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Role of Adenosine 3′,5′-Monophosphate (Cyclic AMP) in Actions of Catecholamines

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THERE is at present little evidence that would link adenosine 3′,5′ monophosphate (cyclic AMP) to the regulation of catecholamine metabolism in any part of the nervous system. However, there is abundant evidence that links the formation and subsequent action of cyclic AMP to certain aspects of tissue regulation performed by catecholamines and many other hormones in a wide variety of organs. The role of cyclic AMP in the regulation of nervous system function is only slowly beginning to emerge, owing in large part to the enormous degree of cellular inhomogeneity and to our relative ignorance concerning the humoral and other factors that influence cyclic AMP metabolism in nervous system tissues. In this brief dissertation I hope to lay the ground work for serious consideration of the possibility that cyclic AMP may play some part in the regulation of catecholamine metabolism in the sympathetic nervous system. In so doing, it will be necessary to summarize some of the important information and problems regarding the general role of cyclic AMP in hormonal regulatory mechanisms as well as to summarize some of the information and problems regarding the role of cyclic AMP in the nervous system.

Role of Cyclic AMP in Actions of Catecholamines and Other Hormones

Cyclic AMP was discovered in the course of investigations on the mechanism of the glycogenolytic action of epinephrine and glucagon in liver (44, 60). This substance was formed by particulate fractions of liver homogenates in the presence of the hormones and adenosine triphosphate (ATP), and was capable of stimulating the accumulation of the active forms of phosphorylase catalyzed by supernatant fractions that were insensitive to the hormones. From this and other evidence, the proposition was advanced that cyclic AMP was an intracellular "mediator" of the glycogenolytic action of epinephrine in liver and other tissues by virtue of increasing the concentration of the physiologically active species of glycogen phosphorylase (43, 61). Concurrent and subsequent investigations in a number of laboratories have provided evidence for the involvement of cyclic AMP in the actions of a large number of polypeptide and amine hormones in a wide variety of tissues from a diverse array of animal

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species. This subject has been rather intensively reviewed in the past few years (4, 15, 21, 32, 47, 62), and not all such publications have been cited here. In addition, this topic has been the subject of four major symposia in the span of less than 2 years, the proceedings of which have been published (17, 19, 41, 49). Finally, the reader is referred to the thoughtful and authoritative monograph by Robison *et al.* (48) for literature citations and discussion of many of the statements not fully documented in this abbreviated dissertation. Throughout this article, I will refer frequently to a publication summarizing and reviewing the topic under discussion rather than to original articles. The reader will need to consult these publications for more complete literature citations.

In considering the evidence linking cyclic AMP to the hormonal regulation of glycogenolysis in liver, lipolysis in adipose tissue, steroidogenesis in the adrenal cortex, cardiac contractile force and water and electrolyte movements in the toad bladder, Sutherland *et al.* (59) formulated what has become known as the "second messenger concept." In this view, hormones (or "first messengers") would interact with tissue-specific sites in the plasma membrane and produce an activation of the enzyme, adenyl cyclase, also in the plasma membrane. The augmented level of cyclic AMP so produced would then proceed to influence a variety of cell structures by way of sequences of events that for the most part are unknown. Upon removal of the hormone the system would "relax" owing in part to the conversion of cyclic AMP to 5′-AMP by a special phosphodiesterase that was inhibitable by methylxanthines. Cyclic AMP could thus be viewed as a kind of "trigger," setting in motion rather stereotyped responses determined by the programming in the individual differentiated cell. This view has been enormously useful in suggesting experimental approaches for the dissection of regulatory effects of a large number of hormones and in suggesting ways in which chemical agents and other environmental factors might influence hormone action (62).

Subsequent investigations by a mounting number of workers have served both to expand the number of hormones, tissues and cellular processes that can be linked to the regulatory function of cyclic AMP and to provide greater insight into the mechanism of action of cyclic AMP. These latter investigations have revealed the existence of a family of protein kinases in a variety of tissues that are stimulated by cyclic AMP and that transfer phosphate groups to a variety of cellular proteins resulting in some cases in a marked change in biological properties (18, 28, 65). One substrate for these enzymes is the form of phosphorylase *b* kinase that is essentially inactive at physiological pH (65). The product of this reaction would then be capable of converting phosphorylase *b* to phosphorylase *a*, the species active in the absence of 5′-AMP. Other substrates include the histones, and this and other observations form the basis for the hypothesis that cyclic AMP-dependent "histone kinases" are involved in certain adaptive changes in enzyme levels involving protein synthesis *de novo* (18, 28). This aspect of cyclic AMP function will be important later in this discussion for the development of a view of adaptive changes in catecholamine metabolism.

Investigations subsequent to 1965 have also served to provide warning that a

rigorous or simplified application of the second messenger formulation will not allow adequate explanation of certain observations. First, there have been a number of instances in which the application of cyclic AMP or its derivatives on intact cell preparations has not faithfully reproduced the effects of the hormone in question. While the absence of any effect may be explicable in terms of difficulty of penetration into some cells or in terms of the deleterious effects of high concentrations of *any* adenine nucleotide (48), it is sometimes more difficult to understand those instances in which some, but not all of the hormonal effects are reproduced by exogenous cyclic AMP. One interesting example is the failure of N^6-butyryl cyclic AMP to bring about release of K^+ ions while efficiently inducing amylase release from rat parotid slices (1). Very recently, Batzri *et al.* (2) have published evidence indicating that the release of amylase is mediated by stimulation of *beta*-adrenergic receptors (and by cyclic AMP) while the release of K^+ ions involves stimulation of *alpha*-adrenergic receptors and thus would not be expected to be mediated by increased levels of cyclic AMP. These data have preserved the cyclic AMP-second messenger view as far as *beta*-adrenergic receptor function is concerned (48), but in more general terms they call attention to the possibility that hormones may exert effects not mediated by cyclic AMP by setting in motion parallel sequences of events that may or may not interact with those initiated by the formation of cyclic AMP. In some instances it may not be so easy to determine whether two different populations of receptors are involved. Rasmussen (46) has emphasized the point of view that hormones might simultaneously increase both the penetration of Ca^{++} and the formation of cyclic AMP and that the total hormone action represents an interplay between the two "second messengers." This view may have some unique value in certain situations, but suffers considerably when it is extrapolated globally. The relevance of these comments to the subject at hand is simply to point out the impossibility of taking any short cuts in attempting to establish a particular role for cyclic AMP in the nervous system.

Another problem with the "classical" second messenger view is the implication that the adenyl cyclase and associated receptors functioning in regulatory processes are localized in the plasma membrane. There is, of course, evidence in a number of tissues that the vast majority of adenyl cyclase resides in the plasma membrane fraction. In addition, it is necessary teleologically to visualize adenyl cyclase in the membrane with receptor sites facing out and catalytic sites facing in when one is dealing with a large polypeptide hormone like adrenocorticotropic hormone (ACTH). This is especially true in the face of the observation that ACTH retains biological activity even when coupled to polysaccaride complexes visible microscopically (52). However, there is much less urgency to place all the functional adenyl cyclase and receptors in the plasma membrane when one is dealing with biogenic amine hormones that are known to enter both neuronal and extraneuronal structures to be stored or metabolized. There have been reports that adenyl cyclase is a component of nuclear membranes in the liver (57) and prostate (29) or of the sarcoplasmic reticulum in skeletal (38) and cardiac muscle (10). While it is difficult to exclude completely the possibility that the

adenyl cyclase activity found in cell fractions is due to contaminating fragments of cell membranes, in the case of cardiac muscle the specific activity of the sarcoplasmic reticulum fraction was no lower than that of preparations of the sarcolemma despite differences in other enzymatic properties of the two fractions (10). Breckenridge and Bray (5) observed that adenyl cyclase accumulated proximal to an occlusion of the chicken sciatic nerve and concluded from parallel morphological studies that a portion of nerve adenyl cyclase is associated with intracellular organelles. Thus it is important to keep in mind that in some cells, including neurons, a portion of the adenyl cyclase may be located in internal membranous structures and may be subject to regulation by humoral agents of low molecular weight penetrating from the outside or by other changes in the internal cellular environment.

Still another problem with an overly rigorous interpretation of the second messenger formulation is the implication that changes in the cellular function subject to regulation by cyclic AMP should be accompanied by changes in the intracellular concentration of this compound. Very often it has been possible to observe increases in cellular levels of cyclic AMP only in the upper regions of the physiological dose-response relationship of a hormone. In some cases, maximal physiological response has been associated with only a 20 to 40% increase in cyclic AMP (48). One practical reason for these problems is that the amount of cyclic AMP found under base-line conditions is high relative to the amount that might be expected to exert a biological effect. For example, the lowest level of cyclic AMP that was observed in rat diaphragm muscle incubated under "control" conditions was somewhat greater than 0.3 nmole/g (9), while the apparent K_m of rabbit skeletal muscle protein kinase for cyclic AMP is about 0.02 nmoles/ml (65). These and other observations have been interpreted as indicating the existence of a sequestered, physiologically inactive pool that contributes to the analytical "blank." Exton *et al.* (11) observed a much closer correlation of glucose output with cyclic AMP appearing in the medium of perfused rat livers than with tissue levels of the compound. They felt that the amount released was a better measure of the "free," physiological active nucleotide. Unfortunately, very few tissues are as prone to release appreciable amounts of cyclic AMP as the liver. This is particularly true of brain tissue (23). One important consequence of this state of affairs is the difficulty in ruling out the participation of cyclic AMP in a regulatory phenomenon simply because increases in tissue levels of this compound cannot be observed. This is especially true when the response under consideration requires many hours or even days to develop.

Role of Cyclic AMP in the Nervous System

This topic has been reviewed recently and was emphasized heavily in a recent symposium (17, 40). While the subject of a great deal of interest and effort, there is a relatively small amount of information available that speaks directly to the issue. Accordingly, analogies to processes regulated in other tissues will be frequently drawn.

It is, of course, much easier to provide arguments that cyclic AMP has *a* role

in regulation of nervous system function than to specify what roles it might play. The high levels of adenyl cyclase found in brain tissue have always been impressive, but the small to non-existent stimulatory effects of putative neurohormones on broken cell preparations of this enzyme have been frustrating. Therefore, most of the information available on the regulation of cyclic AMP metabolism has been derived from experiments with brain slices. Accumulation of cyclic AMP in slices has been followed by direct measurement, or by recovery of radioactive cyclic AMP from tissue prelabeled by incubation with ^{14}C-adenine (26, 42, 50, 55). The two approaches have yielded essentially the same pattern of information with only a few important exceptions. Incubation of brain slices with either norepinephrine (NE) or histamine has produced increases in accumulation of cyclic AMP that have ranged from less than 2-fold to more than 20-fold depending upon the agent, area of brain, species and age of the animal. The receptors involved are pharmacologically distinguishable, and additive or greater than additive effects have been observed when both agents were used simultaneously. The responses to NE have without exception been inhibited or prevented by *beta*-adrenergic blocking agents. There has been one report that responses to NE in slices of guinea pig cerebral cortex were also inhibited by *alpha*-adrenergic blocking agents (7). The significance of this observation is not yet appreciated. In peripheral tissues, stimulation of *alpha*-adrenergic receptors has been invariably associated with either no change or a decrease in cyclic AMP accumulation. Of the other suspected neurotransmitter agents, only serotonin has produced detectable increases in cyclic AMP levels in slices of central nervous system tissue. However, in blocks of rabbit superior cervical ganglia, dopamine was recently reported to produce more than a 6-fold increase in the nucleotide (25). Ganglionic tissue also responded to NE, but only at elevated doses. In addition, the effects of NE, but not of dopamine, were inhibited by *beta*-adrenergic blocking agents; only *alpha*-adrenergic blocking agents were capable of reducing the effects of dopamine. We will return to this system a little later. Thus there is abundant evidence that catecholamines and other biogenic amines influence cyclic AMP metabolism in neural tissue, but only in the case of the sympathetic ganglia is there even indirect evidence that these changes occurred in neurons. It is disquieting to note two recent reports that catecholamines can produce more than 100-fold increases in cyclic AMP levels in cultured glial tumor cells (8, 14). Thus it is possible that the data on catecholamines acquired by using slices may be dominated by responses of glial elements. Speculation on the functional significance of cyclic AMP metabolism in glial cells will not be attempted in this dissertation.

The application of assorted depolarizing stimuli has also produced large increases of cyclic AMP in brain slices. These stimuli have included electrical pulses, 40 to 43 mM K^+ ions, batrachotoxin, veratridine and ouabain (42, 55). The relative effectiveness of these stimuli has varied considerably depending both on the species and the area of brain studied. One extreme example of this perplexing variability is provided by guinea pig brain. In slices of cerebral cortex, both electrical pulses and 40 mM K^+ ions produced approximately a 10-fold

increase in cyclic AMP levels within 5 min; in cerebellar slices, electrical pulses increased cyclic AMP levels about 7-fold while exposure to 40 mM K^+ ions was without detectable effect (67). Omitting Ca^{++} ions or elevating Mg^{++} ion concentration in incubation media has reduced the effect of most, but not all of the depolarizing stimuli. For example, incubating guinea pig cerebral cortex slices with 14 mM Mg^{++} ions completely prevented the effect of 40 mM K^{++} ions, but augmented the effect of electrical pulses (67). Despite the strong possibility that these depolarizing stimuli produce at least a portion of their effects by releasing endogenous neurohormones, the application of these stimuli in the presence of maximal amounts of either NE, histamine or serotonin has invariably produced augmented, rather than diminished responses.

The puzzling effects of depolarizing stimuli have been partially, but far from completely clarified by the discovery of the very large increases in cyclic AMP levels in brain slices produced by relatively small amounts of adenosine (51). As little as 0.01 mM adenosine produced a 5-fold increase within 5 min, and 0.05 mM often caused a 30-fold elevation of cyclic AMP in guinea pig cerebral cortical tissue. The combined addition of adenosine and either NE or histamine led to synergistic responses in many instances, depending upon the brain area or species studied (42). Another similarity between the properties of adenosine and depolarizing stimuli was the inhibitory effects of methylxanthines. As little as 0.5 mM theophylline could prevent completely the effects of 0.05 mM adenosine and could inhibit the effects of the various depolarizing stimuli by 60 to 85%, depending upon the stimulus used (42, 55). This variation in theophylline inhibition argues against the proposition that the depolarizing stimuli have identical modes of influencing cyclic AMP metabolism, namely by the formation and action of adenosine (39). This conclusion is reinforced by the observation that the effect of 40 mM K^+ ions on cyclic AMP levels in guinea pig cerebral cortical tissue is actually augmented in the presence of maximal amounts of adenosine even though under the same conditions electrical pulses produce no further increase. We are left with the picture that depolarizing stimuli influence cyclic AMP accumulation in part through the aegis of a metabolite, adenosine, and in part through essentially unknown means. These could be quite direct effects of membrane depolarization on adenyl cyclase activity or relatively indirect effects involving mediation by unknown humoral factors. We are presented with an array of potentiative interactions among stimuli for the accumulation of cyclic AMP that has no parallel in peripheral tissues. We are also left with the remarkable effects of the metabolite, adenosine, a diffusible substance, free to move out of a cell to act on its neighbors or free to move into a cell to influence internal structures. Again, the action of adenosine on cyclic AMP accumulation has as yet no parallel in peripheral tissues. It is also comforting to note that cultured rat glioma cells did *not* respond to incubation with adenosine (14).

So far, the information outlined above on the regulation of cyclic AMP metabolism has provided only small clues as to the roles cyclic AMP might play in the nervous system. In this section I will select cellular processes of special importance in the functioning of neurons that have been shown to be subject to regulation by

cyclic AMP in peripheral tissues and discuss briefly any evidence indicating similar modes of regulation in the nervous system. At the outset, we can rapidly dispose of the issue of the regulation of glycogen metabolism, the process classically associated with cyclic AMP. In slices of guinea pig brain, the concentration of phosphorylase *a* either does not change or falls at the same time that cyclic AMP levels are rising markedly (24). In rat brain *in vivo*, the concentration of the form of glycogen synthetase that is "independent" of glucose-6-phosphate increases under circumstances in which cyclic AMP levels are also increasing (16). In peripheral tissues, cyclic AMP promotes the disappearance of this form of the enzyme. If cyclic AMP is involved in regulation of glycogen metabolism in nervous system tissues, then the mode of action must be different from that proposed for peripheral tissues.

One important function carried out by neurons is secretion. There is a growing body of evidence linking cyclic AMP to the regulation of release of a number of protein hormones and enzymes. These include amylase from the parotid gland, insulin from pancreatic islets and various trophic hormones from the anterior pituitary. (In addition to reference 48, the reader is referred to references 3, 6, 27, 46 and 54 for discussions of this topic.) It has been known for some time that epinephrine can be observed to facilitate neuromuscular transmission, producing both an increased frequency of miniature end-plate potentials and an increased amplitude of the end-plate potential. Both dibutyryl cyclic AMP and methylxanthines have been found to mimic the effects of catecholamines (56). In view of the marked effects observed with the methylxanthines by themselves, one is tempted to conclude that pools of cyclic AMP turning over during nerve excitation normally play a role in acetylcholine release. However, caution is necessary because of the long known effects of methylxanthines on mobilization of Ca^{++} ions (see discussion in reference 45). Even if cyclic AMP cannot be linked to the instigation of transmitter release, these observations suggest that it may be involved in modulating this process.

Another basic function that is extremely important in the operation of nervous system tissue is the regulation of ionic fluxes across cell membranes. Cyclic AMP has been observed to mimic the ability of catecholamines and glucagon to bring about transient release of K^+ and Ca^{++} ions from the perfused rat liver (13, 36). These fluxes are apparently associated with a significant hyperpolarization of the liver cell membrane (58). The mechanism or significance of these findings are yet to be determined. In the case of the toad bladder, there is convincing evidence the actions of vasopressin are mediated by cyclic AMP (20). These actions involve increased sodium transport and osmotic water flow across the epithelial cells. Similar effects produced in amphibian skin by oxytocin and *beta*-adrenergic agents also appear to involve cyclic AMP (34). In the rabbit ileal mucosa, cyclic AMP and theophylline have been found to mimic the effects of cholera toxic in severely reducing inward sodium flux and setting in motion an outward chloride flux (12). These and other observations have established that cyclic AMP can be involved in the regulation of membrane permeability and ion transport. In the central nervous system, an important series of experiments have strongly sug-

gested that cyclic AMP mediates the characteristic inhibitory effect produced by the iontophoretic application of NE onto rat cerebellar Purkinje cells (22). There are a number of interesting and important facets to this work. Aside from providing evidence for a heretofore unsuspected adrenergic inhibitory input to Purkinje cells, these observations showed the existence of an unusual type of inhibitory potential involving an increase in both membrane potential and membrane resistance. A similar mechanism apparently underlies the slow inhibitory potentials generated in rabbit sympathetic ganglia either by preganglionic stimulation or by application of NE or dopamine (30, 31). Although the electrophysiological effects of applying cyclic AMP have not been assessed (as they have in Purkinje cells), the fact that *alpha*-adrenergic blocking agents prevent both the generation of the synaptic potential and increased levels of cyclic AMP argue strongly for the mediation of the nucleotide (25).

The final cellular process I wish to consider is that of selective "induction" of synthesis of proteins. In addition to the function of cyclic AMP that has been established in catabolite repression and enzyme induction phenomena in microorganisms (37), there is a growing body of evidence for the mediation by the nucleotide of certain hormonally induced increases in enzyme levels in mammalian liver. Cyclic AMP or dibutyryl cyclic AMP has been shown to mimic the ability of catecholamines and glucagon to increase the concentration of such enzymes as tyrosine transaminase, phosphoenolpyruvate carboxykinase and the arginine synthetase system in cultured fetal rat liver (53, 66). These effects are distinct from those of corticosteroids which produced additive or greater than additive effects when combined with maximal amounts of the other hormones or the cyclic nucleotides. In addition, theophylline can usually be shown to potentiate the effects of catecholamines, glucagon or cyclic nucleotides, but not those of glucocorticoids. These effects can be blocked by cyclohexamide or actinomycin D and, at least in the case of tyrosine transaminase, there is direct evidence that synthesis of the enzyme is increased. There is no evidence available concerning a similar role for cyclic AMP in the nervous system. However, analogy with its role in liver forms the basis for the speculation that follows.

Concluding Remarks

The speculation I wish to advance is simply that cyclic AMP may play a part in the increased levels of tyrosine hydroxylase and dopamine-β-hydroxylase found in sympathetic ganglia after certain experimental interventions. These interventions include the administration of reserpine and phenoxybenzamine, and the effects appear to be mediated by increased nerve activity since surgical decentralization and nicotinic ganglion blockers prevent the increases in enzyme activity (35, 63, 64). In addition to these observations, any hypothesis dealing with possible cyclic AMP mediation would have to deal with the reports that increases in ganglionic cyclic AMP are *not* prevented by hexamethonium, are blocked by phenoxybenzamine and are *not* produced by antidromic stimulation (25, 33). Therefore one consequence of such a hypothesis would be that the large increases in ganglionic cyclic AMP produced by dopamine or preganglionic

stimulation cannot be related to tyrosine hydroxylase "induction." This would require postulating functionally separated pools of cyclic AMP. Without detracting from the conclusion concerning cyclic AMP mediation of ganglionic hyperpolarization, it might also mean that nearly all of the observable change in cyclic nucleotide level took place in glial rather than neuronal elements. Expressed another way, it is possible that the alteration in neuronal cyclic AMP metabolism involved in the hyperpolarization response might not lead to visible increases in the accumulation of the nucleotide. A second consequence of the hypothesis would be that acetylcholine acting either directly through nicotinic cholinergic receptors or indirectly through some consequence of repeated depolarization produced an increase in cyclic AMP metabolism in the "correct" part of the neuron. Again, it is necessary to postulate that any such change in cyclic AMP metabolism does not necessarily yield detectable increases in accumulation of the nucleotide. If simply changes in electrical activity of a neuron were sufficient to alter cyclic AMP metabolism in the proper locale, it could be imagined that changes in the internal concentrations of certain ions or metabolites might constitute the signal. The conversion of as little as 0.1 % of the ATP of the cell to adenosine could be one such signal.

The value of this speculation may be somewhat reduced by the difficulty in proving or disproving key elements of the hypothesis. Perhaps its greatest value lies in providing a perspective somewhat different from the classical second messenger formulation with which to view the possible function of cyclic AMP in the nervous system.

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