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COMMENTARY

CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE ROLE IN THE PHYSIOLOGY AND PHARMACOLOGY OF THE CENTRAL NERVOUS SYSTEM

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The physiological role of cyclic AMP in a variety of cells and tissues has been the subject of extensive investigation during the past two decades. As a result of this research, a wealth of knowledge has accumulated concerning the properties and regulation of the enzymes that form and degrade the cyclic nucleotide and on the fundamental enzymatic processes involved in translating an intracellular accumulation of cyclic AMP into a change in biochemical or biophysical properties of the cell. However, definition of the subtle relationships between the level and duration of accumulation of cyclic AMP and the modulation or alteration of one or more physiological functions in any particular cell remains a formidable challenge. Similarly, it it difficult to establish whether pharmacologically active agents, which do alter cyclic AMP levels in cells, elicit their pharmacological response, solely or even partially, through cyclic AMP-dependent mechanisms.

FORMATION, DEGRADATION AND ACTION OF CYCLIC AMP

Cyclic AMP is generated in intact cells by the catalytic action of membranal adenylate cyclases. The activity of these enzymes is increased many fold upon interaction of receptor sites with various hormones. A typical example would be a norepinephrine-sensitive adenylate cyclase whose activity would be increased by interaction of its receptor unit with a β-adrenergic agonist, such as norepinephrine or isoproterenol. The so-called β-adrenergic antagonists prevent the activation of the cyclase enzyme by the β-agonist. In brain tissue, activation of receptors by α- or β-adrenergic agonists, histamine, serotonin or adenosine elicits accumulations of cyclic AMP [1]. Thus, pharmacologically active agents that are agonists or antagonists at such receptors might owe their central activity to alterations in the levels of cyclic AMP (Table 1).

Degradation of cyclic AMP is catalyzed by the action of soluble and membrane-bound phosphodiesterases. A number of active inhibitors of these enzymes have been developed (Table 1). Such inhibitors have proven useful in the study of cyclic AMP-dependent mechanisms, since they often greatly increase the intracellular accumulation of cyclic AMP elicited by physiological or pharmacological activation of adenylate cyclases. However, in brain a variety of phosphodiesterases are present and each may exhibit a different inhibition profile [2]. Thus, any correlations between the central activity of a compound and its potency as an inhibitor of a particular phosphosiesterase must be made with caution. In addition, drugs often affect more than one biochemical parameter. For example, theophylline, a classical phosphodiesterase inhibitor, blocks adenosine-elicited accumulations of cyclic AMP in brain tissue [3].

Physiological effects of the cyclic nucleotides are generally believed to be mediated by the activation of

Table 1. Pharmacologically active agents with possible *in vivo* effects on cyclic AMP-dependent mechanisms

Agents which can elicit accumulations of cyclic AMP in brain tissue	Agents which can block accumulations of cyclic AMP in brain tissue	Phosphodiesterase inhibitors	Activators of protein kinases
Norepinephrine	α-Adrenergic antagonists	Theophylline	*N*-Butyrylcyclic AMP
Dopamine	β-Adrenergic antagonists	Isobutylmethylxanthine	8-Benzythiocyclic AMP
Serotonin	Phenothiazines	Papaverine	
Histamine	Butyrophenones	Phenothiazines	
Adenosine	Antihistamines	Benzodiazepines	
Prostaglandin E_1	Theophylline	Imipramine	
Glutamate	Local anesthetics	3,4-Dialkoxybenzyl-imidazolidinones (RO 20-1724)	
Depolarizing agents			

protein kinases. The activated kinases catalyze the phosphorylation of intracellular protein, thereby effecting an alteration in the functional role of this protein(s) in the cell. Dephosphorylation of the protein by phosphoprotein phosphatases would terminate the effect of a "pulse" of cyclic AMP. Protein kinases can be activated in intact cells either by endogenous cyclic AMP or by exogenous cyclic nucleotides. Thus, in brain, intraventricular or intracerebral administration of cyclic AMP or of active synthetic analogs (Table 1) would be expected to activate cyclic AMP-dependent kinases. The resultant pharmacological effects of the exogenous cyclic nucleotide may well reflect the sequelae to kinase activation. Such an approach to the study of the physiological role of cyclic AMP in brain is, however, quite imprecise. The exogenous cyclic nucleotide would be expected to activate cyclic AMP-dependent mechanisms to differing and unpredictable extents in a variety of cell types, resulting in a composite pharmacological response.

The futility of attempts to rigorously establish the role of cyclic AMP in brain by tabulating correlations between central effects of different drugs and their effects on components of the cyclic AMP system becomes all too apparent when one considers the complexity of the functioning cyclase–phosphodiesterase–kinase–phosphatase system in an intact cell or tissue. Questions pertain in intact cells as to the site of generation of cyclic AMP in relation to the intracellular localization of phosphodiesterases, protein kinases, substrates of protein kinases, phosphoprotein phosphatases and the availability of ATP both as a precursor of cyclic AMP and as a cosubstrate for protein kinases. It would appear likely that accumulation of low levels of cyclic AMP might only activate protein kinases intimately associated with the adenylate cyclase system. Such kinases might serve to regulate membrane properties proximate to a neuronal cyclase enzyme. Accumulation of high levels of cyclic AMP might activate more distant protein kinases. Certain of the latter protein kinases could be involved in a feedback inhibitory regulation of cyclic AMP generation, a phenomenon which appears to pertain in many cell types [4], or might be involved in regulation of protein synthesis via phosphorylation of histones at nuclear sites.

The question remains: How can such subtle mechanisms be investigated and defined in a system as complex as a living cell? Even in isolated homogeneous cell populations in which cyclic AMP functions are relatively well-defined, these are inter-relationships that are difficult to investigate. In a tissue such as brain, where the physiological roles of cyclic AMP remain to be established and where complex communication between a variety of different cell types is fundamental to the function of the system, an attempt to establish correlations between the over-all elevation of levels of cyclic AMP in the tissue elicited by various substances and alterations in physiological parameters in specific cells and/or compartments appears to be a virtually hopeless task. Nonetheless during the past five years, an ever-increasing literature has appeared related to the role of cyclic nucleotides, in particular cyclic AMP, in the central nervous system. For example, a recent review of this literature [5] includes over 400 references to papers published since 1967. However, the sites and functional significance of the accumulations of cyclic AMP that are elicited in brain tissue by biogenic amines such as norepinephrine, dopamine, serotonin and histamine, by electrical stimulation, by prostaglandins, by adenosine and by glutamate are still the subject of controversy. According to one proposal, the accumulations of cyclic AMP elicited by putative neurotransmitters occur in neurons and the cyclic AMP then serves a post-synaptic role in regulating the excitability of the neuronal cell. An opposing proposal is that accumulations of cyclic AMP elicited in brain tissue by catecholamines occur primarily in glial cells, where cyclic AMP then presumably regulates certain important metabolic functions. A corollary to this second proposal is that adenosine and prostaglandins elicit accumulations of cyclic AMP in neuronal cells. Undoubtedly, cyclic AMP plays a regulatory role in both neuronal and glial cells, but the relative importance of these two cell types to the accumulations of cyclic AMP elicited by various substances in brain tissue remains as yet a debatable point. A critical evaluation of published data pertaining to the sites of cyclic AMP formation in brain tissue provides some insights and directions pertinent to further research in this area.

SITES OF GENERATION OF CYCLIC AMP IN THE CENTRAL NERVOUS SYSTEM

Cell-free preparations. Brain tissue contains extremely high levels of adenylate cyclases, phosphodiesterases and protein kinases. The levels vary in different brain regions and appear particularly high in gray matter, and low in white matter. In homogenates of brain tissue, high levels of adenylate cyclases, phosphodiesterases, protein kinases and phosphoprotein phosphatases have been found in particulate fractions generally referred to as "synaptosomes" [6–9]. These consist of pinched-off nerve-endings in the form of resealed entities containing mitochondria and transmitter storage vesicles, along with an adhering postsynaptic membrane. Thus, all the components of the cyclic AMP system are present as part of the synaptic complex. It would then seem "simple" to isolate synaptosomes from different brain regions and subfractionate and characterize them, perhaps into discrete populations showing specific responses to norepinephrine, histamine, serotonin, adenosine or prostaglandins. Such an approach has provided evidence for populations of synaptosomes with specific uptake mechanisms for either norepinephrine, dopamine or γ-aminobutyric acid [10]. Unfortunately, the adenylate cyclases in homogenates of brain tissue retain relatively little of the receptor-mediated regulation of activity

which is almost always seen in cell-free preparations from other tissues. Thus, although biogenic amines, adenosine and prostaglandin elicit significant accumulations of cyclic AMP in intact cells of brain slices, they have either no effect or only small effects on the activity of adenylate cyclases in homogenates of brain tissue [3, 11–13]. The magnitude of the small effects of such agents on adenylate cyclases in homogenates is dependent upon the methods of homogenization and assay, probably accounting for the lack of correspondence of results obtained by different groups of investigators. In view of the present problems in retaining hormonal responsiveness of adenylate cyclases in homogenates it would appear premature to attempt to define the loci of hormone-responsive systems present in the intact tissue by classical fractionation techniques. Fractionation of brain into neuronal and glial-enriched populations followed by homogenization and assay of norepinephrine and histamine-sensitive cyclases in the resultant cell-free preparations has been carried out [14]. Marginal stimulations by the biogenic amines pertained in neuronal or glial preparations from a variety of brain regions. Similar assessment of hormone-elicited accumulations of cyclic AMP in populations of intact cells, isolated from brain, might be even more profitable.

Homogenization and fractionation techniques which afforded a cell-free particulate preparation from brain tissue that retains a hormone-responsive cyclic AMP-generating system have recently been reported [15]. The nature of the vesicular entities in this preparation that respond to norepinephrine, histamine and adenosine remains to be defined.

Brain slices. The discovery by Kakiuchi *et al.* [16–18] that putative neurotransmitters and electrical stimulation elicit accumulations of cyclic AMP in cells of brain slice preparations provided the impetus for ever-expanding research on this model system. One of the primary aims of such research has been to define the nature of the morphological compartments associated with the hormone-elicited accumulations of cyclic AMP. Through use of adenine-labeled brain slices, it was discovered that only a small fraction of the total adenine nucleotides serve as substrates for adenylate cyclase and that these compartmentalized nucleotides were preferentially labeled during incubation of slices with radioactive adenine. Thus, specific activity of cyclic AMP that accumulates after stimulation of the adenine-labeled brain slice is 3- to 8-fold higher than that of ATP in the slice [19–21], indicating that these highly labeled compartments contain only a small fraction of the total nucleotides of the slice. Equilibration of these nucleotides with the remainder of the ATP of the slice does not occur to a significant extent. Radioactive adenosine is not incorporated as specifically into the cyclic AMP-generating compartments [22].

Characterization of these nucleotide compartments has proven a difficult task. Brain slices, of course, represent a heterogenous tissue containing neurons of various types. Morphological compartments within neurons could consist of cell bodies, dendritic processes, axons and synaptic terminals. In addition, glial cells of various types are present in brain tissue. Certain of these have long processes which may represent morphological compartments. It appeared possible, indeed likely, that in brain tissue histamine regulates cyclic AMP formation in one cell type or morphological compartment, norepinephrine in another cell type, serotonin in another, adenosine in another, etc. However, greater than additive effects of certain combinations of agents on accumulations of cyclic AMP clearly indicated that, in some cells, more than one neuroregulatory substance affected the formation of cyclic AMP. Such synergistic combinations included histamine–norepinephrine, adenosine–norepinephrine, adenosine–histamine and adenosine–serotonin [20]. If different cells were indeed involved in the formation of cyclic AMP as elicited by specific agents, it appeared likely that the specific activity of the adenine-labeled nucleotides in these different populations of cells would not be identical. It then follows that the cyclic AMP which accumulates in response to histamine would have a different specific activity from that which accumulates, for example, in response to norepinephrine. Differences in the specific activity of cyclic AMP which accumulates in response to various agents have, however, not been marked [20–23]. Indeed, this relative constancy of the specific activity of the cyclic AMP suggests that similar cell populations or morphological entities are associated with the major cyclic AMP-generating systems in brain slices.

Since nearly half of the adenine-labeled nucleotides present in guinea-pig cortical slices can be converted to cyclic AMP during incubation with certain combinations of stimulatory agents [24], it was obvious that at least one-half of total radioactivity of the slice must be intimately associated with the cyclic AMP-generating compartments. Attempts to define the localization of radioactivity in adenine-labeled slices have, however, not been particularly successful. In preliminary radioactographic studies, it appeared that a major portion of the radioactivity was associated with synaptic regions (F. Bloom, M. Huang and J. Daly, unpublished results, reported in part at the First International Conference on Physiology and Pharmacology of Cyclic AMP, Milan, Italy, July 20–23, 1971). Subsequent studies revealed that from 50 to 60 per cent of the total radioactivity in the slice had been lost during fixation. Hence, no firm conclusions were possible. Attempts to minimize loss of radioactivity during fixation were not particularly successful.

Efforts to define the localization of adenylate cyclase, phosphodiesterase or kinase activity by histochemical techniques are subject to similar problems. For example, although phosphodiesterase activity in brain slices was found to be associated primarily with postsynaptic membranes, a very large proportion of enzyme activity had been lost during preparation of the slices for assay [25]. Histochemical localization of

these enzymes or of adenine phosphoribosyl transferase, the enzyme responsible for incorporation of radioactive adenine specifically in cyclic AMP-generating compartments, would seem deserving of further effort. Histological localization of cyclic AMP by immunofluorescent assay has also been used to attempt to define the sites of cyclic AMP generation in brain tissue [26].

A variety of evidence suggests that the major cyclic AMP-generating compartments in brain slices are not associated with pre-synaptic terminals: (a) the accumulations of cyclic AMP elicited by norepinephrine or other agents are not decreased when the noradrenergic pre-synaptic terminals are destroyed with 6-hydroxydopamine [27–30] and (b) adenosine to a greater extent than adenine preferentially labels the pre-synaptic adenine nucleotides of "synaptosomes" [31]. The converse (adenine > adenosine) is true with respect to specificity of labeling of the nucleotides of the cyclic AMP-generating compartments [22]. Such results suggest that the adenylate cyclase activity of "synaptosomes" (see above) might in large measure be associated with the post-synaptic membrane which still adheres to the pre-synaptic terminal. Such terminals in brain slices often "innervate" dendritic processes and cell bodies of neurons.

It is obvious that the heterogeneity and complexity of brain slice have impeded progress in defining the nature of the compartments associated with cyclic AMP generation in such systems. Agents or treatments with selective effects on viability of neuronal or glial cells or on levels of ATP in such cells might be profitably used to investigate the relevancy of such cell types to accumulations of cyclic AMP. Such protocols might include anoxia, X-ray irradiation, specific neurotoxins, or metabolic inhibitors. Neurotoxins such as veratridine and batrachotoxin reduce ATP levels in brain slices, and elicit a concomitant accumulation of cyclic AMP, probably via a depolarization-linked release of adenosine from neuronal structures [32–35]. One interpretation of these results is that different neuronal sites are depolarized and depleted of ATP at different rates and that the site containing the cyclic AMP compartment may be relatively resistant to depolarization by such neurotoxins. Conversely, depolarization of neuronal structures might release adenosine and other compounds to elicit accumulations of cyclic AMP in glial structures. Exposure of brain slices to high concentrations of potassium ions has somewhat selective effects on metabolism of glial cells [36], reduces total ATP levels and will in slices from certain brain regions elicit an accumulation of cyclic AMP [24, 37]. High concentrations of potassium ions, however, are not without effects on neurons. It is obvious that more selective agents are needed before this type of approach can be expected to yield meaningful results.

Cultured cells. In an attempt to provide a simpler model system relevant to brain, many researchers have initiated studies with cell lines derived from various neuromas [38–44]. These include cells from neuroblastomas and cells from various gliomas, primarily astrocytomas. The results are of some interest, but it is well known that tumor cell lines often show greatly altered enzyme levels or hormone responsiveness compared to those of the parent tumor cell or to those of normal cells. Hence, results with neuroma cells cannot be readily extrapolated to the intact functional brain tissue. In certain glioma cell lines, norepinephrine and other β-agonists, histamine, adenosine and prostaglandin elicit accumulations of cyclic AMP. In neuroblastoma cells, adenosine and prostaglandin elicit accumulations of cyclic AMP, while biogenic amines have no effect.

More relevant to the responses of normal cells in brain tissues are results obtained with cultured fetal brain cells. In reaggregation cultures, which probably contain proportions of neurons and glia similar to those of immature brain, norepinephrine elicits a modest accumulation of cyclic AMP [45]. In surface cultures, where extensive multiplication of cells, presumably mainly of glial cells, has taken place, norepinephrine elicits a much greater accumulation of cyclic AMP [46]. Further studies with such cultures should provide invaluable information relevant to the types of cells which respond to different hormones in brain tissue. It is of interest that, in brain slices obtained from animals during maturation of the central nervous system, responses of cyclic AMP-generating systems to biogenic amines reach an early maximum perhaps prior to formation of synapses and then decline to adult levels [47–50]. Such factors should be considered when interpreting the results obtained with cultures of fetal brain cells.

Intact brain. The pioneering investigations by Siggins *et al.* [51] have provided a key insight into the function of cyclic AMP in the central nervous system. This group has studied the effects of exogenous cyclic AMP and of agents known to stimulate its formation or block its degradation on the electrical activity of individual neurons in the central nervous system. Such studies have firmly established that norepinephrine released from pre-synaptic terminals causes a inhibitory hyperpolarization of cerebellar Purkinje cells. Furthermore, in many experiments exogenous cyclic AMP and analogs were shown to mimic the inhibitory effects of norepinephrine on these and other neuronal cells. The latter results have been challenged by others [52] who could not reproduce the inhibitory effects of the cyclic nucleotides. One of the most convincing pieces of evidence for the involvement of cyclic AMP in the effect of norepinephrine on Purkinje cells has, however, not been challenged. This is the observation using an immunofluorescent assay for cyclic AMP, that norepinephrine on topical application to cerebellum elicits a specific and pronounced increase in levels of cyclic AMP in Purkinje cells [26]. Immunofluorescent assay of cyclic AMP in other brain preparations after treatment with various stimulatory agents might be expected to provide further definition of the sites of formation of cyclic AMP in brain.

An inhibitory role for cyclic AMP has also been established in a peripheral system, the superior cervical ganglion. In that structure, release of dopamine from small interneurons results in activation of dopamine-sensitive adenylate cyclases in the noradrenergic cell-body and a subsequent inhibitory hyperpolarization of the membrane [53].

CONCLUSIONS

The cumulative evidence provided by many groups of scientists working with different types of brain preparations strongly implicates cyclic AMP in post-synaptic events, governing neuronal interactions and spontaneous neuronal activity in the central nervous system. Such evidence does not preclude a role for cyclic AMP in neurone–glia interations.

Attempts to correlate cyclic AMP-dependent mechanisms with the physiology and pharmacology of the central nervous system have paralled the investigation of the sites of cyclic AMP formation in brain tissue. For example, the behavioural effects of intraventricular cyclic nucleotides have been documented [54], as well as the anti-anxiety effect of parenterally administered phosphodiesterase inhibitors [55]. Unfortunately, much more sophisticated approaches are needed to establish specific roles for cyclic AMP in the integrated function of the central nervous system. Attempts to correlate responsiveness of specific cyclase systems with spontaneous and drug-elicited behavioral parameters represent one such approach. Two examples of such studies might be mentioned: (a) the striking correlation of responses of norepinephrine-sensitive cyclic AMP-generating systems in cortex and midbrain-striatum with spontaneous motor activity in various strains of rats [56], and (b) the equally striking correlation of responses of norepinephrine-sensitive cyclic AMP-generating systems in brain with the degree of hyperactivity in chronically reserpinized rats [57]. Since interruption of synaptic input results in a compensatory hypersensitivity of responses of cyclic AMP-generating systems [27–30] lesioning of specific brain tracts, followed by assay of responses of cyclic AMP-generating systems in slices from regions innervated by such tracts, should provide evidence as to the role of cyclic AMP in many pathways in the brain. Such approaches would appear to indicate productive directions for research on the physiological and pharmacological roles of cyclic AMP in the central nervous system.

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