THE cAMP CASCADE IN THE NERVOUS SYSTEM: MOLECULAR SITES OF ACTION AND POSSIBLE RELEVANCE TO NEURONAL PLASTICITY

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I. THE cAMP CASCADE: UNIVERSAL YET SPECIFIC

Cyclic adenosine 3′,5′-monophosphate (cAMP) is a ubiquitous cellular regulatory signal. Yet alterations in its level can often produce different and specific modulations in cellular activity. This transformation from universal to unique is inherent in the mode of operation of the cAMP cascade. A brief review of the molecular components of this cascade is therefore a prerequisite for further analysis of the biological activity of cAMP in general and its role in neuronal plasticity in particular. (For more extensive reviews of the biochemistry of the cAMP cascade, the reader should consult References 1 to 8.)

The discovery that cAMP is synthesized in cells in response to extracellular signals, for example, hormones, was made by Sutherland and colleagues in the 1950s.[9] An enzyme system responsible for the formation of cAMP in the presence of ATP, Mg^{2+}, and other cofactors was identified and named adenyl cyclase (EC 4.6.1.1., also adenylate cyclase or adenylyl cyclase).[10,11] Another enzyme, cAMP phosphodiesterase (PDE, EC 3.1.4.c), capable of cAMP hydrolysis, was also identified.[12] Krebs and colleagues, following earlier findings by G. and C. Cori, Sutherland, Krebs, Fischer and co-workers, discovered that cAMP acts by activating a cAMP-dependent protein kinase (cAMP-dPK, EC 2.7.1.37);[13] this together with the work of Greengard and colleagues ultimately led to formulation of the hypothesis that the diverse effects of cAMP throughout the animal kingdom are mediated via activation of cAMP-dPK and the subsequent posttranslational modification of substrate proteins by phosphorylation.[1,3,4,6,13,14] The "protein kinase hypothesis" serves to date as the central dogma of research on cAMP and its physiological functions (exceptions to the dogma were, however, suggested; see Section IX below).

The signal discoveries of the aforementioned and many other prominent research groups[1-9,10-15] have led to the following schematic picture of the structure and mode of operation of the cAMP cascade: an extracellular signal (hormone, neuromodulator, neurotransmitter, probably also ions) modulates the activity of the enzyme adenylate cyclase.[11,16] The hormones, neuromodulators, or neurotransmitters trigger this modulation by binding to specific membrane receptors, which face extracellularly. Ions may regulate the receptors, too, and also exert direct effects on other components of the adenylate cyclase complex[11] (see below). The activation of the receptor leads to activation of a guanine-nucleotide binding regulatory subunit (termed G or G/F or N). G proteins form a family,[11,16,17] two members of which are involved in regulation of adenylate cyclase. One, designated Gs, when activated by the appropriate receptor, stimulates the catalytic subunit of the enzyme; the other, designated Gi, when activated by the appropriate receptor, inhibits the enzyme. G proteins are themselves

multisubunit complexes, and their detailed and flourishing biochemistry and molecular biology are beyond the scope of this discussion.[11,16-18] G proteins thus either increase or decrease the activity of the catalytic subunit of adenylate cyclase. Whether there exists only one basic type of catalytic unit of adenylate cyclase or several, which differ in their mode of regulation (e.g., by Ca^{2+}-calmodulin), is not yet resolved.[19-22]

When cAMP was first assigned a universal role in cellular regulation, it was termed a "second messenger", the extracellular signal being the "first messenger".[6,7] Recent data on the role of G proteins in signal transduction[17,23-26] suggest that subunits of the latter may themselves be regarded as second messengers, shuttling information between receptors and target proteins (not necessarily in systems which generate cAMP), while cAMP itself fulfills the role of an intracellular messenger downstream the cascade.

Total cAMP level in the cell is regulated not only by its rate of synthesis by adenylate cyclase, but also by its rate of hydrolysis by cAMP-phosphodiesterase (cAMP-PDE). Again, multiple forms of the enzyme are known, differing in their molecular weight, subcellular distribution, and affinity for cAMP.[12,27,28]

An increase in the level of cAMP leads to activation of cAMP-dPK, which catalyzes the transfer of the γ-phosphoryl group of ATP to the hydroxyl group of a seryl or threonyl residue of a substrate protein.[5,6] cAMP-dPK is a multisubunit enzyme, composed of two types of subunits, catalytic and regulatory. There are two cAMP binding sites on each regulatory subunit and none on the catalytic subunit. The catalytic subunit is myristylated[29] and was reported to contain phosphoryl groups.[30] The physiological function of both residues is not known. Only a single gene was found to code for the catalytic subunit in the mouse; the gene was recently cloned.[31]

Two major classes of cAMP-d-PK are known, originally designated by convention Type-I and Type-II on the basis of their elution from DEAE-cellulose.[32] These two enzyme forms contain similar catalytic subunits, but distinct regulatory subunits, designated R-I and R-II, respectively. The two regulatory subunits have different biochemical properties, for example, R-I has a smaller molecular weight and a higher affinity for cAMP than R-II and is not a substrate for phosphorylation by the catalytic subunit, whereas R-II can undergo such an intramolecular-complex autophosphorylation.[5,6,33-36] R-II, at least in some nonneural tissues, may also undergo catalytic subunit independent phosphorylation and dephosphorylation.[36a] Bovine R-I has been cloned and appears to be a product of a single gene.[37] Biochemical and immunological techniques revealed tissue and species heterogeneity of R-II,[38-45] for example, R-II from bovine cerebral cortex displays higher affinity for certain binding proteins than R-II from bovine cardiac and skeletal muscle[45a] (see Sections VII and VIII.C below). It is not yet clear how much of these heterogeneity results from posttranslational modification, e.g., proteolysis. It was, however, reported, on the basis of differences in immunological properties and in primary structure, that brain R-II represents a unique gene product.[45] Such "neuronal" regulatory subunit may not be as specific to the nervous tissue as previously thought: its presence in rat ovary was recently detected.[46]

The cAMP-dPK holoenzyme consists, in both Type-I and Type-II cAMP-dPK, of a regulatory subunit dimer and of two catalytic unit monomers. The regulatory subunits inhibit the catalytic subunit, and thus the $(R)_2,C_2$ form of the enzyme is inactive. cAMP activates the enzyme by binding to R, leading to dissociation of two active catalytic subunits. All four cAMP binding sites on the holoenzyme are involved in the process in a positively cooperative manner.[5,6,33-36] The catalytic unit of cAMP-dPK has broad substrate specificity, a property which will become important in the course of our discussion (Sections IX and XIII). The amino acid sequences around the phosphorylated site of several substrate proteins have been characterized in recent years and in most cases known to date are of the type arg-arg/ser-X-ser/thr(P).[5,6] The same site serves as a specific target for protein phosphatases (PP; EC 3.1.3.-), which, together with the protein kinase, regulate the phosphorylation state

of substrate proteins. Few major classes of broad-specificity PP have been identified.[2,47] Specific endogenous polypeptide inhibitors exist which regulate the activities of cAMP-dPK or of the PPases; at least one of the latter is itself subject to regulation by cAMP-dPK (see Section VI below).

The phosphorylation of substrate protein(s) by cAMP-dPK brings about, or initiates, cellular response to the extracellular signal that has triggered the cAMP cascade. The properties and role of several such substrates in neural tissue, and the established or postulated physiological outcome of their phosphorylation, are the subject of the present review.

From the very brief review of the general properties of the cAMP cascade presented above, it is evident that specificity of metabolic and physiological responses can be superimposed on the common cellular denominator (i.e., the modulation of cAMP level) at various sites along the cascade: in the availability of the extracellular ligands; in the specificity of adenylate cyclase-coupled receptors for these ligands; in the specificity of the regulatory subunits of cAMP-dPK, PP, and PDE and the availability of ligands that regulate them; and in the availability of specific substrate protein(s) in the appropriate subcellular microcompartment. In addition, specificity could result from temporally integrated interaction of different ligands with a component of the cascade (e.g., neurotransmitter and Ca^{2+}-calmodulin in the case of adenylate cyclase) and from interaction with other regulatory cascades (e.g., the Ca^{2+}-calmodulin or the C-kinase cascade). Some of these specificity-endowing mechanisms will be discussed below within their physiological context.

II. MEDIATION, MODULATION, AND THE MACROMOLECULAR TOOLS OF NEURONAL PLASTICITY

Neurons sense a substantial part of the information from their milieu in general and from other cells in particular via specific receptors. Some types of such receptors are coupled downstream to the cAMP cascade. In such cases, by definition, the cAMP cascade takes part in *mediating* information transfer in the network. In some instances, the information thus mediated may cause only a solitary, transient event in the target cell. However, often the information so mediated results in *modulating* the response of the target cell to later stimuli. Such modulation results from phosphorylation (and hence alterations in the properties) of receptors, channels, enzymes, enzyme and receptor regulators, and structural proteins and/or from changes in the availability of these cellular components. The short- and long-term alterations thus created in the interactive and integrative properties of neurons will be defined, for the purpose of our discussion, as *neuronal plasticity*. Such plasticity is assumed to underlie modifications in the output of the nervous system or its parts, in development and/or in response to external stimuli, e.g., during learning.

As will be detailed below, some processes which underlie neuronal plasticity may indeed involve cAMP-mediated phosphorylation of identified channels, enzymes, receptors, and structural proteins. Moreover, as detailed below, cAMP-mediated processes probably play a role also in long-term modification of neural structure and function by modulating gene expression.

The cAMP cascade is of course not confined to neurons or glia, but organisms make very extensive uses of it within the context of their nervous system. That this is the case is already evident from the highly active cAMP-dependent protein phosphorylation system and the high specific activity of the components of the cAMP cascade in neural tissue.[6,8,48-54] This refers to many types of adenylate cyclase-coupled receptors and to the regulatory and catalytic units of adenylate cyclase itself,[11] to cAMP-PDE,[27] and especially to cAMP-dPK. High levels of this enzyme exist in all brain parts,[15,55-58] in both neurons and glia.[58] Type-II cAMP-dPK appears, however, to be more enriched in neurons, whereas Type-I may be more enriched in glia.[60,61]

In contrast with other tissues, where most of cAMP-dPK is localized in soluble fractions, approximately 50% of the cAMP-dPK in brain is associated upon homogenization with membrane fractions. In both the latter and the soluble fraction, there is more R-II than R-I (up to threefold more in cortex and striatum membranes).[57] Especially enriched are fractions of synaptic origin,[57,62] mainly synaptic junctions.[62] Upon homogenization, soluble R-II is often associated with specific proteins, e.g., microtubule associated proteins (MAPs),[45b,63-65] calmodulin and the Ca^{2+}-calmodulin-dependent protein phosphatase calcineurin,[66] and a 75-kilodalton phosphoprotein termed P75.[45a,67] These associations will be discussed further in Sections VII and VIII below. As previously mentioned, there is structural heterogeneity in R-II subunits, and again, although heterogeneity hints at differential regulatory roles, its physiological significance awaits clarification.[38-45]

Among the components of the cAMP cascade, the target of phosphorylation, i.e., the substrate proteins, might be expected to display an especially high degree of cell and task specificity. Scores of such substrate proteins have been identified in neural tissue from various sources, and the number seems to grow as the sensitivity of the resolving and detecting techniques increases. The work of Walaas et al.[68,69] can serve as a representative example of a study intended to identify substrates for the cAMP-dPK by searching for incorporation of ^{32}P into polypeptides under the appropriate assay conditions; the labeled peptides were resolved on SDS-acrylamide gel electrophoresis and detected by autoradiography.

About 30 distinct phosphoproteins (and many faint bands which were not further analyzed) were thus detected in a particulate fraction prepared from rat brain. These phosphoproteins were classified into three categories:[68,69]

1. Phosphoproteins found in all parts of the brain in essentially equal amounts (among these were a microtubule-associated protein of 280 kilodaltons (MAP-2,[64,65] see Section VII below), the synaptic vesicle associated proteins Synapsin Ia and Synapsin Ib of molecular weights 80 and 86 kilodaltons[70] (see Section VIII below), Protein IIIa of molecular weight 74 kilodaltons,[71] and the approximately 55-kilodalton R-II of cAMP-dPK.)
2. Phosphoproteins which were widely distributed within the brain, but showed large regional variations (among these were the synaptic protein Protein IIb of molecular weight 52 kilodaltons[41] and a high molecular weight protein of 265 kilodaltons.)
3. Phosphoproteins which showed highly restricted regional distribution (among these were proteins specific for the cerebellum (later found to be localized to Purkinje cells)[72] and for dopaminergic pathways.)

In a parallel study, the same methodology was employed to identify approximately 40 endogenous substrates for cAMP-dPK in a soluble fraction prepared from the same tissue. Again, the phosphoproteins revealed on the autoradiographed electrophoretograms were classified into three categories. In Category 1, the MAP-2, as well as other polypeptides of 130, 55, 52, and 43 kilodaltons were observed. In more recent work by the same group, a dopamine and cAMP-regulated phosphoprotein of molecular weight 32 kilodaltons (abbreviated DARPP-32) was identified which could also be classified either into Category 2 or into Category 3 phosphoproteins, being enriched in dopamine innervated neurons, mainly in the basal ganglia.[73] DARPP-32 will be discussed further in Section VI below. Several other phosphoproteins localized specifically in the basal ganglia, of molecular weights 21 to 98 kilodaltons, were classified by Walaas et al. as Category 3 phosphoproteins.[68,69]

Taken together, the results demonstrate the existence in mammalian brain of a rich variety of phosphoproteins which are substrates for cAMP-dPK and some of which display regional specificity. Of the most abundant, widely distributed phosphoproteins, some could be easily detected in nonmammalian nervous systems as well, for example, the R-II subunit of the cAMP-dPK.[38,74-76]

The aforementioned studies,[68,69] as well as additional ones,[77] also demonstrate that the overall distribution of the cAMP cascade in the brain does not overlap the distribution of two other phosphorylation cascades, namely, the Ca^{2+}-calmodulin-dependent phosphorylation cascade and the diacylglycerol-dependent (C-kinase) phosphorylation cascade. The different cascades are, however, often colocalized within the same cellular compartment and can work in concert, as will be discussed later.

In our discussion of the involvement of the cAMP cascade in neuronal plasticity mechanisms, we will keep returning to several of the phosphoproteins which were revealed by the direct search for substrates of cAMP-dPK in the brain. It should, however, be emphasized that the physiological function of most of the phosphorylated polypeptides revealed in brain homogenates is not yet known. It might therefore prove advantageous to assess the role of cAMP-dependent phosphorylation on neuronal physiology by a somewhat different approach: instead of searching for previously unknown phosphoproteins by virtue of their ability to be phosphorylated by cAMP-dPK, one can characterize the phosphorylation of proteins with an established function and the effect of phosphorylation on known cellular responses. The overall scheme of our review is indeed largely governed by the latter strategy. Thus, we will concentrate on a few classes of proteins which are known to fulfill a central role in regulation of neuronal properties, survey the evidence for their modulation by cAMP-dependent phosphorylation, and then ask how might the neuron utilize such macromolecular modulation in its short- and long-term plastic responses, either as an individual cell or as a member of a neuronal network.

Finally, those few experimental systems in which concrete molecular alterations were correlated with physiological and/or behavioral plasticity are discussed; common denominators emerging from such systems, as well as criteria employed in assessing the role of the cAMP cascade in vivo, are outlined. Some research trends are also briefly mentioned.

III. cAMP-DEPENDENT PHOSPHORYLATION OF ION CHANNELS

Those are gated ion channels that turn neurons excitable.[78] It is customary to classify gated ion channels on the basis of their gating mechanism. Such classification distinguishes between voltage-gated channels that participate in and contribute towards the generation of action potentials and chemically gated channels, i.e., channels that generate postsynaptic potentials in response to neurotransmitters and neuromodulators, or receptor potentials in response to sensory signals that initiate an intracellular chemical cascade within sensory cells (light, odorants). However, in recent years it has become evident that neurotransmitters can also lead to chemical gating of voltage-gated ion channels. One mechanism for such chemical modulation of ion channels, and hence of neuronal excitability, is cAMP-dependent phosphorylation.[79-82]

A. Na^+ Channels

1. Molecular Studies

Sodium channels are the only voltage-gated ion channels to date which have been purified to homogeneity and their gene cloned and sequenced. The voltage-sensitive Na^+ channel, which is responsible for the regenerative Na^+ permeability increase during the initial rising phase of the action potential in nerve and muscle, has been purified from mammalian muscle,[83] mammalian brain,[84] chick heart,[85] and the electroplax of the electric eel, *Electrophorus electricus*.[86] The channel from the latter source was subsequently cloned and sequenced,[87] thus paving the way to isolation of channel genes from other sources, too. The acetylcholine-gated Na^+ channel, which is part of the acetylcholine receptor complex, was also purified from electric organ tissue and the genes coding for its subunits were cloned.[88-90] Only the voltage-gated channel will be discussed here; the properties of the

acetylcholine-gated channel will be described in Section IV below, in the context of the acetylcholine receptor complex.

Several drugs and toxins interact with high affinity with the voltage-gated Na^+ channel and affect its function. Among them are tetrodotoxin and saxitoxin that block conductance by binding to external sites on the channel molecule; local anesthetics that block conductance by binding to internal sites; veratridine and batrachotoxin that cause persistent channel activation; and scorpion and sea anemone toxins that perturb gating and enhance the effects of veratridine and batrachotoxin. Using neurotoxins as a specific molecular probe, the channel from electroplax and heart was identified as a glycoprotein with a molecular weight of approximately 260 kilodaltons, denoted the α-subunit. In mammalian neurons, the α-subunit is attached by disulfide bonds to a smaller glycopeptide of 33 kilodaltons and is also linked noncovalently to glycopeptide of 36 kilodaltons; subunit(s) of 37 kilodaltons are attached to the α-subunit in skeletal muscle.[83-86]

Analysis of the clone isolated from the electric eel revealed a sequence coding for a polypeptide of 1820 amino acids, yielding a molecular weight of 208 kilodaltons. This polypeptide consists of four domains of approximately 300 amino acids each which display high homology between themselves and are connected and flanked by shorter segments of nonhomologous residues. On the basis of the sequence data, a topological model for the channel was proposed that views each homologous domain as containing six segments. Four of them are α-helices that are depicted as spanning the membrane, and the other two are depicted as being located intracellularly. It was suggested that the walls of the transmembrane pore are formed by segments from each of the four homologous domains. In addition, the model suggests, on the basis of the sequence, the existence of four clusters of negatively charged residues, and it was proposed that these act in concert with a segment of positively charged residues, present in each of the four homologous domains, to gate the channel.[87,91] Detailed models correlating structure with function, which incorporate electrophysiological data on the channel properties, have been suggested; some of these models attempt to depict charge translocation of identified channel segments in response to depolarization, as underlying voltage-dependent gating.[91-93] These models provide a framework for further analysis of structure-function correlation, including analysis of the effect of site-directed mutagenesis.

Reconstitution of the purified channel into artificial lipid structures[83,84,94,95] yielded functional channels which revealed, upon single channel recordings from patches of the reconstituted membrane, selective ion transport and voltage-dependent gating properties resembling, both qualitatively and quantitatively, the properties of Na^+ channels nerve and muscle.[96] Such preparations also provide a convenient experimental system for future studies of the cloned gene product.

Catterall and colleagues[97] have found that the α-subunit of the Na^+ channel purified from rat brain can be phosphorylated by the catalytic subunit of cAMP-dPK to a level of 3 to 4 mol of ^{32}P per mole of saxitoxin binding site. Similarly, the α-subunit in rat brain synaptosomes can be rapidly phosphorylated *in situ* by an exogenous cAMP-dPK, as detected by immunoprecipitation of detergent solubilized synaptosomes with antichannel antibodies, followed by electrophoresis and detection of cAMP-dependent ^{32}P incorporation by autoradiography.[98] Analysis of phosphopeptides subjected to a tryptic digest revealed five major sites of phosphorylation. The level of phosphorylation by endogenous cAMP-dPK was also measured, using a rephosphorylation method,[99] in which the sites not phosphorylated *in situ* were labeled by the use of an exogenous cAMP-dPK after lysis of the synaptosomes. Incubation of the synaptosomes with a nonhydrolyzable analogue of cAMP rapidly blocked the labeling of the α-subunit by rephosphorylation, indicating cAMP-dependent phosphorylation *in situ*.[98]

The functional properties of the channels in the synaptosomes were studied by measurements of the binding of neurotoxins and by measuring neurotoxin activated ion influx (see

above). Phosphorylation resulted in a decrease in the initial rate of the neurotoxin-activated ^{22}Na influx mediated by the channel, but the binding of neurotoxins themselves was not significantly altered. It was therefore postulated that cAMP-mediated phosphorylation of the Na^+ channel may also lead to functional alterations in channel properties in vivo. The latter hypothesis has yet to be proved. It should be mentioned that treatment of muscle cells with cAMP does not alter the rate of Na^+ channels turnover, although it does lead to an increase in the density of channels;[100] this phenomenon will be discussed further below.

2. Electrophysiological Studies

Only few reports exist of physiological modulation of Na^+ currents by cAMP-dependent phosphorylation, as revealed by electrophysiological measurements. Injection of cAMP into different types of neurons from several mollusk species depolarized the cells in a reversible and dose-dependent manner, and the effect was shown by voltage clamp to be due to a cAMP- (and cGMP-)induced transient increase in a membrane Na^+ current.[101-103] The current, termed $I_{Na(cAMP)}$, was resistant to tetrodotoxin and thus differed in its pharmacology from the fast inward Na^+ current underlying the upstroke of the action potential. The current was also not sensitive to drugs that inhibit the Na^+-K^+ pump and Na^+ transport in epithelial cells.[101]

In mammalian cardiac Purkinje fibers, the pacemaker current, I_{K2},[104] has been shown by voltage clamp analysis not to be a pure K^+ current, but rather an inward current activated during hyperpolarizations of less than -50 mV.[105] I_{K2} is similar in its properties to the I_f current of the sinoatrial node.[106] Both currents disappear in low Na^+ solutions and are blocked by Cs^+ and may have a Na^+ component. Both currents are responsible for the acceleration in pacemaker firing induced by adrenaline,[107] and since the latter ligand is known to activate adenylate cyclase, cAMP might be involved in the modification of the Na^+ current.

In all the above-mentioned electrophysiologically recorded modifications of Na^+ currents, no direct evidence exists for the phosphorylation of ion channels per se, or even of polypeptide(s), which are intimately involved in channel action.

B. K^+ Channels

Detailed molecular studies on K^+ channels polypeptide(s), similar to those performed on the voltage-gated (and the acetylcholine-gated) Na^+ channels, are not yet possible and must yet await the purification and cloning of K^+ channels (some progress in this front has nevertheless been made;[108] see below). On the other hand, ample information exists on the functional modulation of various types of K^+ channels by cAMP, and in certain cases, patch-clamp analysis of single channels has led to a detailed understanding of the alteration in channel properties. Moreover, the role of certain K^+ channels modulations in cellular plasticity has been clearly established.

A substantial part of our understanding of the properties of various types of K^+ channels emerges from studies on invertebrates, especially mollusks. Several kinds of voltage-gated K^+ channels have been found to be modulated by cAMP. Some examples are briefly described here.

An example of multiple K^+-channel modulation brought about by cAMP-dependent phosphorylation, which results in a remarkable physiological modification and initiation of a characteristic part of an innate behavioral repertoire, is provided by the work of Strumwasser and colleagues on the bag cells in the sea hare, *Aplysia*.[109-113] The bag cells are an ensemble of several hundred neurosecretory cells which are located in the abdominal ganglion of *Aplysia*. They secrete neuroactive peptides that control egg-laying and other components of reproductive behavior. Peptide secretion is associated with long-lasting repetitive firing of the cells, known as afterdischarge. It has been demonstrated that cAMP is involved in this

afterdischarge by decreasing one transient and two (fast and slow) delayed-rectifying depolarization-evoked outward K^+ currents.[109-111] Since K^+ currents play a role in repolarization of the action potential, this multiple K^+ channel inhibition results in marked enhancement of both the amplitude and duration of the action potential in the bag cells. Application of forskolin (an adenylate cyclase activator) and injection of the catalytic unit of cAMP-dPK produce similar enhancement of the action potential, and injection of a specific protein kinase inhibitor blocks the enhancement in excitability brought about by forskolin or by electrical stimulation.[111-113]

An anomalously rectifying K^+ current, modulated by cAMP-dependent phosphorylation, is involved in a characteristic physiological response of another neuron in *Aplysia*. This is the oscillating firing activity of the neuron R-15, a large and identifiable neuron which is located in the abdominal ganglion.[114] R-15 can be described as a pacemaker: its normal pattern of activity consists of bursts of action potentials, separated by interburst hyperpolarizations. The oscillations are generated endogenously by the concert activity of several ionic currents, but can also be modulated for long periods of time by synaptic stimulation and by application of putative neurotransmitters, including biogenic amines and neuropeptides.[115] Serotonin, which in R-15 stimulates adenylate cyclase, causes R-15 to hyperpolarize and stop firing. This is due to an increase in K^+ conductance. There are at least four different types of K^+ channels in R-15, and one of them is an inward (anomalous) rectifier; this channel is partially activated in the resting cell and is further activated by the biogenic amine.[116]

Using a combination of electrophysiological, pharmacological, and microneurochemical techniques, and taking advantage of the huge size of R-15 which boosted its popularity as a neurophysiological preparation, Levitan and colleagues have been able to demonstrate that the depression of oscillatory activity caused by serotonin could be mimicked by cAMP analogues and the stable GTP analogue Gpp(NH)p, potentiated by phosphodiesterase inhibitors, and blocked by the G-unit inhibitor $GDP_\beta S$ and by the inhibitor protein of cAMP-dPK.[117-121] Moreover, protein phosphorylation induced by synaptic stimulation[122] and by application of serotonin[123] has been identified within single R-15s, and two phosphoproteins, of molecular weights 29 and 70 kilodaltons, have been suggested to be involved in the regulation of serotonin-sensitive K^+ channel in the neuron.[124] However, it is not clear whether these are indeed associated with the anomalous rectifier channel and also whether phosphorylation directly leads to channel opening.[125]

In the same abdominal ganglion of *Aplysia* that contains the bag cells and R-15, there exist also clusters of sensory neurons, which provide us with an additional intriguing example of modulation of a K^+ channel by cAMP-dependent phosphorylation. Here yet another type of K^+ channel is involved: the S channel, which can again be modulated by serotonin (hence the S). The S channel, studied extensively by Kandel and colleagues, is open at resting potential, its gating is affected by intracellular Ca^{2+}, and it is only moderately dependent on membrane potential.[126] As opposed to the inward rectifier in R-15 mentioned above, here phosphorylation closes the K^+ channel. This has been established by using electrophysiological, microneurochemical, and pharmacological tools, including testing the effect of serotonin on endogenous cAMP level, and the effects of injection into sensory neurons of cAMP, of stimulators and inhibitors of the regulatory and catalytic subunits of adenylate cyclase, of the catalytic subunit of mammalian cAMP-dPK, and of the protein inhibitor of the kinase.[127-131]

Closure of the S channel in the sensory neurons causes spike broadening, which results in increased Ca^{2+} influx to sensory neuron terminals, thus permitting more transmitter to be subsequentially released per impulse from the terminals. As will be described in detail in Section XI.A, this phosphorylation-induced modulation of synaptic efficacy plays a major role in the neuronal alterations which underlie learning and short-term memory in a modifiable reflex in *Aplysia*.[132]

The S channel has been studied by single-channel recording techniques.[126] These studies provided insight on how phosphorylation modulates the channel. The overall current (I) conducted by a population of channels can be described as $I = N_t*p*i$, where N_t is the number of functional channels, p is the probability that an individual channel is open, and i is the current that passes through a single open channel. Changes in I can therefore arise from alterations in N_t, or in p, or in i, or in their combinations. Such changes could be detected by patch-clamp techniques for single-channel recording.[133] Using this technique for recording from individual S channels in patches of *Aplysia* sensory neurons, it was found that serotonin acts on the channel in an all-or-none manner, i.e., it does not reduce channel conductance or modifies gating, but closes the channels and hence decreases the number of open channels in the membrane.[126] The change is thus in N_t and not in i; the apparent decrease in N_t might, however, still be due to a drastic reduction in p.[81]

The effect of serotonin on single channels was mimicked by application of the catalytic subunit of cAMP-dPk to the isolated and sealed membrane patch. Addition of F^-, a phosphatase inhibitor, together with the cAMP-dPK catalytic subunit, increased the effect, suggesting again that phosphorylation is involved in the process.[131] However, even in this experimental system, it is not yet clear whether it is the K^+ channel per se that is phosphorylated or whether phosphorylation only alters the properties of other polypeptides that regulate the channel (such postulated regulators, if existing, are present in the membrane patch, and hence might be membrane bound and intimately associated with the channel).

Evidence for cAMP-modulated K^+ S channels was also obtained in *Helix* neurons,[134] where the cAMP cascade is triggered both by biogenic amines and by a neuropeptide, Phe-Met-Arg-Phe-NH_2 (FMRF-amide).[135,136] In *Aplysia* sensory neurons, too, the cAMP cascade that culminates in modulation of the S channel can be triggered by the molluscan cardioexcitatory neuropeptides SCP_A and SCP_B.[137]

In another mollusk, *Hermissenda crassicornis*, which is employed by Alkon and colleagues to elucidate the cellular and molecular basis of plasticity in a neuronal network that executes a modifiable behavior, iontophoretic injection of the catalytic subunit of cAMP-dPK was reported to cause a decrease in a delayed voltage-dependent K^+ current, I_B. The same treatment was also reported to have a smaller effect on an early, rapidly inactivating, voltage-dependent K^+ current I_A.[138] The latter currents were later shown to be affected also by a Ca^{2+}-dependent protein kinase and a C-kinase.[139,139a]

A Ca^{2+}-dependent K^+ conductance which can be enhanced by cAMP-dependent phosphorylation has been demonstrated in *Helix* neurons. The neurons were internally perfused with a solution containing the catalytic subunit of cAMP-dPK; a Ca^{2+}-dependent increase in K^+ current was detected.[140] No increase was obtained when the catalytic subunit of the enzyme was chemically inactivated prior to the perfusion. It should be noted that the perfusion technique permits the introduction of a given concentration of an exogenous ligand into the cell, which is particularly important when dealing with kinases that at too high a concentration might phosphorylate substrates nonspecifically. The results of the cell perfusion experiments were later corroborated and extended by single channel analysis of membrane patches from the same neurons and of a channel-containing membrane preparation embedded in artificial phospholipid bilayers.[141] Individual Ca^+-dependent K^+ channels were observed by the latter technique. Analysis of the data thus obtained indicated that cAMP-dependent phosphorylation increased p, the probability for channel opening, and that this might have resulted from an increased affinity of the K^+ channel for its regulatory Ca^{2+}.[141]

Experiments done with dialyzed squid axons revealed a decrease in K^+ currents in the absence of exogenous ATP and reversal of the effect upon addition of ATP.[142] The effect on K^+ current was complex and was interpreted as suggesting alteration in the relative proportion of two populations of K^+ channels. It was proposed that the effect involves phosphorylation, since it required Mg^{2+}, and a nonhydrolyzable ATP analogue was not

effective. Addition of the catalytic subunit of cAMP-dPK was not necessary for the ATP effect, yet potentiated it. This could be interpreted as resulting from high levels of endogenous cAMP-dPK, or from an effect additive to that of another protein kinase. More data is therefore required to establish the role of cAMP-mediated phosphorylation, if any, in this system.

Evidence for K^+ channel modulation resulting from activation of the cAMP cascade emerges not only from studies of invertebrate systems. In pyramidal cells of the rat hippocampus, noradrenaline (acting via a β-adrenergic receptor, which is coupled to adenylate cyclase) facilitates excitatory synaptic responses.[143-148] By recording from CA1 pyramidal cells in hippocampal slices, Madison and Nicoll[146-148] have demonstrated that noradrenaline and cAMP block Ca^{2+}-activated K^+ channel, resulting in a reduction in spike-adaptation that normally occurs with depolarizing stimuli and hence in an increase in the number of spikes elicited by such stimuli.

In conclusion, it appears that many types of K^+ channels may be regulated by cAMP-dependent phosphorylation. Activation of the cAMP cascade results in channel closure or in channel opening, depending on the type of channel, on the neurotransmitter or neuromodulator that initiates the cascade, and on the cell type. The same transmitter or modulator may lead to different effects on K^+ channels in different cells. Such a rich arsenal of K^+-channels modifying tools provides neurons with many degrees of freedom in their specific responses to stimuli and to combinations of stimuli. It should be noted in this context that K^+ channels, including serotonin-gated channels, are also known which are modulated by transmitter-regulated G-unit, though cAMP is not involved.[148a]

C. Ca^{2+} Channels

Voltage-gated Ca^{2+} channels seem to be present in every excitable membrane. They play central roles in diverse types of excitability, including generation of action potentials, contractility, and processes involved in synaptic transmission and neuronal growth.[149-153]

Perhaps the most studied Ca^{2+} channel to date is the one that mediates the slow inward current in mammalian cardiac muscle (and also in cardiac muscle of other vertebrates) and is modulated by β-adrenergic drugs.[154] Adrenaline and noradrenaline, or their analogues, increase $I_{Ca^{2+}}$, leading to increased cardiac muscle contractility. The effect is mediated by cAMP: it can be mimicked by injection of cAMP[155,156] or of the catalytic subunit of cAMP-dPK into myocardial cells.[157,158] Also, injection of the regulatory subunit of cAMP-dPK shortens the action potential duration, and the effect of the latter is reversed by adrenaline.[157]

What is the mechanism by which the cAMP-dependent phosphorylation leads, either directly or indirectly, to Ca^{2+} channel modulation in cardiac muscle? As previously mentioned, three factors determine the amount of overall current carried by a population of channels: the number of functional channels (N_t), the probability that an individual channel opens (p), and the unitary current (i). Reuter and colleagues performed single channel recordings in cell-attached membrane patches in cultured neonatal heart cells and found that a stable cAMP analogue led to increased p: the average duration of channel opening was prolonged and the closure intervals between successive openings were shortened.[159,160] Further analysis suggested acceleration of the on-rate of channel opening.[160] Whether any change also occurs in the total number of functional channels during β-adrenergic activation is not yet clear.[81,160]

Can the Ca^{2+} channel itself be phosphorylated by cAMP-dPK? Recently, Lazdunski and colleagues[161] and Catterall and colleagues[161a] presented evidence for the phosphorylation of a polypeptide which they propose to be a putative voltage-dependent Ca^{2+} channel from mammalian muscle. The putative channel protein was identified in and purified from the T-tubular system of rabbit skeletal muscle,[162a] on the basis of its high-affinity binding to dihydropyridines (e.g., nifedipine or nitrendipine).[163-165] Dihydropyridine-sensitive $^{45}Ca^{2+}$ flux in skeletal muscle cell culture was indeed found to be stimulated by cAMP and its

analogues, by cAMP-phosphodiesterase inhibitors, and also by isporoterenol, a β-adrenergic agonist.[166] Curtis and Catterall suggested that although two of the channel subunits, (mol wt 165,000 kilodaltons) and β (mol wt 53,000 kilodaltons), are phosphorylated by cAMP-dPK in vitro, only the latter polypeptide is a substrate for cAMP-dependent phosphorylation *in situ*. Lazdunski and colleagues later reported that limited proteolysis of the putative channel preparation revealed a peptide fragment of approximately 5.4 kilodaltons, which can serve as a phosphorylation site for both cAMP-dPK and Ca^{2+}-calmodulin kinase.[161] In another preparation, i.e., cardiac microsomes, nitrendipine was reported to modulate phosphorylation of a 42-kilodalton peptide;[167] a dihydropyridine channel activator and a channel inhibitor did not, however, have any effect on phosphorylation of the purified dihydropyridine-binding site preparation.[161]

Flockerzi et al.[166a] succeeded in reconstituting the dihydropyridine-binding site into phospholipid bilayer membranes. The reconstituted preparation displayed conductance similar to that displayed by cellular Ca^{2+} channels. Phosphorylation by cAMP-dPK prolonged the open-channel lifetime and shortened the shut intervals between openings.[166a]

β-Adrenergic agonists have multiple effects on heart cells, one of them being the induction of cAMP-dependent phosphorylation of phospholamban, a 22- to 27-kilodalton proteolipid activator of the Ca^{2+} pump in cardiac sarcoplasmatic reticulum; this results in an acceleration of Ca^{2+} uptake.[168,169] The cAMP-dependent phosphorylation of a similar protein, of molecular weight 23 kilodaltons, named calciductin, was reported to be associated with an increase in depolarization-induced Ca^{2+} uptake into sarcolemmal vesicles prepared from dog heart.[170] The authors suggest that calciductin is either a slow Ca^{2+} channel or a channel-bound activator protein and that its adrenaline-induced phosphorylation increases Ca^{2+} influx.[170] The relevance of calciductin to Ca^{2+} channel protein was, however, questioned.[171]

As noted above, most of the information currently available on the effect of cAMP-dependent phosphorylation on Ca^{2+} channels stems from studies performed on mammalian muscle, especially cardiac muscle. Some Ca^{2+} currents revealed in other systems appear to be regulated by cAMP, too.

Voltage-activated, dihydropyridine-sensitive CA^{2+} channels have been studied in an excitable cell line derived from a rat pituitary tumor.[171a] These channels might be similar to mammalian muscle channels. Recent experiments indicate that one type of these channels must be phosphorylated by cAMP-dPK to respond to membrane depolarization.[171a] Taken together with the aforementioned results, these data thus suggest that an increase in cAMP can inject Ca^{2+} rapidly into the cell, and thus that Ca^{2+} channels serve as a convergence locus between cAMP and Ca^{2+} signals (see Section XII below).

In neurons from the snail, *Helix pomatia*, the conductance of Ca^{2+} channels in the somatic membrane was found to be closely related to the intracellular level of cAMP.[172,173] Washing out of the cell content by intracellular perfusion, and activation of cAMP phosphodiesterase, led to decline in Ca^{2+} current, which could be restored by exogenous cAMP administered together with Mg^{2+} and ATP. Addition of F^-, a phosphatase inhibitor and an adenylate cyclase activator, increased Ca^{2+} current amplitude.[172] Perfusion of the exogenous catalytic subunit of cAMP-dPK also led to restoration of Ca^{2+} conductance, whereas heat-inactivated catalytic subunit did not.[173] These results resemble the aforementioned data obtained for Ca^{2+}-dependent K^+ channels by using a similar internal perfusion technique in *Helix* neurons (Section III.B).[140]

A voltage-sensitive Ca^{2+} current enhanced by serotonin (and possibly by the cAMP cascade) was reported in LB and LC neurons in the abdominal ganglion of *Aplysia*.[174] It is of interest also to note that recently Ca^{2+} channels have been described which are inhibited by a transmitter-regulated G unit, though cAMP is not involved[26,175] — similar to the situation for K^+ channels mentioned in Section III.B above.

D. Junction Channels

Gap junctions between cells, excitable cells included, contain sieve-like arrays of channels, through which ions and other types of molecules of molecular weight approximately less than 1 kilodalton can pass.[176] The channels are comprised of hemichannels (connexons) contributed by each cell and arranged in register. Connexons are made of a major polypeptide of molecular weight approximately 27 kilodaltons.[177] Gap junctions are gated by pH, cations including Ca^{2+}, and sometimes by voltage.[158] In several tissues, the junction is also modulated by cAMP.

In the horizontal cells of the carp[178] and the turtle[179] retina, which are electrically coupled via the junction channels, dopamine decreases the lateral spread of a potential evoked by light; the biogenic amine can be also shown to restrict the spread of injected Lucifer yellow. Dopamine's effects are very probably mediated via the cAMP cascade, since they could be mimicked by injected cAMP[179] or by extracellular application of the permeable analogue, dibutyryl cAMP,[178] and also by the adenylate cyclase activator forskolin and by inhibitors of cAMP phosphodiesterase.[178]

cAMP also modulates the junctional conductance in mammalian cardiac fibers, but in opposite direction to its effect on the retinal horizontal cells: intracellular injection of the cyclic nucleotide enhanced electrical coupling in canine Purkinje fibers.[180] Several reports exist of the effect of cAMP on gap junctions in nonexcitable cells,[181,182] including a report which describes the jucntional deficiency in mutant hamster ovary cells that lack a regulatory subunit of cAMP-dPK, and correction of this genetic deficiency by exogenous regulatory subunit.[181] In rat liver cells, glucagon increases junctional conductance, and the effect is blocked by intracellular injection of the protein inhibitor of cAMP-dPK; moreover, the 27-kilodalton major connexon polypeptide is phosphorylated in these cells in a cAMP-dependent manner, and the isolated gap junction from the same source can serve as a substrate for the purified catalytic subunit of cAMP-dPK.[182] The results, taken together, thus suggest that hormones that activate the cAMP cascade can modulate the electrical coupling and the flow of molecules between cells.

IV. cAMP-DEPENDENT PHOSPHORYLATION OF TRANSMITTER-GATED CHANNELS AND OF RECEPTORS FOR NEUROTRANSMITTERS

A. The Acetylcholine Receptor

The nicotinic acetylcholine receptor from *Torpedo* electric organ was the first neurotransmitter receptor to be purified to homogeneity[183-186] and subsequently cloned.[88-90] Purification and cloning of nicotinic receptors from muscle, from tumor cells, and from mammalian brain later followed.[187-195] The electric organ receptor is a transmembrane pseudosymmetric glycosylated protein pentamer, of molecular weight approximately 290 kilodaltons, composed of four different subunits denoted α, β, γ, and δ, which are present in the stoichiometry of $(2\alpha)\beta\gamma\delta$. The subunits are coded by different genes and display strong homology in their structure. An additional subunit, denoted ϵ, was discovered in calf muscle; ϵ is closest in its structure to the γ-subunit and can substitute for it functionally.[194] Nicotinic receptors from muscle and brain are different, but strongly homologous proteins.[195,196]

The nicotinic acetylcholine receptor complex is not only a receptor for the neurotransmitter acetylcholine, but also a Na^+ and K^+ channel which is gated by the neurotransmitter (and see III.A above). Each subunit contains putative transmembrane segments, and at least one of these was suggested to contribute directly to the formation of the channel; the lining of the channel pore that is formed in between the subunits apparently contains both positive and negative charges.[197,198] The cloned cDNAs encoding the α-, β-, γ-, and δ-subunits of the *Torpedo* receptor can be expressed to produce the functional receptor in *Xenopus* oocytes, and so can the analogous subunits plus the ϵ-subunit from calf muscle, as well as hybrids

of mammalian and electric fish subunits.[199-201] Single channel recording of the injected oocytes reveals that the fish and mammalian channels form Na^+ channels of similar conductance, but with different gating properties; the average duration of the elementary units of the calf channel was found to be an order of magnitude longer than that of the *Torpedo* channel, and the data suggested that functional domains of the α- and β-subunits of the two species are responsible for the difference, with the δ-subunit possibly determining the channel-closing step.[201]

That the electric organ nicotinic acetylcholine receptor is a substrate for protein kinases was observed long before the molecular structure of the receptor was elucidated.[202-205] *Torpedo* postsynaptic membrane fragments, which are highly rich in acetylcholine receptor, contain endogenous protein kinases that phosphorylate the receptor.[206-210] These include a cAMP-dPK,[207] as well as a C-kinase[209] and a tyrosine-specific kinase.[208] The catalytic subunit of cAMP-dPK purified from bovine heart phosphorylated the purified *Torpedo* receptor on the γ- and the δ-subunits, with a stoichiometry of approximately 1 mol of phosphate per subunit.[207] The phosphorylation site for the cAMP-dPK was later deduced to be ARG-ARG-SER-SER from the amino acid sequences of the receptor subunits.[208] This was later confirmed by demonstrating that antibodies against the a synthetic peptide of the same sequence inhibit specifically the cAMP-dependent phosphorylation of both the γ- and the δ-subunit; in addition, the antibodies recognized the expected sequence in the tryptic digest of the phosphorylated receptor.[211]

What is the functional outcome of the nicotinic receptor phosphorylation in general and cAMP-dependent phosphorylation in particular? Several reports indicate that the phosphorylation state of the receptor is intimately associated with its rate of desensitization by agonist.[212-214] In the rat soleous muscle endplates, the adenylate cyclase activator forskolin reversibly increased the rate at which the receptor desensitized upon exposure to acetylcholine.[212] In addition, in this preparation forskolin also reversibly increased the decay rate of the miniature endplate currents, suggesting that increased cAMP — and hence cAMP-dependent phosphorylation — decreases the channel open time.[212] Inhibitors of cAMP-phosphodiesterase potentiated the forskolin effect.

The experiments on the rat receptor clearly dissociated between the time course of desensitization and decreasing channel opening time: the first was rapid; the latter was slow. It was therefore suggested that relatively rapid phosphorylation of part of the phosphorylated sites leads to desensitization, while slower phosphorylation of another phosphorylated site results in modification of gating time. Evidence cited in Reference 208 claims that in addition to the rapid phosphorylated sites on the γ- and δ-subunits, mentioned above, there is also much slower phosphorylation of the α-subunit. Another possibility is that the desensitization rate of the receptor molecule increases with its overall level of phosphorylation, and when enough sites are phosphorylated, the desensitization rate becomes comparable to the normal channel closing rate. Therefore, when phosphorylation of receptors is extensive, many receptors switch directly from the open state to the nonconducting desensitized state; the slowly developing increase in decay rate of miniature endplate currents, which is induced by forskolin, may thus reflect the rate of onset of desensitization from the open state.[212] Activation of C-kinase also seems to increase desensitization of nicotinic receptors in muscle cells.[215]

Phosphorylation was also shown to regulate the rate of receptor desensitization in *Torpedo*. Huganir et al.[214] purified both phosphorylated (by cAMP-dPK) and nonphosphorylated receptor and reconstituted it into phospholipid vesicles. The covalent modification did not alter the dissociation constant for acetylcholine, but did increase by seven- to eightfold the rate of desensitization (measured by the transmitter-induced $^{86}Rb^+$ transport).[214] The data indicated that this was due to phosphorylation of either the γ-subunit, or the δ-subunit, or both. In this system, preliminary experiments (cited in Reference 214) revealed no effect of phosphorylation on the single channel conductance or on the mean channel open time.

It is of interest to note that the phosphorylation sites for cAMP-dPK (as well as those for C-kinase) are situated in the putative major intracellular loops adjacent to the membrane-spanning α-helices that are postulated to form the channel pore (and see above). It therefore appears that the intracellular loops are involved in the desensitization mechanism. The exact nature of this mechanism has yet to be determined; the detailed information available on the nicotinic receptor, including its ligand binding sites and the postulated channel pore, and the variety of powerful techniques available — especially the combination of electrophysiology with molecular biology, for example, site-directed mutagenesis and reconstitution of modified receptor[200] — all these make it very likely that the structure-function relationship of phosphorylation of the nicotinic receptor will be soon clarified in much greater detail.

Phosphorylation of the nicotinic receptor might be correlated with developmental stage. Adult receptor in a membrane fraction prepared from *Torpedo* was reported to be more phosphorylated than neonatal receptor.[212] It is not, however, clear how the phosphorylation relates to this developmental change. The synthesis of muscle nicotinic receptor is also regulated by cAMP,[217,218] but this probably is related to regulation of gene expression and not to the phosphorylation of the receptor molecules per se. The effect of cAMP on gene expression is discussed in Section IX below.

The number of muscarinic receptors, as detected by specific binding of quinuclidinyl benzilate (QNB) in rat brain cells and membrane preparations, is reduced following incubation under phosphorylating conditions (Mg^{2+}, ATP, and cAMP), and the effect may involve both cAMP- and calmodulin-dependent phosphorylation.[219-222] Muscarinic QNB-binding sites in brain exist in different states of affinity for their ligands, and phosphorylation was also reported to increase the proportion of the super-high-affinity agonist-binding sites; the effect could not be explained just on the basis of loss of total antagonist (i.e., QNB) binding sites. The authors, therefore, concluded that phosphorylation brings about both a loss of total receptor sites and an apparent conversion of high-affinity into super-high-affinity agonist-binding sites. In the same system, the GTP analogue Gpp(NH)p converted super-high- and high-affinity muscarinic agonist-binding sites into low-affinity binding sites; this effect was partially inhibited by incubation under phosphorylating conditions.[222]

B. β-Receptor and Rhodopsin

Aside from the nicotinic receptor, the most studied neurotransmitter receptor is the β-adrenergic receptor. As opposed to the nicotinic receptor, which contains its integral ionophore, the β-adrenergic receptor mediates its physiological effect by stimulation of adenylate cyclase.[11] The β-receptor has been purified to homogeneity from various vertebrate tissues,[223,224] reconstituted with the components of the adenylate cyclase system in vitro[225,226] and cloned.[227,228] It has significant homology with rhodopsin and like the latter contains multiple membrane-spanning regions.[226,227] The receptor in various tissues appears to be phosphorylated by several types of protein kinases, including cAMP-dPK,[229,230] Ca^{2+}-calmodulin kinase,[230] and C-kinase.[230-232] However, even when phosphorylated by a cAMP-dependent process, e.g., in turkey erythrocytes, the receptor is still a relatively poor substrate for the purified catalytic subunit of cAMP-dPK.[229]

Incubation of cells that contain β-receptors with their agonists leads to desensitization, and this was shown to be associated, in different types of cells, with phosphorylation. When cAMP is effective in bringing about desensitization, it is not the sole mediator of the effect, and desensitization is heterologous, or "nonselective", i.e., there is a general decrease of the β-receptor responsiveness to a wide variety of ligands. Recently, a special kinase was discovered that phosphorylates only the agonist-occupied β-receptor. The action of this β-receptor-kinase may underlie homologous desensitization, i.e., an attenuated response solely to the desensitizing agent.[233] The β-receptor kinase activity is insensitive to cAMP (as well as to other ligands that interact with known species of protein kinases, such as Ca^{2+},

calmodulin or phospholipids, and phorbol esters). The phosphate is incorporated into serine residues only. The first step in the desensitization process may involve agonist-promoted translocation of the kinase from cytosol to the plasma membrane.[234]

A similar kinase was also found that phosphorylates only the light-bleached form of rhodopsin.[235,236] The β-receptor kinase can phosphorylate rhodopsin in a light-dependent fashion and rhodopsin kinase can phosphorylate the agonist-occupied β-receptor.[236]

The cAMP-dPK phosphorylation of the β-receptor, as well as phosphorylation by C-kinase, leads to uncoupling of the receptor from the stimulatory G regulatory unit, resulting in attenuation of the cell response to the β-agonist.[231,232,237] This was also suggested as a possible mechanism for the desensitization resulting from phosphorylation of the agonist-occupied receptor by the β-receptor-kinase.[233] Phosphorylated rhodopsin, in an analogous manner, displays reduced ability to interact with transducin, an alteration which might underlie light-induced desensitization.[236]

The existence of a kinase that phosphorylates only an agonist-occupied β-receptor does not necessarily mean that many different receptors have their own "private" kinases. The existence of many specific kinases (or different functional states of a small number of receptor kinases) cannot be indeed excluded, but, on the other hand, it is also possible to envisage a situation in which kinases like the β-receptor-kinase catalyze the phosphorylation of different receptors provided that they interact with common molecular entities, for example, the subunits of the adenylate cyclase system; indeed, prostaglandin E, which promotes homologous desensitization of its own receptor, also promotes translocation of the β-receptor kinase to the membrane.[234] Nevertheless, the discovery of receptor-specific kinases does necessitate a more cautious evaluation of the physiological role of cAMP-dPK phosphorylation of receptors, especially since cAMP-dPK has a broad specificity and can also phosphorylate nonphysiological substrates under certain experimental conditions. This point is discussed again below.

In the meantime, much less is known about the role of phosphorylation in regulation of other receptors for neurohormones. Desensitization of the α-adrenergic receptors is also associated with phosphorylation, though by a C-kinase.[238] In another receptor, the GABA-receptor complex of mammalian brain, it is not the receptor itself, but a protein inhibitor that regulates receptor activity (termed GABA-modulin), which is phosphorylated by different kinases (cAMP-dPK included); the cAMP-dependent phosphorylation (but not the Ca2+-dependent phosphorylation) results in loss of receptor inhibition by the endogenous inhibitor.[239]

In conclusion, it appears that phosphorylation of receptors is a ubiquitous mechanism for attenuating the sensitivity of cells, neurons included, to extracellular signals. It is especially associated with the phenomenon of agonist desensitization, and in several cases, cAMP-dPK may be involved either solely or in concert with other broad specificity or highly specific protein kinases.

V. cAMP-DEPENDENT PHOSPHORYLATION OF ENZYMES

Ample information is available on the regulation of enzymes by cAMP-dependent phosphorylation, and the subject has been extensively reviewed, both in general[1-6,240] and with a special emphasis on the nervous system.[6,48,50,52] Selected cases of cAMP-dependent phosphorylation of enzymes are listed in Table 1. Only two examples will be discussed further here in the context of the present review.

A. The Autophosphorylation of cAMP-dPK

cAMP-dPK, like many other protein kinases,[6,33] can undergo autophosphorylation.[45,257-262] The catalytic subunit of the kinase phosphorylates R-II in an intramolecular reaction. (R-I

Table 1
SOME EXAMPLES OF ENZYMES WHICH WERE REPORTED TO BE REGULATED BY cAMP-DEPENDENT PHOSPHORYLATION

Type of enzyme	Ref.
Nucleic acid and protein synthesis	
RNA polymerase	241
Aminoacyl-tRNA synthetase and synthetase phosphatase	242
Nucleotide synthesis	
Carbamyl phosphate synthetase II, aspartate transcarbamylase, and dihydro-orotase (CAD)	243
Amino acid metabolism	
Phenylalanine hydroxylase	244
Tyrosine hydroxylase[a]	245
Carbohydrate metabolism	
Glycogen phosphorylase	reviewed in 2,5
Phosphorylase kinase	reviewed in 2,5
Glycogen synthase	reviewed in 2,5
Fructose 2,6-bisphosphatase	reviewed in 2,5
Pyruvate kinase	reviewed in 2,5
Lipid metabolism	
Hormone-sensitive triglyceride lipase	246
Acetyl-CoA carboxylase	247
Cholesterol ester hydrolase	248
Cyclic nucleotide metabolism	
Adenylate cyclase[b]	249—251
Guanylate cyclase	252
cAMP phosphodiesterase	253—255
Protein kinases	
cAMP-dPK[a]	reviewed in 1,5,6,33
Myosin light chain kinase	256

Note: For extensive reviews see References 1 to 8.

[a] See also references in the text.
[b] The data presented in References 249 and 250 could be interpreted as resulting from activation of adenylate cyclase by guanyl nucleotide, but the report in Reference 251 indicates that the enzyme can indeed be phosphorylated.

Table is modified from Nestler, E. J. and Greengard, P., *Protein Phosphorylation in the Nervous System*, John Wiley & Sons, New York, 1984, Table 8.1. (Reference 6.)

can apparently be phosphorylated by cGMP-dPK, but not by cAMP-dPK).[263] Two moles of phosphate are incorporated, at different rates, per mole of R-II,[264,265] at sites which are in proximity to the two cAMP-binding sites.[266] The autophosphorylation results in decreased affinity of the regulatory subunit for the catalytic subunit.[259,262] This decrease in affinity is not a must for dissociation of R from the catalytic subunits (i.e., for activation of the kinase)

— but it may facilitate it. The catalytic subunit itself was also reported to incorporate 2 mol of phosphate per mole subunit when incubated with MgATP in the absence of other protein substrates.[30,267] In spite of its effect on the affinity of R to the catalytic units, the physiological role of the autophosphorylation of the regulatory subunit is not yet clear, nor is the physiological role of the phosphoryl residues in the catalytic subunit.

Autophosphorylating protein kinases have recently gained popularity, mainly due to the suggestion that they serve as memory-storage devices. Intramolecular autophosphorylation creates a memory of the signal that has activated the kinase, provided the autophosphorylated kinase molecule differs in its activity from the nonphosphorylated molecule. Intermolecular autophosphorylation may serve as a memory system which is immune to molecular turnover.[268,269] This topic is discussed further in Section XI.

B. Phosphorylation of Tyrosine Hydroxylase

Tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of catecholamine neurotransmitters. It might thus play a key role in regulating neuronal function. The enzyme is a tetramer of subunits of approximately 60 kilodaltons each.[270] Neuronal impulses as well as neurotransmitters are known to increase catecholamine synthesis via activation of the enzyme.[50] An increase in cAMP was shown to lead to enzyme activation, and cAMP-dPK was shown to catalyze the incorporation of 1 mol of phosphate per mole subunit in tyrosine hydroxylase from brain and from tumor cells.[245,271-277] Phosphorylation was reported to lead to several modifications of the enzyme, including an increase in the V_{max}, and an increase in the affinity for tyrosine, for a pterin cofactor, and for feedback inhibitors.[275-277] Thus, cAMP-dependent phosphorylation may be suggested here as a mechanism by which cellular stimulation leads to an increase in transmitter synthesis.

However, the role of cAMP-dependent phosphorylation in the in vivo activation of tyrosine hydroxylase is not yet well understood. Tyrosine hydroxylase can serve as a substrate for several types of kinase in addition to the cAMP-dPK.[277-284] In the case of Ca^{2+}-calmodulin kinase, tyrosine hydroxylase is phosphorylated at a primary site different from that which is phosphorylated by the cAMP-dPK (and by the phospholipid-dependent kinase),[281,282] and the increased activity may result from a facilitated interaction of an activator protein with the hydroxylase.[283] In some cases, activation of tyrosine hydroxylase following depolarization is additive to subsequent activation by cAMP-dependent phosphorylation.[284] This may arise from rapid dephosphorylation — but also from distinct phosphorylation by other kinases, as mentioned above.[277-284] The postulated causative role of cAMP-dependent phosphorylation of tyrosine hydroxylase, possibly in concert with other kinases, in bringing about enzyme activation in response to stimulation under physiological conditions, therefore awaits further clarification.

VI. cAMP-DEPENDENT PHOSPHORYLATION OF ENZYME AND RECEPTOR REGULATORS

One of the mechanisms by which cAMP-dependent phosphorylation affects cellular activity is modulation of enzyme regulators. The aforementioned autophosphorylation of the cAMP intracellular receptor, i.e., the R-II subunit of the cAMP-dPK complex, can be regarded as such a mechanism. However, the most studied example is the phosphorylation of protein phosphatases inhibitors.

Protein phosphatase-1 (PP-1) is one of the major, broad specificity PP forms that is capable of dephosphorylating seryl and threonyl residues in cells.[2] Its activity can be inhibited by a heat-stable, acid-soluble protein of molecular weight approximately 32 kilodaltons, named inhibitor-1.[285-290] The latter protein is active at very low concentration provided that a specific threonyl residue is phosphorylated by cAMP-dPK.[287-290] Thus, elevation of cAMP level may

lead not only to increased phosphorylation due to activation of cAMP-dPK, but also to decreased dephosphorylation due to inhibition of protein phosphatase.[2]

A form of inhibitor-1 exists in the mammalian brain and is concentrated in dopaminoceptive nerve terminals; it is named DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein of molecular weight 32 kilodaltons), and as the name implies, it is also activated by cAMP-dependent phosphorylation.[73,291-295] DARPP-32 displays physical properties and amino acid composition very similar to those of inhibitor-1[293] and is a potent inhibitor of PP-1.[291] It is a soluble protein, localized primarily in the cell body, axons, dendrites, and terminals of those dopaminoceptive cells that possess D-1 dopamine receptors, which are known to be coupled to adenylate cyclase.[292,293] DARPP-32 is also present in certain glia cells that might contain dopamine-sensitive adenylate cyclase.[292] It was found in amniote vertebrates, but not in anamniote vertebrates and in invertebrates.[294]

Both dopamine and cAMP analogues have been shown to increase phosphorylation of DARPP-32 in caudate nucleus slices.[295] It is therefore plausible to assume that one of the outcomes of dopamine stimulation of dopaminoceptive cells that contain D1 receptors is phosphorylation of DARPP-32, leading to inhibition of PP-1 and decreased dephosphorylation of some substrate protein(s). DARPP-32 can be dephosphorylated by PP-2B, i.e., calcineurin, the Ca^{2+}-calmodulin-dependent phosphatase which is abundant in the nervous system;[296] it is thus a locus of convergence between the cAMP cascade and the Ca^{2+}-calmodulin cascade (see Section XII).

It is of interest to note that another inhibitor-1-like protein, the G-substrate, which is localized in Purkinje cells in the cerebellum, is regulated by cGMP-dPK.[297] The newly encountered members of the inhibitor-1 family in the brain emphasize the important regulatory role that protein phosphatases play in neuronal regulation and modulation.[2,6,240] Indeed, the interrelationship between cAMP-dependent phosphorylation and dephosphorylation may even be more intimate than previously thought: there is a report which suggests that the regulatory subunit of cAMP-dPK, R-II, may itself inhibit protein phosphatase from muscle.[298]

An example of cAMP-dependent modulation by a regulator of a receptor for a neurotransmitter was mentioned in Section IV in the context of receptor regulation: this is the cAMP-dependent phosphorylation of GABA-modulin, which decreases receptor inhibition by the regulator.[239]

VII. cAMP-DEPENDENT PHOSPHORYLATION OF NEURONAL CYTOSKELETAL ELEMENTS

Phosphorylation of cytoskeletal elements by both cAMP-dependent and cAMP-independent kinases is a widespread phenomenon in neuronal as well as in nonneuronal tissue. Of the various types of neuronal cytoskeletal elements,[299,300] the cAMP-dependent phosphorylation of microtubule-associated proteins (MAPs) was most extensively studied.[301] Microtubules (sometimes called neurotubules when in neurons) are protein tubes of an outer diameter of approximately 25 nm which are especially prominent in axons and dendrites where they exist as longitudinal arrays.[300] They are composed of polymerized tubulin and several additional proteins, one of which, of a molecular weight of approximately 270 kilodaltons, is denoted MAP-2.[301,302] MAP-2 is the most prominent MAP in brain tissue. It appears to bind to and extend from the entire length of the microtubule and is assigned a key role in promotion and regulation of microtubule assembly, as well as in cross-linking microtubules to other cytoplasmic organelles.[301,302]

An endogenous cAMP-dPK is associated with MAP-2.[303-305] Phosphorylation of MAP-2 both in vivo and in vitro occurs on multiple sites, only part of which are substrates for cAMP-dPK.[69,300,301,305] The cAMP-dPK is bound to the long arm of MAP-2 via the R-II subunit, which might be a special isoform of the regulatory subunit; actually MAP-2 is

among the most abundant R-II-binding proteins in mammalian brain, approximately one third of total cytosolic cAMP-dPK being associated with it[304] (Section II above). Phosphorylation of MAP-2 by cAMP-dPK was reported to increase the rate of both assembly and disassembly of microtubules; phosphorylation results in microtubules in which the MAP-tubulin interactions are altered in such a way as to facilitate the rate of subunit addition and loss at the microtubule ends, i.e., increasing subunit flux.[306-308] In vivo, under certain conditions, purified phosphorylated MAP-2 inhibited the rate and the extent of MAP-stimulated microtubule assembly,[306-308] reflecting a reduction in microtubule nucleation centers, resulting in fewer, yet longer, microtubules at steady state.[308] Phosphorylation of MAP-2 was also reported to decrease its actin cross-linking activity.[309]

All together, the picture that emerges is that phosphorylation of MAP-2 may contribute to increased dynamics and reshaping of cytoskeletal elements. This, as is discussed in Section XI.A, may play a role in regulating dendritic organization during development; indeed, it has been reported that cAMP-dependent phosphorylation of MAP-2 in the cat visual cortex is strongly modulated by exposure of the animal to light and that this may be an important factor in determining the state of plasticity of the cortex;[310] see also Section XI.B on the postulated roles of cAMP in neuronal development.

Many other cytoskeletal elements are substrates for protein kinases, though in most of the cases not of cAMP-dPK.[6] Some can be phosphorylated in vitro by cAMP-dPK, for example, a neurofilament subunit which can be phosphorylated by the MAP-2-associated cAMP-dPK.[311] It is not yet known whether such cAMP-dependent phosphorylation of neurofilaments also occurs in vivo, but there is evidence that phosphorylation in general alters the structural properties of neurofilaments.[312]

VIII. OTHER NEURONAL SPECIFIC PROTEINS

Several neuronal proteins were first discovered due to the fact that they are major substrates for cAMP-dPK in the nervous system. Among these the most studied ones are synapsin I (Protein I) and protein III.[6] Two additional brain phosphoproteins, termed P75 and PCPP-260, are also briefly mentioned here.

A. Synapsin I

Synapsin I in mammalian brain is an elongated, acid-soluble, basic protein that appears on SDS-gel electrophoresis as a doublet of molecular weight 80 and 86 kilodaltons. The molecular weight of synapsin in other species may differ.[313-315] Synapsin I was found only in neurons, is concentrated in presynaptic terminals, and is associated with synaptic vesicles. It is a ubiquitous synaptic protein and its ontogenesis in the brain follows synapse formation.[70,316-318] cAMP-dPK phosphorylates a single serine residue (site I) which can also be phosphorylated by a Ca^{2+}-calmodulin protein kinase; two other serines (sites II and III) can be phosphorylated only by a Ca^{2+}-calmodulin kinase.[319]

The state of phosphorylation of synapsin I in various in vivo and in vitro neuronal preparations is altered by physiological and pharmacological manipulations that alter cAMP level, as well as by manipulations that alter Ca^{2+} level.[99,320-323] In brief, serotonin and cathecolamines depolarizing agents and nerve stimulation increase the level of the phosphorylated form of synapsin I. Since synapsin I is specific to neurons, widespread in nerve terminals, and associated with synaptic vesicles, it was postulated to play a key role in transmitter release.[6] Pressure injection of synapsin I into the preterminal of the squid axon giant synapse was indeed found to alter transmitter release: injection of dephosphosynapsin decreased the amplitude and rate of rise of the postsynaptic potential, whereas injection of phosphosynapsin (phosphorylated by Ca^{2+}-calmodulin kinase II) or heat-treated dephosphosynapsin had no effect.[314]

On the basis of the aforementioned and additional experiments, the following picture was suggested: in the resting state, dephosphosynapsin I is bound to the cytoplasmic surface of the synaptic vesicle, preventing fusion of the vesicle with release sites on the presynaptic membrane. The invading action potential increases Ca^{2+} level in the presynaptic terminal, leading to Ca^{2+}-mediated phosphorylation of site II in synapsin by the Ca^{2+}-calmodulin kinase, and resulting in dissociation of synapsin from the vesicle, which renders the latter ready for release.[314] This postulated mechanism does not account for the cAMP-dependent phosphorylation of the cAMP-dPK sensitive serine (site I), but a coordinated, additive or synergistic interaction between Ca^{2+}- and cAMP-generating stimuli may be involved in the modulation of transmitter release (and see below). The mechanism by which the postulated interaction of synapsin with the vesicle, with the membrane, and with other cellular components take place is also not yet clear. Some evidence links synapsin to spectrin and relates it to protein 4.1 of erythrocytes[324] and some other data link it to microtubules and implicate it in microtubule bundling.[325] However, since synapsin is an extremely sticky protein, any claim that it is associated with a specific cellular protein must be carefully assessed.

B. Protein III

Protein III is another substrate for cAMP-dPK which is concentrated in the nervous system (but is present also in adrenal chromaffin cells). Again, in mammalian brain, protein III is a doublet: protein IIIa is of molecular weight 74 kilodaltons and protein IIIb is of molecular weight 55 kilodaltons. Both protein IIIa and protein IIIb have similar physicochemical properties.[326,327] They are present primarily in nerve endings, in association with synaptic vesicles. cAMP-dPK phosphorylates a single serine residue per mole protein III.[326] As in the case of synapsin, the phosphorylation of protein III is modulated by physiological and pharmacological manipulations that modulate cAMP level, i.e., electrical activity and catecholamines increase the relative amount of the phosphorylated state.[99,328] Protein III thus resembles synapsin I in many respects, and, similar to synapsin, it was postulated to take part in the mechanism of transmitter release.

C. P75

In addition to its association with MAP-2[63-65] (Sections II and VII above) and with calcineurin,[66] a large proportion of cytosolic brain R-II is found to be associated, upon extensive purification, with a polypeptide of molecular weight 75 kilodaltons, termed P75.[45a,67] Brain R-II displays higher affinity to P75 than muscle R-II.[45a] P75 is a calmodulin binding protein. It is capable of forming a complex of one or two P75 molecules with an R-II dimer. The complex so formed retains calmodulin binding capacity. P75 can be detected in both cytosolic and particulate fractions prepared from neuronal as well as nonneuronal tissues. It is especially abundant in cerebral cortex cytosol, but a particulate-associated P75, readily extracted with a detergent, is enriched in liver. P75 is a substrate in vitro for the catalytic subunit of cAMP-dPK.[67] It was suggested that P75 may play a role in linking the cAMP-dependent and the Ca^{2+}-calmodulin-dependent phosphorylation cascades, by directing cAMP-dPK to substrates of the Ca^{2+}-calmodulin kinase.[67] This interesting hypothesis has yet to be substantiated.

D. PCPP-260

Purkinje cell-specific phosphoprotein, molecular weight 260 kilodaltons (PCPP-260), can serve as an example of another neuronal specific substrate for cAMP-dPK.[72] As opposed to synapsin and protein III, which are found in all brain parts in large amounts, PCPP-260, as its name implies, is specific to Purkinje cells in the cerebellum and can thus be classified according to Walaas et al.[68,69] as a Category 3 protein (Section II above). PCPP-260 is an integral membrane-bound phosphoprotein that is phosphorylated by endogenous as well as

by exogenous cAMP-dPK, but not by cGMP-dPK, Ca^{2+}-calmodulin dependent kinase, or C-kinase.[72] Its function is not known.

Numerous additional neuronal protein substrates for cAMP-dPK with a yet unknown function have also been reported in recent years,[6,8,38,329,330] but their function must be established before a physiological role is suggested for their phosphorylation.

E. A Brief Word of Caution

How much physiological significance should be attributed to the observation that a given protein can serve as a substrate for cAMP-dPK, especially if the experiment was carried out in vitro? This question haunts many students of protein phosphorylation throughout their academic career (for a critical evaluation of the problem, see References 6 and 331). The answer, of course, must be assessed in each case ad hoc. We return to this question in Section XII, while evaluating the role of cAMP in neuronal plasticity in general and considering criteria that should be satisfied before a given physiological function of cAMP-dependent phosphorylation is accepted.

Several general points do, however, deserve to be very briefly pointed out already at this stage, before proceeding towards the discussion of additional postulated roles of the cAMP cascade in the nervous system. As already mentioned, cAMP-dPK is a broad specificity protein kinase; under certain experimental conditions it would phosphorylate "unnatural" substrates, especially if present in large amounts or mislocated during homogenization and rupture of microcompartmental barriers. Several additional artifacts may arise from minute incorporation of ^{32}P, which is inadvertently inflated to the status of a major phosphorylation event due to highly sensitive biochemical detection methods and comigration of multiple bands in molecular separation systems, while only one polypeptide is indeed phosphorylated. Great caution must therefore be practiced in attempting to conclude on the basis of in vitro cAMP-dependent phosphorylation alone that a given protein is indeed regulated by cAMP-dPK in vivo. Reinforcing such an assumption by additional techniques which provide physiologically relevant results, i.e., measuring relevant function *in situ*, is therefore a must. In spite of the enormous amount of data on cAMP-dependent phosphorylation of many proteins, well-established physiological data are still the exception, not the rule.

IX. THE EFFECT OF cAMP ON GENE EXPRESSION

All the information reviewed in the preceding sections relates to the most studied effects of cAMP in eukaryotic cells in general and in nervous tissue in particular, namely, the rapid-onset modulation of cellular activity by phosphorylation of existing proteins. However, ample data exist which show that cAMP can also regulate eukaryotic gene expression. Actually, from a phylogenetic point of view, it appears that cAMP regulation of gene expression preceded cAMP regulation of cellular activity by posttranslational modification: in prokaryotes, the sole established function of cAMP is induction and repression of specific genes.[332] This is done by binding of the cyclic nucleotide to a receptor protein, called the catabolite repressor protein or also CAP (cAMP binding protein), followed by interaction of CAP with DNA sequences that regulate transcription at adjacent promotors.[332,333] In eukaryotes, increase in mRNA in response to intracellular increase in cAMP has been reported for several proteins,[334-341] including in excitable tissue[342-344] (see also References 100, 217, 218, and 345 for reports of increased synthesis and processing of transmitter- and voltage-gated ion channels by cAMP and Reference 346 for an example of possible clinical relevance of alteration of neuronal gene expression by drugs that might modulate cAMP levels).

How does cAMP modulate gene expression? Two major possibilities exist: (1) cAMP activates cAMP-dPK by causing the dissociation of the regulatory subunit from the catalytic subunit, leading to phosphorylation of proteins that themselves regulate gene expression;[347]

(2) cAMP binds to the regulatory subunit of cAMP-dPK and the regulatory subunit per se regulates gene activity, similar to CAP. In the latter scenario, phosphorylation by the catalytic subunit of the kinase is not involved, and, if it does, it is in addition to the direct action of the R subunit on the chromosome.[348,349]

A. Regulation of Gene Expression by cAMP-dPK

cAMP-dPK may in theory regulate gene expression by two major processes: either a phosphoprotein, which is a substrate for cAMP-dPK, migrates from the cytoplasm to the nucleus and regulates the genetic material or the cAMP-dPK itself functions in the nucleus. There is evidence that the catalytic subunit of cAMP-dPK can indeed be translocated from the cytoplasm into the nucleus upon dissociation from the regulatory subunit[348,350] (for earlier data, see the review in Reference 347; it was also suggested that the holoenzyme might be translocated into the nucleus.[351]) For example, in bovine epithelial cells, both R-II and the catalytic subunit (C) of cAMP-dPK were found to be associated with the Golgi complex, as determined by immunofluorescence using anti-R and anti-C antibodies. But following cell stimulation by the adenylate cyclase activator forskolin or addition of dibutyryl cAMP, the catalytic subunit accumulated in the nucleus, whereas the regulatory subunit remained associated with the Golgi complex. A few minutes after stimulation ceases, the catalytic subunit was again associated with the Golgi.[350]

Several nuclear substrate proteins for cAMP-dPK have been reported, including H1 histones and also other classes of histones,[347] nonhistone basic nuclear proteins,[352,353] DNA-dependent RNA polymerases,[241,354] high-mobility-group proteins which are associated with nucleosomes,[355] and ribosomal proteins, especially the ribosomal subunit S6, the extensive phosphorylation of which results in enhanced recruitment of ribosomes into polyribosomes.[356] All the aforementioned substrates have been suggested to play a role in regulation of gene expression.

B. Regulation of Gene Expression by the Regulatory Subunit of cAMP-dPK

As mentioned above, the possibility was suggested that the regulatory subunit of cAMP-dPK, when dissociated from the catalytic subunit, may directly regulate gene expression, independently of the catalytic subunit[348,349,351] and similarly to the CAP bacterial protein.[332,333] This, it should be noted, is in contrast to the aforementioned report[350] in which it is the catalytic subunit of cAMP-dPK, not R-II, that is translocated into the nucleus upon stimulation. Nevertheless, the cAMP-binding domains of R-II and CAP are indeed homologous,[357] and there is homology in the 5′ flanking region upstream from the transcriptional promotors in eukaryotic and prokaryotic genes which are regulated by cAMP.[349] These observations, combined with the finding that R-II may be translocated to the nucleus upon stimulations that increase intracellular cAMP (Section IX.A above and Reference 358) and supplemented with phylogenetic considerations, have led to the hypothesis that the primary role of cAMP in regulation of transcription via CAP, which originated in prokaryotes, was conserved in eukaryotes and that the role of R-II, the "eukaryotic CAP", in regulating the catalytic unit of protein kinase, was added only later in evolution to enable prompt physiological responses to extracellular stimuli by posttranslational modification of proteins.[349]

The phospho-form of R-II was also claimed by one group of investigators to fulfill the role of topoisomerase, i.e., an enzyme that alters the degree of DNA supercoiling.[359] Thus, a highly purified preparation of phosphorylated mammalian R-II was reported to relax superhelical turns of DNA from X174, pBR322, SV40, and M13. The presence of cAMP was necessary. Dephosphorylation of R-II abolished the topoisomerase activity.[359] Since topoisomerases have been implicated in DNA replication, transcription, and recombination,[360] it was suggested that the reported topoisomerase activity of R-II underlies at least part of the role of cAMP in gene regulation.[359]

If confirmed, the suggested topoisomerase activity of R-II might offer interesting avenues for future studies of gene regulation by cAMP and a novel physiological role for the autophosphorylation of R-II, which is not obligatory for cAMP-dPK activation,[360-362] and hence for phosphorylation of cellular substrates (Section V.A above). It is worth mentioning that topoisomerases have been reported to serve as substrates for protein kinases,[348-350] and cAMP-independent protein kinase was reported to be tightly associated with *Drosophila* Type-II topoisomerase.[363]

C. An Additional Word of Caution

An additional word of caution is appropriate when dealing with subcellular distribution in general, and translocation in particular, of cAMP-dPK subunits. The latter may redistribute during subcellular fractionation experiments, especially if performed at low ionic strength.[364,365] In addition, protein kinase subunits may redistribute themselves artifactually during histochemical manipulations.[366] The specificity of antibodies used in histochemical investigations might also pose a problem.[367] Interpretation of experiments which indicate translocation of kinase subunits must therefore be treated with the appropriate caution before the proposed molecular and cellular mechanism which is based on the translocation is accepted.

X. NEUROGENETIC EVIDENCE IMPLICATING cAMP IN NEURONAL PLASTICITY

Some information which implicates the cAMP cascade in neuronal plasticity in general and in learning and memory in particular has recently emerged from neurogenetic studies performed on the pet organism of 20th-century geneticists, namely, the fruit fly, *Drosophila melanogaster*. The rationale for the neurogenetic approach to the study of neuronal and behavioral plasticity is quite straightforward:[368-374] genes are assumed to encode the macromolecular building blocks of the molecular apparatuses that are responsible for processes of neuronal and behavioral plasticity, e.g., learning and memory. Alteration of each of the appropriate genes should result in disruption of the physiological and behavioral capabilities. Genetic, biochemical, and electrophysiological comparisons of mutant and normal organisms, which differ in one gene only, may then reveal the identity of the defective gene product and its exact physiological role. Moreover, cloning of the normal counterpart of the affected gene may pave the way to its identification in other organisms as well.

Three *Drosophila* mutations, that affect learning and memory in a relatively specific way, indicate that cAMP is involved in the processes of learning and short-term memory. An additional mutation also implicates phosphorylation in the aforementioned processes.

A. *dunce:* A cAMP-Phosphodiesterase Mutant

dunce (dnc) was the first mutant of *Drosophila* to be isolated by an intentional screen for mutations which disrupts learning and memory.[375] *dnc* displays a fleeting and labile memory in several different conditioning tasks.[376-381] Memory excluded, the behavior of *dnc* appears to be normal, and the flies look like any normal fruit fly does. The mutation, however, does lead to an additional physiological defect: *dnc* females are sterile.[382-384] Indeed, mutations in the *dnc* gene were also independently isolated by a deliberate screen for mutations that affect female fertility.[385] In addition, mutations in the same locus were isolated in a deliberate screen for genes that code for cAMP phosphodiesterase.[386,387] *Drosophila* has at least three forms of cyclic nucleotides phosphodiesterase. One of them, denoted PDE II, is coded by the *dnc* gene.[388-390] This isozyme has a molecular weight of approximately 90 kilodaltons, displays high affinity for cAMP and low affinity for cGMP, and is not affected by calmodulin; it appears to be essential for normal memory and for normal female fertility. The effects on development and behavior can, however, be dissociated: a suppressor mutation exists which suppresses *dnc*-induced sterility without improving learning and memory capabilities.[382]

Mutations in *dnc* lead to increased cAMP content in various tissues of *Drosophila* (up to 600% in some alleles).[386] The *dnc* gene itself was cloned.[391] It was found to code for multiple poly(A)RNAs, which appear to result from alternative splicing and some of which are developmentally regulated.[392,392a] It is not yet known which of the transcripts is essential for normal memory. However, the observation that *dnc* flies look and behave normally in spite of the lesion in cAMP phosphodiesterase led to the assumption that the subpopulation of this enzyme which is coded by the gene functions, at least in the adult fly, in the molecular apparatus that is responsible for memory formation; other physiological functions can apparently do quite well with other isozymes of cAMP phophodiesterase which are not lesioned by the *dnc* mutations.

B. *rutabaga:* An Adenylate Cyclase Mutant

Similar to the original *dnc*, *rutabaga (rut)* was also isolated in a deliberate screen for conditioning mutants among the progeny of chemically mutagenized flies.[378,393] Again like *dnc*, *rut* can form an association between sensory cues, but forgets them very quickly, much, much quicker than normal flies do.[377,378,380,393,394] The development of *rut* and its sensory and motor capabilities appear to be normal.[393,394]

rut was also found to be lesioned in a component of the cAMP cascade, namely, in a subpopulation (or a functional state) of adenylate cyclase.[21,22,393-399] Adenylate cyclase in *Drosophila* homogenates is heterogeneous with respect to the subcellular distribution of the enzyme and to its kinetic properties; the *rut* mutation affects only approximately 30% of the total adenylate cyclase in the body, and this subpopulation (or a functional state) of the enzyme is membrane bound, is stimulated by Ca^{2+}-calmodulin,[21,22,393-400] and has high-affinity sites for forskolin.[398,399] The effect of *rut* is most pronounced in posterior body parts, but this was shown to result from high proportion of the *rut*+ coded (or regulated) adenylate cyclase in these body parts (the absolute level of the *rut* affected cyclase in anterior body parts, e.g., the head, is still high). The *rut* gene was mapped to a small region of the X chromosome[22,393,399] and the region, which very probably includes genes in addition to *rut*, has been cloned.[401]

C. *Ddc:* A Mutation in Aminergic Transmission

The enzyme DOPA decarboxylase (*Ddc*) decarboxylates L-DOPA to dopamine and 5-hydroxytryptophan to serotonin.[402] In *Drosophila*, *Ddc* functions in the metabolic pathway that leads to cuticle formation. In addition, the enzyme is present in the nervous system, where dopamine and serotonin serve as neurotransmitters and neuromodulators. Both monoamines exert at least part of their physiological function via the adenylate cyclase system.[403] Mutations in the gene that codes for *Ddc* were isolated in a deliberate screen for the chromosomal loci that control the enzyme activity.[404] The gene, located in chromomeres 37C1-2, was later cloned.[405] Because dopamine is crucial for sclerotization, lack of *Ddc* is lethal. However, the effects of mutations in the gene that codes for *Ddc* on behavior can be studied by using temperature-sensitive alleles, $Ddc^{ts\,1}$ and $Ddc^{ts\,2}$. Files carrying these mutations are killed if they develop at a high, restrictive temperature (e.g., 29°C), but they grow normally at a low, permissive temperature (e.g., 20°C). If the enzyme activity is subsequently turned off in these flies as adults by shifting them to a restrictive temperature, they survive.[404,406]

Ddc temperature sensitive mutants raised at a permissive temperature were tested for their effects on learning and memory after shifting them as adults to a restrictive temperature. The flies failed to learn in several types of associative learning paradigms, and learning was temperature sensitive in the same way as *Ddc* activity was.[406] Flies heterozygous for a chromosomal deficiency which covers the *Ddc* gene, when raised at a restrictive temperature, displayed intermediate amounts of *Ddc* activity and intermediate learning scores. However,

what had been learned was retained for a normal time span, indicating that lack of *Ddc* affects acquisition, but not memory.[406] Learning excluded, the behavior of the flies was essentially normal. Some decrease, though, was apparent in the response to certain sensory cues.[406]

D. *turnip:* A C-Kinase Defect that Affects the cAMP Cascade?

The mutant *turnip (tur)* was also isolated by Quinn and colleagues in a deliberate screen for conditioning mutants among the progeny of chemically mutagenized *Drosophila*.[407] *tur* displays some learning capability, but memory is very feeble, and the effect of the mutation on memory is dominant (i.e., flies heterozygous for mutation in the *tur* gene can learn essentially normally, but forget rapidly).[407] Preliminary experiments showed that *tur* lacks the high-affinity state of receptors for biogenic amines.[370,372] In *Drosophila*, as in vertebrates, receptors for serotonin and octopamine exist in low- and high-affinity states, and guanyl nucleotides induce a shift into the low-affinity state.[408,409] Since the effect of *tur* was not limited to one receptor, it was suggested that the mutation actually affects a G-regulatory subunit of the adenylate cyclase system.[370,372,393] Further studies revealed a defect in the activity of C-kinase and of a protein substrate for this kinase.[410] It should be noted that C-kinase was reported to phosphorylate and modulate Gi, the inhibitory regulatory subunit of the adenylate cyclase complex.[411] Taken together, the results to date suggest that *rut* affects a phosphorylation process that involves the activity of C-kinase and that this activity might interact with the cAMP cascade.

Several additional *Drosophila* mutants which affect behavioral plasticity were reported to be affected in the cAMP cascade. A series of temperature-sensitive mutants, selected on the basis of developmental sensitivity to theophyline (a cAMP-phosphodiesterase inhibitor) and propranolol (a β-receptor ligand), was reported to have aberrations in motor activity and learning.[412,413] Detailed information on these mutations is, however, lacking. There is some suggestive evidence that *amnesiac*, isolated in a screen for conditioning mutants,[407] has a somewhat higher than normal activity of adenylate cyclase,[393,395] but this was not pursued further. The mutant per^1, isolated as a circadian rhythm mutant,[414] was reported to display impaired experience-dependent modification of male courtship.[379,415] Another allele of the same gene, per^0, was found to have reduced levels of tyrosine hydroxylase and octopamine[402] (the latter neurohormone, as mentioned above, may exert its physiological activity via adenylate cyclase); yet per^0 learns and remembers normally.[379,402,415] An allele of *ebony (e)*, another mutation which affects a monoamine, in this case dopamine,[416] was briefly reported to reduce learning,[417] but again, this finding was not further pursued. In conclusion, substantial evidence for the role of cAMP in the physiological apparatus that enables behavioral plasticity exists for three mutations, namely, *dnc*, *rut*, and *Ddc*, and some suggestive evidence for the role of cAMP emerges from studies on additional mutations.

E. Why are the Mutants Stupid?

Heuristic working hypotheses have been raised to explain the failure of *dnc*, *rut*, *Ddc*, and also *tur* to learn and remember normally.[370-374] The inability of *Ddc* to acquire novel information was attributed to lack of monoamines, which prevents transmission of signals in the appropriate synapses. These monoamines fulfill modulatory functions, but not vital mediatory roles, therefore, the flies can behave normally, yet behavioral plasticity is defective (and see, in this respect, Reference 418). The inability of *rut* to form and sustain short-term memory was attributed to a low level of cAMP in some subcellular loci and, specifically, to the lack of Ca^{2+}-calmodulin regulation of adenylate cyclase. The latter regulative property of cAMP formation was suggested to permit the integration of two stimuli, one extracellular (transmitter) and the other intracellular (Ca^{2+}), in the adenylate cyclase system; this molecular conversion has been proposed to play a role in the neuronal mechanism that enables asso-

ciation of sensory stimuli during learning[21,373,419-422] (see Section XI.A.1 as well for discussion of *Aplysia* studies). The inability of *dnc* to form and sustain short-term memory was attributed to the lack of an isozyme of cAMP phosphodiesterase, resulting in increased cAMP at crucial neuronal loci; this might lead either to an increased "noise level", preventing the proper recognition of new information, or, alternatively, to enhanced dephosphorylation and hence immediate erasure of the acquired information.[371,373,394,423]

All the aforementioned heuristic explanations assume that the lesions indeed affect the molecular apparatus that must function during acquisition, retention, and retrieval, but do not affect neuronal wiring. Indeed, subtle defects in the latter, caused by the mutations, cannot yet be excluded, but the data to date do favor the assumption that what is lesioned is the molecular cascade(s) that goes into action during memory formation. For example, pharmacological data, showing direct effects of drugs that interfere with the cAMP cascade on learning and memory in *Drosophila*, support the latter view[424,424a,425] and so is the lack of detectable developmental and physiological effects in some of the conditioning mutants and the dissociation by genetic tools of effects on learning from effects on development, where the latter do occur (*vide supra*). The analogy with the *Aplysia* model of learning and short-term memory is also of interest (see Section XI.A.1 below and discussion in References 372 and 373). It therefore seems that subpopulations of the ubiquitous components of the cAMP cascade may play specific roles within the context of specific physiological tasks, without affecting the ability of the organism to carry out other, vital roles. Conditioning mutants of *Drosophila* may thus provide us with concrete examples of the ways by which the nervous system employs the universal cAMP cascade to execute specific tasks and may reveal heterogeneity within what first appears to be a single type (or a small family) of enzyme. Such molecular diversity in cellular signaling cascades seems to be a widespread phenomena, which clearly emerges from some molecular biological studies (and see, in this respect, the example discussed in Reference 426).

XI. THE cAMP CASCADE IN THE NERVOUS SYSTEM: THE TIME SCALE OF MOLECULAR AND PHYSIOLOGICAL EVENTS

In the previous sections, we reviewed evidence indicating that cAMP can modulate the activity of neurons at many molecular and cellular loci. Such modulations were considered as potential candidates for molecular and cellular mechanisms that underlie certain aspects of neuronal plasticity. It seems appropriate, at this stage, to consider the temporal characteristics of the various actions of cAMP within the cell, their postulated physiological significance, and their integration into neuronal activity.

A. Events in the Seconds to Hours Range: Immediate Cellular Responses and Early Memory Phases

As briefly described in Section I, the cAMP cascade in multicellular organisms is triggered by binding of an extracellular ligand — be it a hormone, a neurotransmitter, or a neuromodulator — to membrane-bound receptor coupled to adenylate cyclase.[11,16] (In bacteria and in fungi, different mechanisms of cAMP action exist, which are outside the scope of our discussion, but were briefly mentioned above in Section IX; see also References 332 and 427 to 431 and Section XI.B.2 below). It is of interest to note that in recent years it became evident that the extracellular signal stimulating the cAMP cascade might be not only a chemical signal sent by other cells, but also a sensory signal: at least some odorants are capable of activating adenylate cyclase in vertebrate olfactory epithelium;[432] see also discussion in Reference 17).

From a methodological point of view, it is convenient to consider the molecular stations, the modification of which underlies cAMP-dependent modulation of neuronal properties, as

being situated from the cell membrane downstream into the cell. In such a picture, the first cellular loci in the cascade are the membrane-bound receptors facing the extracellular milieu. From the discussion in Section IV, it is evident that phosphorylation can regulate the sensitivity of receptors, and that in several cases cAMP-dPK is the executive molecular agent.

In the case of the nicotinic acetylcholine receptor, and of adrenergic receptors and their close relative, rhodopsin, phosphorylation brings about desensitization, either by affecting a built-in ion channel (nicotinic receptor) or by affecting the coupling between receptor and G-regulatory protein (β-receptor) (see Section IV and references therein). Desensitization, a general phenomenon encountered in hormone-receptors, usually takes place within seconds to minutes and alters the responsiveness of the cell to its modulating signal.[433] Changeux and associates have suggested that neurotransmitter receptor desensitization may be involved in memory formation. They based their theory on studies carried out on the nicotinic acetylcholine receptor, but the theory could be applied to other receptors as well. The basic postulate of the model is that the receptor exists in at least two states of affinity for the agonist, one susceptible to activation and the other desensitized.[434] If the amplitude of the postsynaptic response is a function of the amount of receptor in the responsive state, then the ratio between the responsive and the desensitized state would determine synaptic efficacy, and this, in turn, would contribute to a behavioral alteration, i.e., formation of the learned response. The rate constants for the transition between the different activity state would determine the time course of memory.

However, the role of cAMP-dPK in bringing about desensitization in vivo is not yet clear. For example, in the case of the β-receptor, cAMP-dependent desensitization is heterologous, and homologous receptor desensitization is carried out (as in the case of rhodopsin) by a specific receptor-kinase.[232,236] Here again enters the argument raised above in Section VIII: cAMP-dPK is a broad substrate kinase and may phosphorylate under nonphysiological conditions substrates which are untouched in the course of normal physiological events. Alternatively, cAMP may be used here to induce more general alterations in cellular activity, i.e., a sort of a "state indicator" (the cellular analogue of the role of catecholamines in the brain),[418] whereas specific receptor alterations are brought about by specific kinases or by the concert action of several protein kinases. In addition, to date there is no evidence that receptor desensitization, be it cAMP-dependent or independent, indeed participates in any sort of learning which manifests itself in the behavioral level. The phenomenon of hormone-receptor desensitization is thus well documented, seems to play a role in modification of cellular response, and is a prime candidate for a crucial molecular mechanism for the induction of short-term neuronal plasticity, but its integration into plastic responses of neuronal networks has yet to be clarified.

In the nicotinic acetylcholine receptor, desensitization induced by phosphorylation results from alteration in the properties of the neurotransmitter-gated ion channel.[212,214] Ample evidence indicates that cAMP-dependent phosphorylation of other types of ion channels, namely, voltage-gated channels (Section IV above and references therein), underlies short-term neuronal plasticity. It is of interest to discuss in some detail how cAMP-dependent phosphorylation of ion channel might underlie alterations in neuronal properties and how such modification is employed by the nervous system to bring about rapid plastic changes in the properties of a neuronal network and ultimately in the behavior of the organism.

1. The Integration of cAMP-Dependent Phosphorylation of Voltage-Sensitive Ion Channels in Rapid-Onset Plastic Modifications of a Neuronal Network: A Case Study

The work of Kandel, Schwartz, Catelluucci, Carew, and Siegelbaum and colleagues and of Byrne and colleagues on the modification of defensive reflexes in *Aplysia californica* can serve as an example for a research project which reveals how channel modification is integrated into neuronal alterations that underlie short-term behavioral plasticity.[132,435]

Aplysia has an external respiratory organ, the gill, which is housed in a respiratory cavity (the mantle cavity) and is covered by a fold of tissue called the mantle shelf. At its posterior end the mantle shelf forms a fleshy spout named the siphon. The siphon normally protrudes out of the mantle cavity. If one applies a weak or moderate tactile stimulus to the siphon or mantle shelf, a two-component defensive reflex is elicited. The first component is the contraction of the siphon and its withdrawal behind the parapodia. This is called the siphon-withdrawal reflex. The second component is the contraction of the gill and its withdrawal into the mantle cavity. This is called the gill-withdrawal reflex.[435]

The gill and siphon withdrawal reflex, although innate, can be modified by experience in both a nonassociative as well as in an associative way. For example, the reflex can be sensitized: a noxious stimulus applied to the head or tail will result in enhanced subsequent gill and siphon withdrawal; this is a nonassociative behavioral modification. In addition, a light tactile stimulus to the siphon, which produces only a weak gill withdrawal reflex, can be used as a conditioned stimulus (CS), and a strong electric shock to the tail can be used as an unconditioned stimulus (US). Specific temporal pairing of the CS and US markedly enhances subsequent reflex in response to the CS alone.[419,420] This is an associative behavioral modification.

A neural circuit that controls the withdrawal of the gill and a large part of the withdrawal of the siphon is located in the abdominal ganglion, and its cellular components have been identified. There are more than a dozen identified central motor cells (some for the gill, some for the siphon, and at least one for both the gill and the siphon) and about 30 peripheral motor cells for the siphon. The motor cells are activated by 2 populations of sensory neurons, each containing about 24 cells. One population innervates the siphon skin; the other innervates the mantle shelf. The sensory neurons synapse onto interneurons and motoneurons. The motoneurons synapse directly onto the gill or siphon muscle.[435] However, although quite a substantial number of neurons are involved, the monosynaptic component of the gill and siphon withdrawal reflex can actually be schematically reduced, for the purpose of our analysis, into three main elements only, each representing one type of cell mentioned above: a sensory neuron, that receives stimuli from the mantle or siphon; a motorneuron, that innervates the contracting muscle; and an interneuron, that synapses onto the presynaptic side of the sensory neuron to motoneuron synapse and conveys sensory information from other body parts, for example, the tail or the head.

Cellular analysis of the modified reflex revealed that sensitization of the gill withdrawal reflex is largely due to presynaptic facilitation, which takes place in the sensory neuron to motoneuron synapse, i.e., the amount of transmitter released by the sensory, presynaptic terminal onto the postsynaptic, motoneuron side is increased as a result of activity in the sensitizing pathway.[436] The following molecular mechanism has been suggested to account for the presynaptic facilitation:[419,420] the sensitizing stimulus (i.e., the noxious stimulus applied to head or tail) releases a neurotransmitter from the interneuron onto the presynaptic side of the sensory neuron to motoneuron synapse. This neurotransmitter is a biogenic amine, for example, serotonin, and/or a peptide.[137] The transmitter or modulator binds to a specific receptor which is coupled to adenylate cyclase; binding results in cyclase activation and in an increase in the intracellular level of cAMP.[127] A cAMP-dPK then phosphorylates either a K^+ S channel (Section III.B above) or a polypeptide which regulates the S channel, resulting in channel closure.[126,128-131]

The decrease in K^+ current, according to the model, causes prolongation of subsequent action potentials and increased Ca^{2+} influx, ultimately resulting in increased transmitter release, i.e., in the presynaptic facilitation observed on the cellular level — and in the enhancement of the withdrawal response observed on the behavioral level.[132,436,437] Recently, it became evident that in addition to closing the K^+ S channels, the neurotransmitter(s) released from the facilitatory neuron can alter the availability of Ca^{2+} within the cell and

enhance the mobilization of transmitter, a mechanism which may involve recruitment of the C-kinase cascade by the cAMP cascade. This second, mobilizing component of facilitation probably comes into play when the synapse is depressed (and may account for dishabituation).[438] This can thus serve as an additional example of the integrated action of multiple phosphorylation cascades.

Classical conditioning of the gill-withdrawal reflex was suggested to be an elaboration of the mechanism of sensitization of the reflex. It results, according to the model, from amplification of the presynaptic facilitation by the temporally paired activity in the sensory neuron (mediating the CS) and the interneuron (mediating the US). The following molecular mechanism was suggested to account for the activity-dependent facilitation[419-421] (see also Reference 439 for a similarly modified defensive reflex, the tail-withdrawal reflex). The action potential that invades the sensory neuron terminal (= CS) leads to Ca^{2+} influx; the firing of the interneuron (= US) leads to neurotransmitter-induced increase in the level of cAMP in the same sensory neuron terminal. A yet unknown mechanism integrates the two stimuli, and if they do converge within the appropriate temporal window (e.g., within 500 msec, CS starting before US), an enhanced activation of the cAMP cascade results, leading again to phosphorylation of the K^+ S channel (or an intimately associated component), to channel closure, etc. A Ca^{2+}-calmodulin-stimulated adenylate cyclase was proposed as a site of convergence of the two stimuli, i.e., the Ca^{2+} and the neurotransmitter;[21,419-422] the role of this enzyme in neuronal plasticity was indeed independently suggested also by the aforementioned *Drosophila* studies (Section X).

Thus, in elementary mechanisms of both associative and nonassociative learning, modification of a K^+ channel by cAMP-dependent phosphorylation is proposed to play a crucial role. Learning in the above-mentioned model is phosphorylation of the appropriate ion channel; short-term memory is the elevated level of cAMP (given the experimental finding that the phosphorylated state of the substrate protein immediately after learning is labile);[440] and memory readout, at least during the immediate phase after training, is the altered ion currents due to the phosphorylated, blocked S channel.[439-441]

Phosphorylation of ion channels, resulting in long-term modified excitability, was implicated in elementary learning and short-term memory not only by the *Aplysia* studies, but also by data obtained from other experimental systems. Thus, in *Hermissenda*, phosphorylation of early and delayed K^+ currents in a photoreceptor was suggested to generate the cellular correlates of an associative conditioning; Ca^{2+}- and diacylglycerol-dependent phosphorylation may, however, play here a more prominent role than cAMP-dependent phosphorylation.[138,139,139a]

It is also worth noting at this point that cAMP has been suggested to play a role in sensitization in the leech[442] and other invertebrate species[443] (see also the *Drosophila* data in Section X). However, whether ion channels are among the substrate proteins in the latter cases is not yet known.

Several groups have suggested a correlation between cAMP-dependent phosphorylation and learning and short-term memory in mammalian brain.[444] Alterations in synaptic, cytosolic, and nuclear phosphoproteins have been reported by these groups to be correlated with behavioral modifications in rodents.[445,446] The lack of information on the specific neuronal elements involved in the highly complex behavioral responses make such experiments prone to alternative interpretations and question the causative relevance of the observed changes to the behavioral alterations.

Extensive efforts have been directed towards the elucidation of the molecular mechanisms that underlie a more discrete plastic alteration in the mammalian brain, namely, long-term potentiation (LTP) in the hippocampus[447] and the modification of the nictitating membrane and eyelid response, which may involve an LTP-like process.[448] LTP is a lasting increase in the amplitude of evoked potentials in hippocampal cells following brief trains of high-

frequency stimulation.[447] It may last for weeks, but within the context of the present discussion, only the mechanisms underlying its generation and short-term sustention are considered. Protein phosphorylation in general has been implicated in LTP,[449] but so were molecular mechanisms which do not involve phosphorylation or involve it only indirectly, for example, Ca^{2+}-stimulated proteolysis and increase in the availability of amino acid receptors.[450] A mechanism of phosphorylation recently suggested to play a role in LTP is translocation of C-kinase from cytosol to synaptic membranes, resulting in enhanced phosphorylation of substrate proteins, for example, a 47-kilodalton synaptic protein, which was implicated in regulating synaptic efficacy.[451]

Catecholamines, which are known to activate adenylate cyclase, have been shown to be capable of activity-dependent modulation of LTP, but, as opposed to the *Aplysia* case (see above), there was no enhancement of synaptic transmission in the absence of high-frequency modulation.[452] There is an indication that modification of subtypes of K^+ channels may play a role here, too,[453] but the nature of the protein kinase involved has yet to be established. It is therefore possible that at least part of the acquisition and short-term memory mechanisms in LTP resemble mechanisms revealed in mollusks. Nevertheless, it should be emphasized that the role of protein phosphorylation, of the cAMP cascade (if at all), and even the identity of the cellular locus (or loci) of LTP, must yet be clarified.[454]

2. Biogenic Amines, and Possibly cAMP, as Rapid and Reversible Switches of Innate Behavioral Programs

In the gill withdrawal reflex of *Aplysia*, biogenic amines (in combination with neuropeptides), acting at least partially via the cAMP cascade, were portrayed as modulators of modifiable synapses which take part in reflexive memory. The biogenic amines actually modulated the state of excitability of the sensory-to-motor *Aplysia* synapse. Modulation of excitability state appears as a general role of biogenic amines in both vertebrates and invertebrates, not only in the context of modifiable behaviors, but also in the execution of innate behaviors.[418] An example of the action of biogenic amines, and possibly also cAMP, in reversibly setting into action alternate, innate behavioral programs can be taken from studies performed on the lobster, *Homarus americanus*.[455-457]

Kravitz and colleagues[455-457] discovered that when serotonin or octopamine are injected into freely moving lobsters, the animal assumes characteristic static poses that may last for hours. With serotonin injection, the lobster stands in a flexed posture high on the tips of its walking legs, with the claws spread apart and slightly open in front of the animal and the abdomen loosely tucked underneath it. This is similar to the pose that the lobster assumes when startled, at the beginning of a fight or when winning a fight, and during part of the male mating behavior. With octopamine injection, the lobster lies close to the substrate in an extended posture, with its walking legs and claws pointed forward and lifted off the substrate and the abdomen arched upward. This is similar to the posture lobsters assume when losing a fight, during part of the female mating behavior, and in young animals in some threatening situations.[457]

Kravitz and colleagues found that the biogenic amines exert their striking effects on the innate behavioral repertoire by combined activities on the flexor and extensor muscles and on excitatory and inhibitory motoneurons that innervate these muscles.[455] Activation of the appropriate aminergic cells releases amines as neurohormones from peripheral endings of these neurons and as specific synaptic neuromodulators from central endings. Both amines prime peripheral muscles to react more vigorously when stimulated. Their opposite actions on behavior reside in their effects on the motoneurons that innervate the muscles. By interacting with "command" neurons, serotonin presumably directs the readout of a central motor innate program causing flexion and octopamine of a central innate motor program causing extension. Both serotonin and octopamine are known to exert their effects in in-

vertebrates at least partially via adenylate cyclase-coupled receptors.[403,458,459] At least in the neuromuscular loci, the effect of monoamines in the lobster may be mediated by cAMP: a 29-kilodalton soluble polypeptide was detected, which was phosphorylated in a serotonin- and cAMP-dependent manner in intact tissue (and also in a cGMP-dependent manner in nerve-muscle homogenates).[456] Octopamine was only a weak agonist of the phosphorylation of this protein. The 29-kilodalton phosphoprotein was detected in different tissues and was suggested to be related to metabolic aspects common to several types of cells — and in the case of the postural reflexes, related to the serotonin-induced changes in nerve and muscle physiology, probably at presynaptic sites.[456]

The observation that serotonin and octopamine administered exogenously induce specific and opposite behavioral responses is not unique to the lobster: it was also been made in the crayfish,[457] in the crab,[460] and in the nematode, *Caenorhabditis elegans*.[461] Numerous other examples exist which suggest a role for serotonin and octopamine in priming innate behavioral programs in invertebrates and for serotonin, norepinephrine, and dopamine in priming innate, or mainly innate, responses in vertebrates (e.g., see discussion in References 418, 458, and 459). Some of these physiological effects may be mediated by cAMP, but a causative role has not yet been established. Invertebrates often offer relatively simple behavioral repertoires and relatively simple nervous systems and could therefore be expected to yield clearer answers as to the relationship between molecules, neurons, and behaviors, but even in an extensively studied system like the lobster postural control system, the physiological role of the cAMP cascade in the behaviors elicited by serotonin and octopamine must yet be proven. For a review of the role of cyclic nucleotides in invertebrate physiology in general, see Reference 458.

B. Events in the Days to Years Range: Long-Term Memory and Development

Since cAMP-dependent phosphorylation is a posttranslational modification of proteins, alterations in cellular properties which result from the activation of cAMP-dPK should be expected to last as long as the phosphorylated serine or threonine are not dephosphorylated by the appropriate phosphatase or, at most, as long as the modified protein is not degraded. For the time course of the cellular modification to outlast the life span of the modified protein, some mechanism must be recruited which does not depend on the integrity of single copies of protein molecules. Three possible classes of such mechanisms could be considered:

1. Initiation of a molecular cascade which transforms the cell, or subcellular compartments, into a new structural (or metabolic) equilibrium
2. Initiation of an autocatalytic molecular cascade which leads to replenishment of the modified protein by favoring the modification of newly synthesized protein molecules
3. Alteration of gene expression

All three postulated mechanisms might be involved in cAMP-dependent events which last more than days. From the data reviewed in Sections I to IX above, it is clear that in principle, cAMP is capable of pushing a cell in general, and a neuron in particular, into each of the three avenues. For example, cAMP-dependent phosphorylation of cytoskeletal elements (Section VII) may lead to reorganization of cellular shape, serving as a crystalization event for novel cellular specialization, such as neurite extension or precipitation of submembrane particles that take part in synaptic contacts (see, in this respect, Reference 462 for the correlation between synapse structure and function). Such "step function" changes might then last long after the initial phosphorylation event in the original cytoskeletal element took place.

1. Long-Term Memory as a Developmental Process

Especially favorable in recent years in attempting to explain how posttranslational modifications of proteins might outlast the life span of the protein themselves are autocatalytic molecular switches. The concept was already mentioned earlier in the context of the autophosphorylation of protein kinases.[268,269] For example, Lisman[269] proposed a bistable molecular switch, the operation of which is based upon the competing activities of an autophosphorylating kinase and a protein phosphatase. The kinase (denoted for the purpose of discussion kinase-1) can be activated either by phosphorylation by another molecule of its kind (intermolecular autophosphorylation) or by a separate protein kinase (denoted for the purpose of discussion kinase-2). Suppose kinase-1 is at first nonphosphorylated (i.e., the molecular switch is "off"). An initial phosphorylation by kinase-2 (a "teaching signal") activates kinase-1, which then phosphorylates other molecules of its kind. If the teaching signal is strong enough, the level of activity of kinase-1 will remain high enough to persistently phosphorylate, and hence activate, newly formed kinase-1 molecules, in spite of removal of the original teaching signal and the action of the phosphatase. The cellular switch will remain in the "on" position, and the modification will outlast the life span of individual kinase-1 molecules.

Despite its appeal, there is no evidence that such bistable molecular switches are responsible for the long-lasting effects of posttranslational modification of proteins in general and of the cAMP-dPK in particular. cAMP-dPK is capable of autophosphorylation, though intramolecular and not intermolecular[257-262] (see Section V.A). Intramolecular autophosphorylation of other protein kinases is known as well;[463-467] such a process frees the activated enzyme from dependence on the presence of the activating ligand, but activity would not outlast the life span of the modified protein.

The third possibility mentioned above, namely, regulation of gene expression by cAMP, is certainly possible if the actions of cAMP on gene expression are considered (Section IX above). Again, in the case of memory, there is no direct evidence that cAMP indeed participates in consolidation. The idea that it does is based on the observation that cAMP appears to be involved, in some systems, in learning and short-term memory (Section XI.A above); that under the appropriate training conditions, short-term memory matures into long-term memory; and that cAMP can, as just mentioned, regulate some neuronal genes. Goelet et al.[468] have suggested a molecular framework for the transformation of short-term into long-term memory, which postulates a multiphasic process, consisting of three main stages. At first, there is short-term memory, based on posttranslational modification of proteins — such as the cAMP-dependent phosphorylation event(s) which result in modulation of the K^+ S-current in *Aplysia* (the experimental system from which examples to support the suggested framework were drawn).[132] Here the time course of memory is determined by the stability of the phosphorylated state of the modified protein(s). Following the initial memory phase, so suggest Goelet et al.,[468] there is a gene induction phase during which trans-acting regulators modified by the second messengers involved in the early memory phase, activate early effector genes. The time course of memory is determined here by the half-life of the induced mRNA and proteins. The early genes then lead to alteration in the expression of late effector genes; long-term memory is sustained by the altered transcription of these latter genes and the resulting modification in cellular or subcellular properties.

Of special interest in the aforementioned postulated molecular framework is the suggestion that short-term memory matures into long-term memory by passing through a stage in which the early molecular events, that have been initiated during acquisition, induce transient gene induction. Data emerging from both vertebrate and invertebrate experimental systems indicate that long-term memory can often be disrupted by certain drugs and by anesthesia if administered during acquisition or immediately afterwards, but not later.[469-472] Effective drugs are transcriptional and translational inhibitors.[469,470,472] There is thus a time window (extending

from fraction of an hour to few hours), which is open during and after learning and during which transcriptional and/or translational activity is necessary for consolidation. This suggests that some proteins and genes must be transiently active for long-term memory to be established and that their activation is induced by the molecular signals which function during acquisition.

Early genes that regulate the expression of later genes are known in several cases, for example, in developmental processes induced by ecdysone in insects.[473] Proto-oncogenes such as *c-fos* and *c-myc*, or genes with similar properties, were suggested as the type of possible candidates for participation in the transient memory consolidation phase.[468] This is because such genes were reported to encode nuclear proteins that affect transcription or mRNA processing, the activity of their induced products is short-lived, and they are known to induce long-lasting cellular effects.[474-476] cAMP can possibly induce *c-fos*[342] and *c-myc*,[477] thus indicating that the same intracellular signal that leads to alteration of K^+ current during acquisition in *Aplysia*[132] may in principle activate genes with the postulated properties. Additional studies are required to establish whether transiently expressed regulatory genes indeed participate in consolidation and account for the protein-inhibitors-sensitive time window characteristic of this phase and, if they do participate, whether cAMP regulates their expression directly (e.g., via CAP-like proteins, see Section IX above) or indirectly (via phosphorylated substrates, *vide supra*). Experimental systems in which these questions can be tackled on the cellular and molecular level, relating the molecular biology to physiology, are already available.[472] All that can be presently said is that what is currently known on the cellular activities of cAMP does not turn the aforementioned molecular postulate unfeasible.

2. Differentiation of Tumor Cell Lines

In many respects, long-term memory may be regarded as a developmental process, in which the morphogen is the appropriate sensory information, which selects and stabilizes a neuronal state.[478] Ample evidence does indicate that cAMP is involved in neuronal development and in developmental plasticity (for reviews of the general role of cAMP in cellular development, see References 479 and 480; for the role of cAMP as a morphogen in simple systems, see reviews in References 332 and 427 and also in 428 to 430 and references therein).

Evidence was reported suggesting that cAMP may be involved in neural induction. When stable analogues of cAMP, or cAMP together with a cAMP phosphodiesterase inhibitor, were added to explants of undetermined presumative epidermis from three species of amphibia, morphological changes occurred that led to the generation of cells with some characteristic morphologies of neurons and glia; no criteria in addition to morphological similarity were used to determine induction of neural properties.[481] However, most of the studies on the role of cAMP in neuronal differentiation were carried out on partially differentiated neurons and glia cells and mostly on neuroblastoma and glioma cells in culture and on neuroblastoma somatic hybrid cell lines derived from fusion of neuroblastoma with other cells.[482] Many such studies are reviewed in References 483 and 484. Only a few observations are mentioned here.

Neuroblastoma, glioma, and neuroblastoma hybrid cells respond to cAMP analogues and to agents that increase intracellular cAMP level by displaying morphological and biochemical characteristics of differentiation. These include extension of neurites, enlargement of soma, specializations of neurofilamentous structures, and increase in the activity of enzymes that take part in neurotransmitter synthesis and metabolism, such as tyrosine hydroxylase, dopamine β-hydroxylase, and choline acetyltransferase. Also induced are transmitter uptake and release mechanisms, surface glycoproteins, and appearance of cAMP-binding proteins.[42,344,387,483-488] In general, the conclusion was drawn that cAMP is involved in arrest of cell division and in cell differentiation[479] for supporting evidence from neuronal as well as

nonneuronal tissues). In many studies similar effects on differentiation were obtained by employing agents that are not known to modulate cAMP level, and therefore an additional conclusion was drawn that whatever cAMP-mediated mechanism is involved in acquisition of differentiated traits in neural and glia tumor line cells in culture, it is not exclusive.[479,483] Moreover, in some cases doubt was cast on the specificity of the effect of the cyclic nucleotide and on its physiological meaning, due to the differentiating effect of other agents and to medium-dependent variability.[489] Another point which must be taken into consideration is the observation that manipulating the level of cAMP may lead to intracellular increase in the level of cGMP, and cellular changes might have also been attributed to the latter.[490]

The aforementioned studies on neuroblastoma and related tumor cell lines concentrated on morphological and molecular traits of single neurons and of cellular conglomerates, but the effect of cAMP on the development of a most characteristic neuronal trait, i.e., synapse formation, was often not addressed, and when addressed, it was at first difficult to elucidate. Nirenberg and associates have used cocultures of neuroblastoma hybrid cells and myotubes to investigate this point. They found that increasing cAMP levels in the hybrid cells for several days resulted in increased percentage of myotubes innervated by the hybrid cells and in the rate of spontaneous secretion of acetylcholine from synapses.[484] In addition, there was a gradual acquisition by the hybrid cells of functional voltage sensitive Ca^{2+} channels, as revealed by depolarization-dependent Ca^{2+} fluxes and by Ca^{2+} action potentials assayed by intracellular recording. It was suggested that cAMP regulates synaptogenesis in the tumor cells-myotubes coculture at least in part by regulating the expression of voltage-sensitive Ca^{2+} channels, which are required for stimulus-dependent secretion of transmitter at synapses. Further studies on gene expression in the hybrid cells, and especially studies using DNA probes for the implicated channels (probes which are not yet available), are required to verify or refute this hypothesis.

3. Sympathetic Neurons: To Be or Not to Be Adrenergic

At least part of the postulated roles of cAMP in differentiation of tumor cell lines of neural and glia origin could be discussed in the context of the known effects of cAMP on cellular metabolism and structure as revealed in other systems and could be heuristically attributed to such effects, for example, to regulation of transcription[344] (Section IX above). The level of neuronal and glial enzymes was also reported to be elevated by cAMP or by agents that increase cAMP in primary cultures and in organ cultures of neuronal origin, for example, increase of tyrosine hydroxylase in mouse superior cervical ganglion in culture which was sensitive to protein synthesis inhibitors and hence might have involved protein synthesis.[491-493] cAMP and its analogues were reported to initiate neurite elongation in dorsal root ganglia embryonic cultures[494,495] and in clonal pheochromocytoma cells.[496] cAMP was also implicated in the mechanism of action of nerve growth factor (NGF)[497,498] and induction of transiently acting nuclear genes have been reported,[342] but the effect of cAMP and NGF could be clearly dissociated.[496,499]

Cell cultures of dissociated neonatal rat sympathetic neurons have served to investigate the role of cAMP in one of the basic manifestations of neuronal development: the transmitter choice made by the neurons. It has been observed that during development, neurons from the neural crest can express either the gene that codes for tyrosine hydroxylase, which is required for noradrenaline synthesis, or the gene for choline acetyltransferase, which is required for acetylcholine synthesis.[500,501] This crucial decision, to be or not to be adrenergic, has been studied in detail by Patterson and colleagues in cultured sympathetic neurons.[501-505]

Adrenergic differentiation, begun by the cells in vivo, can be completed in vitro, but if grown in the presence of nonneuronal cell types, the decision may be reversed and the neurons express cholinergic features and lose their ability to synthesize catecholamines.[501,502]

Chronic depolarization with K^+ or veratridine directs the neurons to complete adrenergic differentiation in spite of the presence of the environmental factors that otherwise direct the neurons toward a cholinergic course.[501,502] The question was raised whether cAMP is involved in the developmental effects of depolarization.[504] Chronic K^+ depolarization was indeed found to increase cAMP (but not cGMP) level. Short (2 day) exposures to cyclic nucleotides did not have an effect on transmitter synthesis, indicating that the effect is a long-term one and cannot be explained merely by phosphorylation and activation of tyrosine hydroxylase (and see Section VI.B above). So far, the data were compatible with a postulated role for cAMP in depolarization-induced adrenergic development. However, the effects of increasing cAMP levels were never as big as the effects of depolarization, and theophylline, which increases cAMP, reversed transmitter choice.[504]

It should be noted that theophylline and other methylxanthines have effects in addition to cAMP phosphodiesterase inhibition, for example, they interact with adenosine receptors[506] and with Ca^{2+} stores in the endoplasmic reticulum.[507] Taken together with additional experiments on the effect of manipulation of Ca^{2+} concentrations in the developing neurons,[505] the results suggested that cAMP plays a role in the developmental transmitter-choice decision made by the sympathetic neurons, but also that the cAMP cascade does not play exclusive or even a major role in this decision — as opposed to Ca^{2+}, which presumably does play a major role.[505]

4. Does cAMP Play a Role in Developmental Plasticity of the Visual Cortex?

Restriction of visual input can alter permanently the properties of neurons in the mammalian visual cortex.[508] Thus, transient monocular eyelid closure in a kitten during a critical period that extends from the first few weeks to the first few months of age results in domination of cells in the visual cortex by the previously open eye, leaving only few cells with the capacity to be binocularly driven.[508,509] Kasamatsu and Pettigrew reported that intraventricular injection[510,511] or cortical microperfusion[512] of the neurotoxin 6-hydroxydopamine (6-OHDA), which is known to destroy catecholaminergic terminals and hence to deplete the treated region from catecholamines, preserved binocular responses of neurons in the visual cortex of previously eye-sutured, monocularly deprived kittens. These findings, supported by their additional studies,[513,514] led Kasamatsu and Pettigrew to propose that catecholamines, specifically norepinephrine acting through a β-receptor coupled to adenylate cyclase, play an important role in the maintenance of cortical plasticity during the critical period. The catecholamine-hypothesis of neuronal plasticity of visual neurons during the critical period has since become an issue of intense controversy, yielding numerous attempts to prove or refute it.

Kasamatsu and Pettigrew reported not only that 6-OHDA treatment prevented the development of ocular domination following a transient monocular deprivation,[510-512] but also that local microperfusion of areas of the visual cortex with norepinephrine restored neuronal plasticity in kittens previously treated with 6-OHDA and to a certain degree also in adult cats which were no longer susceptible to brief monocular deprivation.[513] Microperfusion of dibutyryl cAMP was also reported to restore plasticity (i.e., enabling ocular dominance to occur) after 6-OHDA treatment, although the effect was not restricted to the hemisphere perfused with the cyclic nucleotide.[514] In addition, the same investigators reported that the recovery of binocular cortical cells from the effects of a brief monocular deprivation (i.e., a week of eye-suture followed by usage of both eyes still during the critical period) was delayed by microperfusion with 6-OHDA and accelerated by microperfusion with norepinephrine.[512]

The finding that administration of 6-OHDA by intraventricular injection or by local cortical microperfusion decreases plasticity in the visual cortex was at least qualitatively reproduced by several groups.[515-517] Inability to reproduce the intraventricular effects of 6-OHDA[518,519]

could be explained as resulting from small samples shadowing the modest magnitude of the effect.[517] However, the interpretation that norepinephrine depletion is responsible for the decrease in plasticity following neurotoxin treatment was questioned.[517,520,521] Systemic injections of 6-OHDA, which produced permanent and substantial reduction in cortical norepinephrine, did not alter the cortical response to monocular deprivation.[516,520] Chemical, 6-OHDA induced, as well as electrolytic lesions of the dorsal noradrenergic pathway going from the locus coereleus to the telencephalon, resulting in substantial reduction in the level of norepinephrine in the visual cortex, also did not alter the plastic response of cortical neurons to monocular deprivation.[521] Moreover, intraventricularly injected doses of 6-OHDA which were found sufficient to produce almost maximal depletion of norepinephrine did not decrease plasticity of the neurons in the visual cortex.[517] The effects of the neurotoxin on plasticity and its effects on norepinephrine level could thus be dissociated.

What is then the mechanism by which 6-OHDA can, under certain conditions, produce a decrease in the plastic response of sensory deprived cortical neurons? One possibility is that 6-OHDA effects on the cortical visual neurons are not directly related to its catecholaminergic toxicity.[517] Another possibility is that these effects are related to the catecholaminergic action, alas not to toxicity, but to neurotoxin-induced supersensitivity of β-receptors.[517] If correct, the latter hypothesis implies that cAMP is still involved in the plastic response of the cortical neurons during the critical period. It is of interest to note in this context that, as previously mentioned (Section VII), cAMP-dependent phosphorylation of MAP-2 in homogenates prepared from the cat visual cortex was found to increase after a relatively brief exposure of dark-reared animals to light.[310] It is also of interest to mention here that maturation of neuronal elements in the newborn rat neocortex is inhibited following a lesion in the locus coereleus,[522] and that visual experience appears to increase norepinephrine levels in chick telencephalon, hinting that this neurohormone might have some role in the process of visual imprinting.[523]

5. Neuronal Regeneration

Peripheral nervous tissue can regenerate after lesion, and also so can some central neurons under certain circumstances. Since cAMP was reported to enhance neuronal development in general and neurite growth in particular (Section XI.B.2 and 3 above), several groups of investigators turned their attention to the possible involvement of cAMP in regeneration, too. Two major experimental systems were used: the sciatic nerve (in rat or frog)[524-530] and the goldfish optic nerve.[531]

Contradictory results were obtained by different groups on the role of cAMP in regeneration of sciatic nerve. In some cases, there were no effects,[525,526] while in other cases, dibutytryl cAMP[524,527] or the adenylate cyclase activator forskolin[530] had a stimulatory effect on regeneration. The discrepancy between experiments might be attributed to the nature of the experimental protocols, differing in the method of lesion (e.g., brief crush vs. prolonged one), in the criteria for regeneration, and also in the time course of the observed effects. Thus, for example, in crush-lesioned rat sciatic nerve, dibutyryl cAMP-treated rats (injected intramuscularly with the cAMP analogue) did not differ from controls until 10 days after a brief (3 sec) lesion, when the return of sensory motor function was used as a criterion for regeneration.[527] Electron microscopy studies demonstrated that the cAMP analogue accelerated breakdown of myelin sheath and increased the number of regenerating myelinated axons in the region distal to the crush.[527] Another group of investigators also used i.m. injection of dibutyryl cAMP near the site of the crush and a sensory motor test for regeneration and obtained positive results already 2 days after the lesion.[524] Yet another group did not observe any effect of injected dibutyryl cAMP up to 9 days after a more prolonged (20 sec) crush.[525] The latter authors questioned the results of other groups which have used a brief crush, raising the possibility that such a brief crush did not sever the nerve completely.

Axonal regeneration following injury was found to be enhanced in nerves that have been subjected to a previous, "conditioning" injury.[532] An increase in adenylate cyclase activity was reported immediately distal to the regeneration locus in the conditioned lesion sciatic nerve in the rat when compared to a nonconditioned lesion, and this was taken as suggesting that an increase in cAMP distal to the lesion may condition the milieu to enhance sprouting or elongation following the test lesion.[529] No information was, however, provided on the activities of other enzymes. Adenylate cyclase was also reported to accumulate proximal (and later also distal) to a constriction placed around the frog sciatic nerve. The enzyme was also reported to accumulate in the proximal transected nerve during the period when Schwann cells proliferated.[528] Again, no information was provided on other enzymes to support the notion that the increase in activity and redistribution of adenylate cyclase were effects specific to this enzyme. Nevertheless, later studies demonstrated that cAMP level in the regenerating nerve stump in the same frog preparation increased twofold coincident with the elongation of regenerating nerve sprouts and that the adenylate cyclase activator forskolin (administered either by injection or by perfusion adjacent to the injury) increased the rate of sciatic nerve generation.[530]

In the goldfish optic nerve, on the other hand, there was a report that dibutyryl cAMP retarded axonal outgrowth in a dose-dependent manner, accompanied by a partial block of neuronal protein synthesis and a reduction in the fast axonal transport of newly synthesized proteins.[531] This latter effect was suggested as a possible explanation for the failure of dibutyryl cAMP to sustain outgrowth of axonal sprouts in some experiments, in spite of its claimed ability to stimulate initial sprout formation.[524-527]

It is of some interest to note here that expression of the proto-oncogenes *c-fos* and *c-myc* was detected in the regenerating goldfish optic nerve.[533] *c-fos*, as mentioned above (Section IX) can be induced in a clonal pheochromocytoma cell line by NGF, and cAMP was implicated in the process.[342] Whether growth factors affect proto-oncogenes during regeneration, and whether cAMP is involved in such a postulated process, remained to be determined.

XII. SOME CRITERIA, TRENDS, AND COMMON DENOMINATORS

In the previous sections, we have surveyed cAMP-dependent modifications of cellular constituents, which may contribute to alterations in neuronal properties that evolve within seconds to weeks and last for periods of seconds to a lifetime. Phosphorylation of neuronal proteins by cAMP-dPK accounts for modifications in cellular properties that last from seconds to probably weeks. These often involve direct attenuation of cellular response to incoming extracellular signals and modulation of neuronal response to later stimuli. On the molecular level, the modulation can be described as homologous (altering future response to the same signal that led to the modulation) or heterologous (altering future responses to stimuli other than the stimulus which has exerted the modulatory effects in the first place). Such modulatory modifications may be achieved by one or more of the following mechanisms: desensitization of receptors for neurotransmitters, opening or closing of voltage sensitive ion channels, activation of enzymes and reorganization of cytoskeletal elements.

Changes that last from days to years may involve regulation of the genetic material, either by substrate proteins for the catalytic subunit of cAMP-dPK or even, possibly, by direct interaction of the chromosomal material with subunit(s) of the enzyme. On the face of it, it seems therefore that the potential molecular tools of neuronal plasticity (Sections I and II) are indeed capable of being manipulated by cAMP and that the operation of the cAMP cascade may contribute to diverse facets of plasticity — ranging from differentiation and development of neurons and nervous systems, through transient alterations in communication of neurons and neuronal ensembles with their milieu, alterations between innate neuronal programs in response to the appropriate signal stimuli, and up to learning and memory.

A major question, which was already raised in Sections VIII and IX, is how much of the effects of cAMP observed in vitro or under special experimental conditions indeed play a significant role in vivo. This question has actually two parts: (1) how much of the molecular events per se do indeed occur in vivo, and if they do, (2) how much do they contribute to neuronal plasticity, if at all?

Krebs and Walsh and colleagues have long ago formulated five criteria that must be satisfied before a given physiological function of cAMP-dependent protein phosphorylation can be accepted. These criteria are the following:[331]

1. A protein is phosphorylated by cAMP-dPK in vitro.
2. This in vitro phosphorylation leads to modified function.
3. A stoichiometric correlation exists between in vitro phosphorylation and modified function.
4. Phosphorylation of the same protein is demonstrated in vivo in response to a cAMP signal.
5. Modified function in vivo is demonstrated in response to a cAMP signal.

In spite of the voluminous data (which keep accumulating at an almost alarming rate), almost all studies on the effects of cAMP in the nervous system have not yet resulted in fulfillment of all the above criteria. In many cases, lack of information on the function of the phosphoprotein makes it impossible to satisfy criteria 2, 3, and 5; our knowledge of function lags much behind our ability to detect very minute amounts of phosphoproteins or even to clone and sequence their genes. Describing a molecular and cellular function without describing an in vivo physiological correlate of the phosphorylating event is also not sufficient for implicating the cAMP-mediated event in neuronal plasticity; for example, as yet there is no evidence that cAMP-mediated functional alterations of Na^+ action-potential channels indeed contribute to neuronal plasticity.

The goal of satisfying the aforementioned criteria is best approached when function is known and a multilevel, interdisciplinary approach is used. Analysis of the effect of cAMP on receptors for neurotransmitters may serve as a partial example. Such studies, combining electrophysiology, pharmacology, biochemistry, and molecular biology, descend from the functional receptor in the intact cell to the cloned receptor and ascend back from the cloned subunits to a reconstituted receptor; they succeed in linking phosphorylation with alterations of protein structure and function on one hand and with alteration in physiological response on the other hand. The results show that cAMP-dependent phosphorylation can lead to agonist-induced receptor desensitization in both the nicotinic acetylcholine receptor and in the β-adrenergic receptor, though by different molecular mechanisms in each case, and at least in the latter case, cAMP-dependent desensitization is heterologous, whereas the more specific, homologous refractoriness is regulated by a specific, cAMP-independent receptor kinase (Section IV). However, the *in situ* physiological role of desensitization and its effect on behavior must yet be scrutinized. Pharmacological studies are suggestive, but ultimately there will be a need for directly proving that the phosphorylation results in a meaningful physiological alteration (and see, for example, the research methodology described in Reference 533a, in another context, for suggesting cause-and-effect relationship between post-translational modification and behavior). Another experimental approach might involve testing the effect of site-directed mutagenesis, preventing phosphorylation of desensitizing sites, on neuronal and behavioral plasticity of neuronal networks or intact organisms.

In other cases, multidisciplinary attempts are being carried out using intact organisms and reduced yet functional pieces of their nervous system, but other chunks of the puzzle — either the exact identity of the phosphorylated substrate or its precise role — are still missing. Studies on the role of cAMP-dependent phosphorylation of the K^+ S channel in sensitization

and short-term associative memory of the *Aplysia* gill (or tail) withdrawal reflex provide such an example. Here, alterations in the properties of a neuronal network (and as a consequence of a specific behavior) can be explained as resulting from cAMP-dependent phosphorylation of a specific type of ion channel in the synaptic membrane (Section XI.A). The channel protein has not yet been isolated, nor was its identity as a phosphorylated substrate established.

Attempts to establish the postulated role of phospho- and dephospho-synapsin in transmitter release is yet another example of a combined molecular and electrophysiological approach, which takes advantage of a purified phosphoprotein, antibodies against it, and a given physiological response. Here, the function of the purified phosphoprotein must yet be convincingly determined.

As all the above examples as well as our expanding knowledge of second (or third?) messenger systems in general attest to, description of cAMP-dependent modification of a given physiological function usually comprises only part of a story. It is now well established that several major ubiquitous phosphorylation systems, responsive to different kinds of extracellular and intercellular messengers, coexist in cells in general and in neurons in particular.[2,6,48,52,240,534,534a] These systems include, in addition to the cAMP-dependent phosphorylation cascade, also the Ca^{2+}-calmodulin-dependent phosphorylation system and the Ca^{2+}-phospholipid-dependent (C-kinase) phosphorylation system. For example, the role of the latter in neuronal plasticity has recently been more and more emphasized.[534a] Other regulatory phosphorylation systems, probably less ubiquitous, may yet be found. Although these systems do not always overlap in the nervous system,[68,69,77] they often clearly interact, either in an opposite or in an additive and even synergystic manner (for some examples of interactions of the aforementioned systems, see reviews in References 2, 6, 52, 240, and 534 and also some additional examples in References 21, 100, 255, 282, 296, 314, 321, 345, and 463).

Special attention has been devoted in recent years to the interaction of Ca^{2+} and cAMP in cellular regulation in general and neuronal regulation in particular.[2,6,48,52,240,534-538] This interaction must be taken into account when cellular modulation such as those involved in differentiation,[505] contraction,[536] secretion,[537] and learning[21,139,419,439] are considered. The complex interaction between the cAMP cascade and the Ca^{2+}-calmodulin cascade is already known in reasonably great detail[2,6,48,52,240,534,535] and appears to involve multiloci reciprocal regulations of the enzymes in each cascade by the other cascade, for example, calmodulin activation of adenylate cyclase,[19] calmodulin regulation and cAMP-dependent phosphorylation of cAMP-phosphodiesterase,[255] as well as coregulation of substrate proteins such as synapsin I[321] and DARPP-32.[296] The picture that emerges here (as in many other facets of cellular function) is a pluralistic one: there is more than one regulative pathway for given cellular processes; different intercellular signals can lead to essentially similar functional outcome. Such multiplicity of regulatory routes is not to be unexpected from a phylogenetic point of view, and in a way, one may conclude that distributed (probably parallel) processing starts in the brain already on the cellular level.

It is very likely that several trends, which have already become apparent in recent years, will dominate research on cAMP in the nervous system in the near future. These include interdisciplinary experimental approaches, incorporating the powerful tools of DNA cloning, site-directed mutagenesis, protein chemistry, and monoclonal antibodies in the study of phosphoproteins of known function, and proper evaluation of the physiological role of the cAMP cascade in each case, taking into consideration the function of other protein kinase systems, either separately or in concert with cAMP-dPK. In addition, it is likely that more attention will be devoted to the possibility that cyclic nucleotides might in some cases regulate cellular function independently of phosphorylation (e.g., Section IX; see also, in this respect, Reference 539 and 540).

Especially rewarding might be those studies that would combine detailed analysis, by molecular-biological and biochemical tools, of the effect of cAMP-dependent phosphorylation on isolated proteins, with physiological analysis of the effect of this phosphorylation in a reconstituted system or in an isolated, well-controlled cellular or subcellular preparation. Such an integrated molecular and cellular approach might yield intriguing knowledge on the detailed mechanisms by which local, cellular alterations in cAMP level contribute to modulation of function and behavior, both innate and acquired, of whole organisms, *Homo sapiens* included.

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