones whose effects are mediated through cyclic AMP, these regulatory agents include cyclic GMP, a variety of neurotransmitters and hormones whose effects are mediated through cyclic GMP, Ca^{2+}, and agents whose effects are mediated through Ca^{2+}, as well as several classes of steroid hormones, insulin, and interferon. For brevity, the numerous peptide hormones whose effects are known to be mediated through cyclic AMP, and the various regulatory agents believed to act through translocation of Ca^{2+}, are not listed individually. It seems likely that some, but not necessarily all, of the biological responses elicited by any given one of the regulatory agents shown are mediated through the protein phosphorylation system; for simplicity, pathways from regulatory agent to biological response that do not involve protein phosphorylation are not shown. (Taken from 16)



attributable to the postsynaptic densities present in these fractions. In agreement with the results of subcellular fractionation studies, immunocytochemical studies also indicate that Protein I is associated with synaptic vesicles and postsynaptic densities (F. E. Bloom, E. Battenburg, T. Ueda and P. Greengard, ms in preparation). A correlation between the time course of synapse formation and the appearance of Proteins Ia and Ib in the brains of developing rats and guinea pigs has provided further evidence that Proteins Ia and Ib are associated with synaptic structures (34). Protein I serves as a substrate for a Type II membrane-bound cyclic (AMP-dependent protein kinase (54, 56 and Lohmann et al., ms in preparation) and a membrane-bound phosphoprotein phosphatase (55).

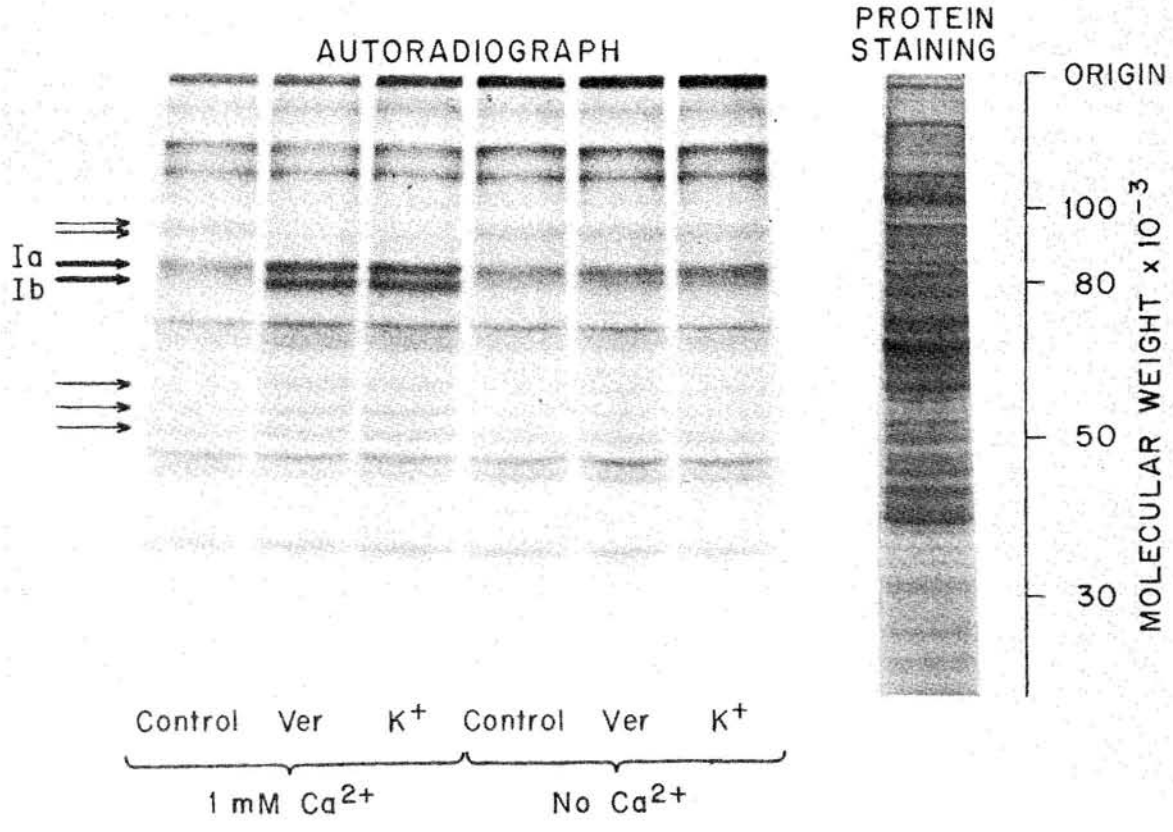
Recently, alterations in the phosphorylation of Proteins Ia and Ib have been observed in intact brain slices (13) and in whole animals (49a) in response to various chemical agents. For instance, several central nervous system depressants (including pentobarbitol, chloral hydrate, and urethane) administered to mice intraperitoneally decrease the state of phosphorylation of Protein I and, conversely, the convulsant drugs pentylenetetrazole and picrotoxin increase the state of phosphorylation of Protein I, in whole brains of mice in vivo (49a). These results indicate that the state of phosphorylation of Protein I, a protein confined to the synaptic region of neurons, can be altered by changes in neuronal activity, supporting the possibility that it may play an important role in the physiology of the synapse.

Biochemical studies of Protein I indicate that this molecule is unusual from a chemical point of view. Protein I is present in the brains of all mammalian species examined. Using bovine brain, in order to have large amounts of starting material, Protein I has been purified several thousandfold to apparent homogeneity. Some of the unusual chemical properties of purified Protein I (53) are summarized in Table 4.

REGULATION OF PROTEIN PHOSPHORYLATION BY CALCIUM, CYCLIC GMP AND STEROID HORMONES

Finally, I would like to discuss protein phosphorylation in a broader context than that of its association with the cyclic AMP system. Some of the studies in our laboratory in the past few years were designed to test the possibility that phosphorylated proteins may play a much wider role in biological regulation than one limited only to

Figure 5. Effects of veratridine and of high K^+, in the absence and presence of Ca^{2+}, on the phosphorylation of endogenous proteins in crude synaptosomal preparation from rat cerebral cortex. The synaptosome fraction was preincubated with $^{32}P_i$ for 30 min in the absence of Ca^{2+}. Aliquots of this suspension were then incubated for 30 sec in the absence (Control) or presence of 100 μM veratridine (Ver) or 60 mM K^+; 1 mM Ca^{2+} was present where indicated. The incubation was terminated by the addition of sodium dodecyl sulfate and the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The molecular weight scale was generated by determining the position of marker proteins of known molecular weight. The positions of Proteins Ia and Ib, whose phosphorylation was stimulated by veratridine or high K^+, are indicated by bold arrows. Light arrows indicate the positions of other protein bands whose phosphorylation was inhibited (upper two arrows) or stimulated (lower three arrows) by veratridine or high K^+. (Taken from 26.)



mediating the effects of cyclic AMP. The results obtained thus far suggest that phosphorylated proteins probably mediate not only the effects of cyclic AMP and those regulatory substances that act through cyclic AMP, but also certain of the effects of a variety of other regulatory agents as well. Thus, it seems that protein phosphorylation may be a final common pathway for the actions of many types of biological regulatory agents. This concept is illustrated schematically in Fig. 4. Experimental data obtained in our own and other laboratories suggest that all of the regulatory agents listed in Fig. 4 achieve at least certain of their effects through protein phosphorylation. This subject was reviewed in some detail recently (16, 17), and therefore I shall limit my discussion to a brief summary of three of the specific regulatory substances that have been studied in our laboratory, namely, Ca^{2+}, cyclic GMP, and the steroid hormones.

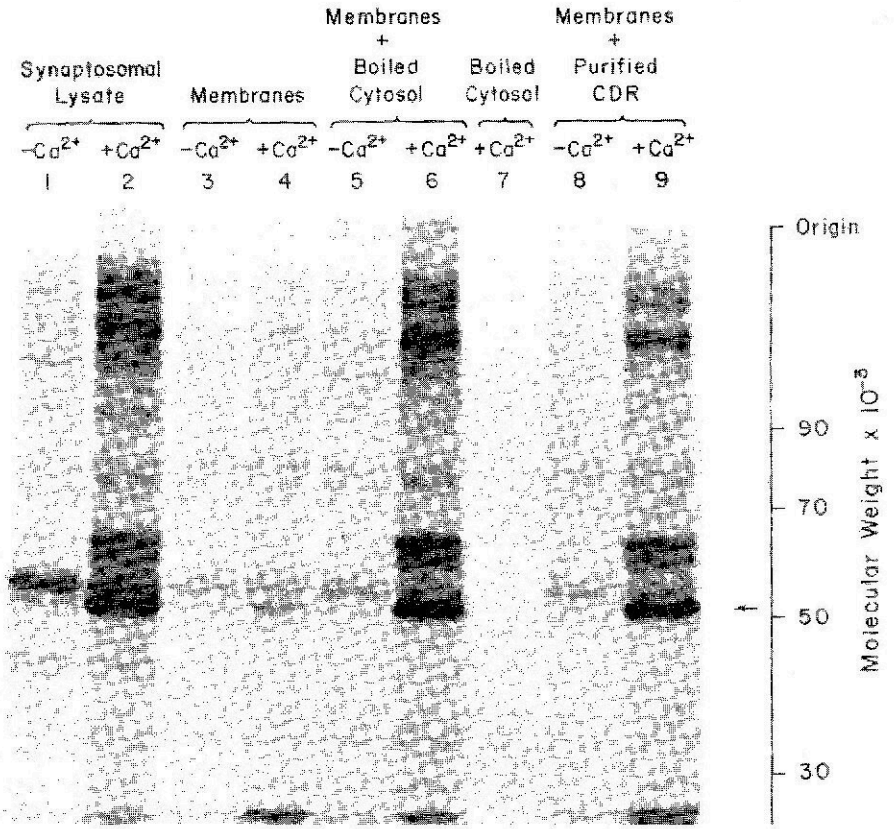
Regulation by Ca^{2+} of protein phosphorylation

When an impulse travels down a nerve axon to the terminal, the wave of depolarization causes an influx of calcium that acts as the immediate trigger for the release of neurotransmitter from the terminal (1, 11, 23, 24). In addition, there is evidence that calcium influx controls the rate of neurotransmitter biosynthesis in the presynaptic terminal (36, 40). It seemed possible that one or more of the effects of calcium in the presynaptic nerve terminal might be mediated or modulated through the phosphorylation of specific proteins. For this reason, Bruce Krueger and Javier Forn studied the effect of calcium influx on protein phosphorylation in intact synaptosomes. They found (26) that agents known to increase Ca^{2+} transport across the plasma membrane of nerve terminals altered the phosphorylation of specific endogenous proteins in intact synaptosomes from rat brain. Synaptosome preparations, preincubated in vitro with $^{32}P_i$, incorporated ^{32}P into a variety of specific proteins. Veratridine and high (60 mM) K^+, which increase Ca^{2+} transport across membranes through a mechanism involving membrane depolarization, as well as the calcium ionophore A23187, each markedly altered the incorporation of ^{32}P into several specific proteins as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. All three agents failed to alter protein phosphorylation in calcium-free medium containing ethylene glycol bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA). The effects of veratridine and of high K^+ on the phosphorylation of proteins in intact synaptosomes are illustrated in Fig. 5.

The data suggested that conditions that cause an accumulation of calcium by synaptosomes lead to a calcium-dependent increase in phosphorylation of specific endogenous proteins. The results support the possibility that these phosphoproteins may be involved in the regulation of certain calcium-dependent nerve terminal functions.

Evidence concerning the mechanism by which calcium entry might stimulate the phosphorylation of synaptosomal proteins has come recently from experiments of Howard Schulman using lysed synaptosomes (46). In these experiments, synaptosomes obtained from rat cerebral cortex were lysed by hypoosmotic shock and assayed for incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into protein. As shown in Fig. 6 (Lanes 1 and 2), calcium markedly stimulated [^{32}P]-phosphate incorporation into many protein bands in the lysed synapto-

Figure 6. Effect of Ca^{2+}, synaptosomal cytoplasm, and purified calcium-dependent regulator (CDR) on endogenous protein phosphorylation of brain membranes. Synaptosomes obtained from rat cerebral cortex were subjected to osmotic shock by homogenization in 10 volumes of ice-cold water (15 ml) and kept on ice for 30 min to optimize lysis. Total membranes were obtained from lysed synaptosomes by centrifugation at 150,000 × g for 30 min. The released synaptosomal cytosol was removed, heated at 95–100 C for 5 min, and used as the source of activator (boiled cytosol). The pellet was resuspended in 15 ml of 5 mM Tris-HCl (pH 7.0), centrifuged as above, and again resuspended in 15 ml of 5 mM Tris-HCl (pH 7.0). This resuspended pellet served as the source of activator-deficient membranes. The reaction mixture for assay of endogenous protein phosphorylation (final volume, 100 μl) contained: 50 mM PIPES buffer (pH 7.0), 10 mM $MgCl_2$, 1 mM dithioerythritol, 0.2 mM EGTA (minus calcium) or 0.2 mM EGTA + 0.5 mM $CaCl_2$ (plus calcium), 5 μM [γ-^{32}P]ATP (5.2×10^4 cpm/pmol), and, where indicated, lysed synaptosomes (50 μg protein), membranes (34 μg protein), boiled cytosol (14 μg protein), or purified CDR (0.25 μg protein). Incubation was carried out for 10 sec at 30 C, the reaction terminated by addition of sodium dodecyl sulfate, and an aliquot of the sample analyzed for protein phosphorylation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. (Taken from 46.)



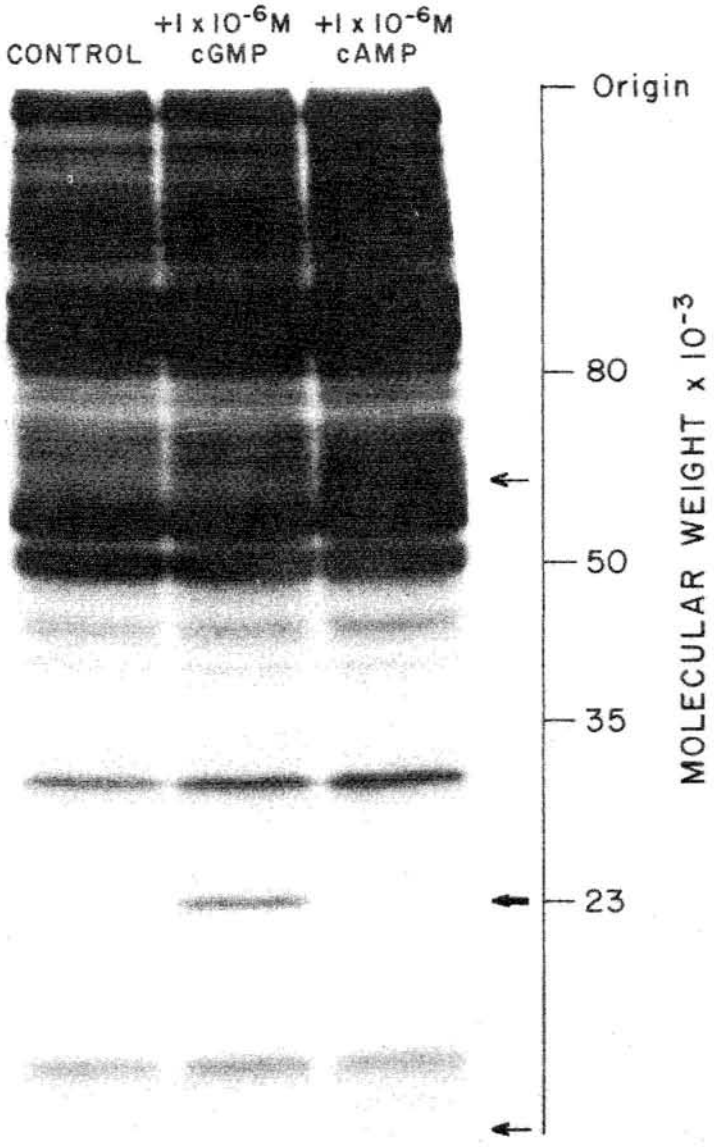
some preparation, which contained membranes (synaptic membranes plus synaptic vesicles) and synaptosomal cytoplasm. The calcium-dependent phosphorylation observed in lysed synaptosomes was lost upon preparation of cytoplasm-free membranes (Fig. 6, Lanes 3 and 4). This phenomenon was not due to inactivation of the calcium-dependent protein kinase activity during experimental manipulations, as it could be regained by addition of an amount of boiled (Fig. 6, Lanes 5 and 6) or unboiled synaptosomal cytoplasm commensurate with the amount of membrane protein present. Boiled cytosol, containing the heat-stable factor that restored calcium-dependent phosphorylation to washed membranes, did not exhibit any endogenous phosphorylation (Fig. 6, Lane 7), nor did it manifest any protein kinase activity using exogenous substrates.

It had been demonstrated earlier that Ca^{2+} plus a calcium-binding protein (calcium-dependent regulator or CDR) activate cyclic nucleotide phosphodiesterase (7, 21) and adenylate cyclase (4) from mammalian brain. The heat stability of the factor in the synaptosomal cytosol that stimulated Ca^{2+}-dependent protein phosphorylation suggested a possible relationship to CDR. Therefore, CDR was purified to homogeneity from bovine cerebral cortex by the method of Teo et al. (51), in order to test its effect in the calcium-dependent protein phosphorylation system. Addition of this protein to washed synaptosomal membrane fractions restored calcium-dependent protein phosphorylation (Fig. 6, Lanes 8 and 9). The pattern of phosphorylation obtained upon addition of heat-treated synaptosomal cytoplasm was indistinguishable from that obtained upon addition of CDR, suggesting that the two factors are functionally equivalent.

Stimulation of protein phosphorylation was dependent on the presence of both calcium and either the cytosol factor or the calcium-dependent regulator (CDR). Thus, calcium in the absence of boiled cytosol and boiled cytosol in the absence of calcium were ineffective, whereas addition of both calcium and boiled cytosol restored phosphorylation (Fig. 6, compare Lanes 3, 4, 5, and 6). Analogous results were obtained with CDR (compare Lanes 3, 4, 8, and 9). The endogenous activator, like CDR, was extremely heat stable, nondialyzable, resistant to DNase and RNase, and sensitive to trypsin (46).

We have recently purified a protein from bovine cerebral cortex based on its ability to stimulate Ca^{2+}-dependent protein phosphorylation in activator-depleted membranes (47). Only a single peak of activity was present throughout the purification procedure. This activity copurified with the phosphodiesterase activator, suggesting that the endogenous activator of the calcium-dependent protein phosphorylation system is CDR. Thus it would appear that regulation by calcium of calcium-dependent protein kinase activity in nervous tissue is mediated by the same calcium-binding protein

Figure 7. Cyclic GMP-dependent endogenous protein phosphorylation in cytosol from rabbit cerebellum. The cytosol fraction was passed over a Sephadex G-25 column to remove endogenous ATP and cyclic nucleotides, and was then incubated with [γ-^{32}P]ATP for 1.5 min at 30 C, in the absence or presence of cyclic GMP or cyclic AMP as indicated. The reaction was terminated by the addition of sodium dodecyl sulfate and the samples heated at 100 C for 1 min. The entire sample was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. Cyclic GMP, but not cyclic AMP, stimulated the phosphorylation of a protein with an apparent molecular weight of 23,000 daltons, indicated by the dark arrow. No specific stimulation by cyclic GMP of phosphate incorporation into other proteins was observed, even when electrophoresis was carried out on a 7.5% polyacrylamide gel, which increased the separation of higher molecular weight proteins. (Taken from 45.)



that regulates cyclic nucleotide phosphodiesterase and adenylate cylcase activities.

These studies of nervous tissue demonstrated that calcium is a regulatory agent some of whose effects may be mediated by protein phosphorylation. In addition, recent studies indicate that many other tissues possess a protein kinase that requires both calcium and CDR for activity, suggesting that this enzyme may be of general importance as a mediator of the regulatory actions of calcium (47).

Regulation by cyclic GMP of protein phosphorylation

Several lines of evidence indicate that cyclic GMP plays an important role as an intracellular regulatory substance (14, 16). Moreover, there are some highly suggestive findings concerning the molecular basis for its actions in various biological systems. A number of years ago, J. F. Kuo and I undertook a study of the mechanism by which cyclic GMP might exert its biological effects. By analogy with cyclic AMP-dependent protein kinase, it seemed reasonable to postulate the existence of cyclic GMP-dependent protein kinase that might mediate the biological effects of cyclic GMP. Through the use of column chromatography, it was possible to demonstrate a cyclic GMP-dependent protein kinase in lobster muscle and to separate it from a cyclic AMP-dependent protein kinase present in the same tissue (29). Subsequently, cyclic GMP-dependent protein kinases have been demonstrated in a variety of other invertebrate (18, 30) and vertebrate (18, 20, 27, 37, 50) tissues. However, in contrast to the situation with the cyclic AMP-dependent protein kinases, where many substrates, some with known and some with unknown function, have been found (see 16, 17, 25, 31, 39, 44 for reviews), efforts to find endogenous proteins that are specific substrates for these cyclic GMP-dependent protein kinases proved difficult. Nevertheless, endogenous substrates for cyclic GMP-dependent protein kinases have been identified in three locations, namely, in membranes of smooth muscle (6), in membranes of intestinal brush border epithelium (10), and in cerebellar cytosol (45). I shall briefly describe our experiments with the cerebellum.

When the cytosol fraction of rat cerebellum was incubated in the presence of [γ-^{32}P]ATP, in the absence or presence of low concentrations of cyclic GMP or cyclic AMP, and then subjected to polyacrylamide gel electrophoresis and autoradiography, results of the type shown in Fig. 7 were obtained. Cyclic GMP stimulated the phosphorylation of a specific protein band with an apparent molecular weight of 23,000 (dark arrow, Fig. 7). An equal concentration of cyclic AMP failed to stimulate the incorporation of [^{32}P]phosphate into this protein. The observation that cyclic AMP stimulated phosphate incorporation into other proteins of lower and higher molecular weight (light arrows, Fig. 7) indicated that cyclic AMP-dependent protein kinase was active in the cytosol preparation.

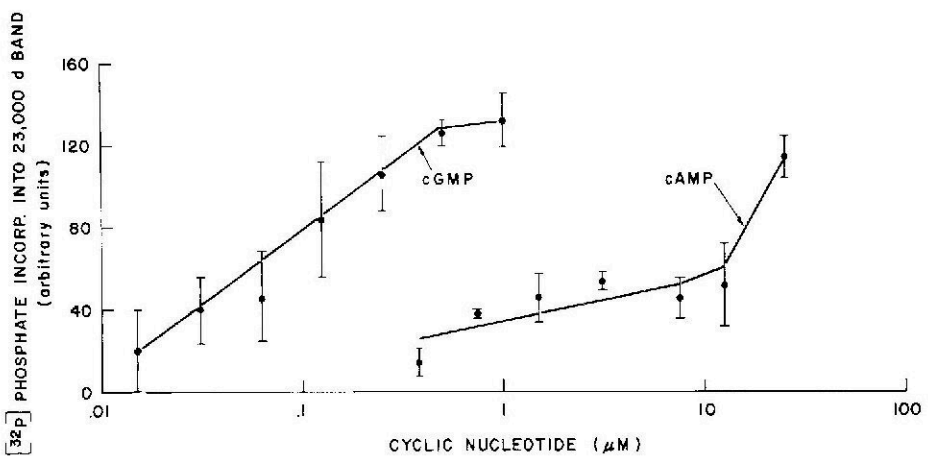
The results shown in Fig. 7 indicated that the 23,000 dalton protein was a substrate for the cyclic GMP-dependent protein kinase rather than for the cyclic AMP-dependent protein kinase that was also present in cerebellar cytosol. This conclusion was supported by studies in which cyclic GMP-dependent protein kinase and cyclic AMP-dependent protein kinase were partially purified from rabbit cerebellum and added to cerebellar cytosol. The effect of various concentrations of cyclic GMP and cyclic AMP on the phosphorylation of the 23,000 dalton protein in the presence of added cyclic GMP-dependent protein kinase is shown in Fig. 8. Cyclic GMP caused a half-maximal phosphorylation of the 23,000 dalton protein at a concentration of 60 nM. When an equal number of units (see 45) of cyclic AMP-dependent protein kinase was added to the cytosol along with cyclic AMP or cyclic GMP, the increase in the phosphorylation of the 23,000 dalton protein was much less.

We are currently attempting to purify and characterize this soluble 23,000 dalton substrate protein for the cyclic GMP-dependent protein kinase from mammalian cerebellum. It is hoped that studies of the biological and chemical properties of this molecule will contribute to an understanding of the role of cyclic GMP and of cyclic GMP-dependent protein phosphorylation in nervous tissue.

Regulation by steroid hormones of protein phosphorylation

Steroid hormones have multiple actions on their target tissues. Many of these actions are either synergistic with, or antagonistic to, the effects of those hormones whose actions are known to be mediated through cyclic AMP. Although steroid hormones have been found to affect cyclic AMP levels and the enzymes (adenylate cyclase and phosphodiesterase) involved

Figure 8. Effect of various concentrations of cyclic GMP and cyclic AMP on the phosphorylation by cyclic GMP-dependent protein kinase of the 23,000 dalton band present in cerebellar cytosol. The incubation mixture contained [γ-^{32}P]ATP, cerebellar cytosol, a partially purified cerebellar cyclic GMP-dependent protein kinase, and the indicated concentrations of cyclic GMP and cyclic AMP. Incubation was for 1 min at 30 C. Other experimental procedures were as described in the legend to Fig. 7. Incorporation of radioactive phosphate was measured quantitatively by densitometric analysis of the autoradiograms. Phosphorylation of the 23,000 dalton protein was calculated as the increase above that observed in the absence of added nucleotide, which amounted to 87 arbitrary units. (Taken from 45.)



in cyclic AMP metabolism in a variety of tissues (42, 43, 48, 57), only a few aspects of steroid-hormone action can be accounted for by the effects of these hormones on cyclic AMP levels (52). It is of significance, therefore, that representatives of all of the major classes of steroid hormones, including the mineralocorticoid, glucocorticoid, estrogen, and androgen classes, affect protein phosphorylation systems in their target tissues (32, 33). In every instance, administration in vivo of the appropriate steroid hormone affects the autophosphorylation of the regulatory subunit of a cyclic AMP-dependent protein kinase by the catalytic subunit of this enzyme. The effect is specific for the respective target tissue, occurs with low doses of the steroid hormones, and can be demonstrated within 1 hour of steroid hormone administration (33, and A. Y.-C. Liu, U. Walter and P. Greengard, ms in preparation). The detailed mechanism by which the steroid hormones affect the autophosphorylation of the regulatory subunit of cyclic AMP-dependent protein kinase is not yet understood. However, the effect of the steroids is indirect and requires de novo protein synthesis, in contrast to the direct effect of cyclic AMP on protein kinases. The effect of steroid hormones and of cyclic AMP on the same cyclic AMP-dependent protein kinase (Fig. 9) may provide a molecular basis for the ability of the steroid hormones to act synergistically (permissive action of the steroid hormones) or antagonistically with those hormones and neurotransmitters whose effects are mediated through cyclic AMP (33, and Liu et al., ms in preparation).

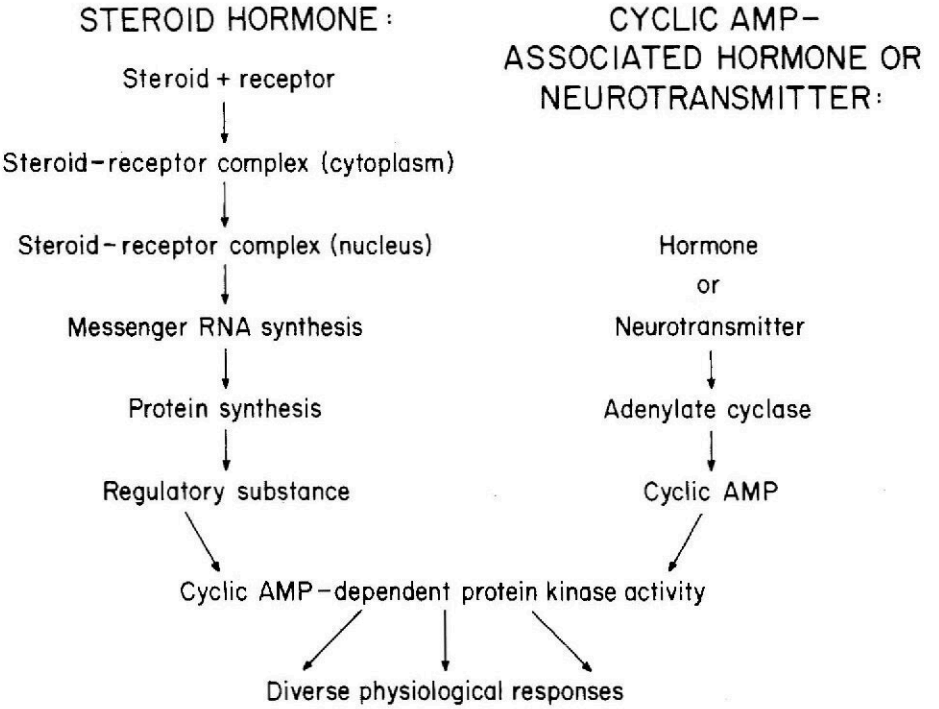


Figure 9. A possible molecular basis for the biological interactions of steroid hormones with those hormones and neurotransmitters whose effects are mediated through cyclic AMP. The steroid hormones, through a mechanism involving de novo protein synthesis but not involving cyclic AMP, regulate cyclic AMP-dependent protein kinases in their target tissues. The cyclic AMP-associated hormones and neurotransmitters, through a mechanism not involving de novo protein synthesis, cause an increase in cyclic AMP that directly affects protein kinase activity. For simplicity, mechanisms of steroid hormone action independent of the cyclic AMP-dependent protein kinase system are not shown. (Taken from 16. Copyright 1978 by The American Association for the Advancement of Science.)

CONCLUSION

In conclusion, a variety of experimental data, summarized here and in greater detail elsewhere (16, 17), indicate that protein phosphorylation represents a final common pathway in biological regulation. Several regulatory agents, including cyclic AMP, cyclic GMP, calcium, and the steroid hormones, have been shown to affect the state of phosphorylation of specific proteins in the nervous system and elsewhere. Clarification of the detailed manner in which these phosphoproteins may regulate function is an exciting area for future research. FP

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