*CYCLIC ADENOSINE MONOPHOSPHATE, CA^{++}, AND MEMBRANES**

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Since the initial discovery of cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) by Rall, Sutherland, and Berthet[1] and its role in glycogenolysis in the liver,[2] evidence has accumulated indicating that this nucleotide is an intermediate in the action of many peptide hormones.[3–9] More recently it has also been implicated in the release of insulin from the pancreas,[10] the release of amylase from the salivary gland,[11] and the action of estrogen on the uterus.[12]

As this evidence has accumulated, no satisfactory theory has developed to explain the cellular or molecular basis of these apparently diverse effects of 3′,5′-AMP. Initially, it was thought that in the case of both epinephrine-induced glycolysis[2] and adrenocorticotropin (ACTH)-induced steroid release from the adrenal cortex[3] the effect of cyclic AMP was confined to an activation of phosphorylase. However, as this nucleotide was identified as an intermediate in the epinephrine-induced lipolysis in adipose tissue[5] and the vasopressin-induced change in water permeability in the toad bladder[6] it became clear that an effect upon the single enzyme, phosphorylase, was insufficient to account for all of the nucleotide's effects. Even in the case of ACTH action, it soon became apparent that cyclic AMP must have effects upon adrenal cell metabolism other than an activation of phosphorylase.[4] Nonetheless, the notion persisted that its major effects were upon enzymes, particularly when it was discovered that it activated phosphofructokinase in certain tissues.[4]

Thus considerable credence was given to the notion that cyclic AMP acted as a modifier of the activities of one or more enzymes in a particular cell.[13] The specificity of its particular effects upon a given cell was explained as being due to the uniqueness of each particular cell type in regard to its enzymatic composition. If this were the case, it would mean that the search for the role of cyclic AMP in each particular cell type would be a separate problem in biochemical investigation. The alternative would be that cyclic AMP has a different function, but that investigative attention as to its site of action had been misdirected.

An alternative mechanism was first proposed when we noted an apparent relationship between 3′,5′-AMP and Ca^{++} in the action of vasopressin on the toad bladder and several other systems.[14, 15] The possible importance of this relationship was further emphasized by the discovery that adenyl cyclase, the enzyme responsible for the conversion of adenosine-5′-triphosphate (ATP) to 3′,5′-AMP, and phosphodiesterase, the enzyme involved in converting 3′,5′-AMP to AMP, were present in high concentrations in the brain,[16, 17] and appeared to be localized primarily in the synaptosome portion of brain homogenates.[18] This discovery was followed by the reports that cyclic AMP also appeared to be an intermediate in the secretion of amylase by the parotid gland[11] and in the secretion of insulin by the β cells of the pancreatic islets.[10] These apparently diverse systems have several striking similarities. A consideration of these common

features leads to a very simple and unifying concept as to one major role of cyclic AMP in cellular activity.

The most important features of these systems can be summarized by comparing the properties of synaptic transmission and the release of insulin from the pancreas. Morphologically, the feature of paramount importance is that in both instances the substance to be released is stored in the respective cell in small membrane-bounded vesicles which, under an appropriate stimulus, fuse with the plasma membrane on the cell surface and discharge their contents extracellularly.[19, 20]

Functionally, the responses are characterized by the following features: (1) 3′,5′-AMP is the probable intermediate between stimulus and release; (2) Ca^{++} is required in the external medium in order to make the appropriate stimulus effective; (3) an elevated Mg^{++} in the external medium inhibits the response; (4) a high concentration of K^+ in the external medium can function as a nonspecific stimulus for release of either substance; (5) this effect of K^+ is also dependent upon the presence of Ca^{++} in the external medium; and (6) ouabain can stimulate either process.[19, 21–25] Less extensive data on amylase secretion by the parotid and pancreas leads to the conclusion that its morphologic and functional characteristics are similar to those enumerated above.[11, 26–31]

These data suggest that an important cellular function of cyclic AMP is that of regulating cellular secretory activity whether it be a macrosecretion from an exocrine gland, a microsecretion from an endocrine gland, or a transmitter release from a nerve ending. The clear implication is (1) that all these processes are controlled in an identical fashion after the initial stimulus, (2) that 3′,5′-AMP and Ca^{++} are the key elements in this process, and (3) that their effects are upon membrane structure and function.

The nature of the changes in membrane structure produced by 3′,5′-AMP and Ca^{++} are not known. Recent considerations of nerve membrane structure have led to the proposal that the membrane can exist in two stable states, the open and the closed,[32–37] which can be converted from one to the other by the appropriate stimulus. Two components, Ca^{++} and ATP, are considered to play key roles in maintaining membrane structure, and the conversion of the membrane from one state to the other is thought to involve a transition from a calcium-associated to a calcium-dissociated state. The question which has not yet been resolved is whether the calcium in the calcium-associated state is coordinated solely to polyphosphates such as ATP, or whether it is also coordinated to macromolecular components as well. In all cases, however, it is assumed that the ATP-Ca complex has a key role in determining membrane structure and function. From this it becomes immediately apparent that the conversion of ATP to 3′,5′-AMP within the membrane converts the adenine nucleotide from a strong chelator of Ca^{++} to an extremely weak one, thereby leading either to the release of Ca^{++} or to the possible complexing of Ca^{++} by other groups, e.g. phospholipids, in the membrane. This would explain why adenyl cyclase is of necessity a membrane-bound enzyme whereas phosphodiesterase is found in the soluble fraction of the cell. This implies that a small pool of membrane-bound ATP is the immediate substrate for adenyl cyclase, and

also accounts for the fact that 3′,5′-AMP is the product rather than 5′-AMP, even though the latter is also a poor chelator of Ca^{++}.[38] In addition, because 5′-AMP is a product of many intracellular reactions as well as being readily rephosphorylated to ADP and then to ATP, the regulation of its concentration in the cell would be difficult. The 3′,5′-AMP on the other hand is quite stable and does not participate in other cellular reactions, either as substrate or product; hence, once formed, it is excluded from the general adenine nucleotide pool. In order to re-enter this pool it must first be hydrolyzed by a specific phosphodiesterase. This phosphodiesterase is found in all cells in which adenyl cyclase is present[4] and is found in particularly high concentrations at the synapse.[17] Thus in this organ there exists the enzymatic capacity to form and destroy rapidly large amounts of cyclic AMP, a biochemical system admirably suited for the control of transmitter release. A similar function for the adenyl cyclase system in neuromuscular transmission has recently been proposed.[39]

In order to understand more fully the relationship between the function of 3′,5′-AMP and the function of Ca^{++}, as well as the influences of high extracellular K^+ and Mg^{++} upon cellular secretion, three questions needed to be answered: (1) In the presence of high external K^+, is cyclic AMP formation increased? (2) In the absence of extracellular Ca^{++}, is cyclic AMP produced by the normal stimulus? (3) In the presence of high external Mg^{++}, is the production of 3′,5′-AMP normal? Answers to these questions were sought in a system employing rat parotid gland slices. It was found that high external K^+ (60 mM) led to an increase in 3′,5′-AMP, as did epinephrine in a low K^+ environment (Table 1). It was also found that in the absence of Ca^{++} or presence of high Mg^{++}, epinephrine induced an increased 3′,5′-AMP, even though amylase secretion was inhibited.[40]

Other evidence indicates that 3′,5′-AMP or its dibutyryl analogue act in these systems at a high enough concentration to bring about the particular physiologic effect,[11] and furthermore that the injection of Ca^{++} alone in the presynaptic terminus does not lead to transmitter release.[41] Thus 3′,5′-AMP must also play an active intracellular role.

It is now generally accepted that an increase of Ca^{++} in the cytosol of muscle cells is the determinant of muscular contraction.[42] Contraction is initiated when free Ca^{++} rises above 10^{-7} M, and the strength of contraction is related to the amount of calcium released. In the heart, epinephrine has been shown to produce a positive inotropic response as well as a stimulation of glycolysis.[2]

TABLE 1. *Effect of changes in the concentrations of extracellular ions upon cyclic AMP production in rat parotid gland slices.*

Additions	3′,5′-AMP (μmoles $\times$ 10^{-5}/100 μg DNA)		
	Control	No Ca	10 mM Mg^{++}
	67.2	80.3	54.0
Epinephrine	240.0	300.0	581.0
K^+	196.4	291.0	

The normal incubation medium was Krebs-Ringer bicarbonate. When incubation was carried out in 60 mM KCl, an equivalent amount of NaCl was removed to maintain isomolarity. Epinephrine was added at a concentration of 5×10^{-5} M. Cyclic AMP was determined by a modification of the Breckenridge method.[39]

The latter effect has been attributed to the activation of phosphorylase-b-kinase by the 3′,5′-AMP produced,[2] but this hypothesis does not account for the positive inotropic effect. Ozawa and Ebashi[43] have recently shown that purified muscle phosphorylase-b-kinase is activated by Ca^{++} in the range between 5×10^{-6} and 1×10^{-5} *M*, but that 3×10^{-6} *M* 3′,5′-AMP does not activate in the absence of Ca^{++}, and its effects in the presence of Ca^{++} represent only a 20 per cent greater stimulation of activity than that seen with Ca^{++} alone. Thus, the important physiologic activator of this enzyme may well be Ca^{++} and not 3′,5′-AMP. In this view, the role of 3′,5′-AMP is that of altering the permeability of cellular membranes to Ca^{++}. This single effect could account for both the positive inotropic effect of epinephrine and the effects of epinephrine upon carbohydrate metabolism.

This interpretation might also account for the effects of caffeine upon muscular contraction.[42] In low concentrations (0.5–1.0 mM), this drug potentiates the muscle twitch and prolongs the active state, but at higher concentrations leads first to reversible contractures, and eventually, above 5 mM, to irreversible contractures. These effects have been attributed to changes in calcium release and/or reaccumulation, and have generally been thought to represent a direct effect of caffeine on the membrane. However, an alternative explanation is that caffeine acts, at least in low concentrations, by increasing 3′,5′-AMP levels, because this drug is known to inhibit the phosphodiesterase and it has been shown to potentiate the inotropic effects of norepinephrine on the heart.[44]

An association of 3′,5′-AMP and Ca^{++} is also apparent in a variety of other situations: (1) vasopressin action upon the toad bladder;[6, 45] (2) melanin-stimulating hormone action upon melanophores;[9, 46] (3) the effect of ACTH action upon the adrenal cortex;[12, 47] (4) the activation of lipase in adipose tissue;[5] and (5) phosphofructokinase in muscle.[48] In all these circumstances, much more precise data is needed before these relationships can be completely defined. Nevertheless, a case can be made in each of these instances for the regulation of these events by alterations in the level of free Ca^{++} in the cytosol of these cells.

Of particular interest is the possible relationship of glucocorticoids, 3′,5′-AMP, and Ca^{++} in the regulation of gluconeogenesis in liver and of lipolysis in adipose tissue. In both instances, glucocorticoids have been shown to play a permissive role in the physiologic response.[49, 50] Thus, adipose tissue from adrenalectomized animals does not respond normally to epinephrine. Also, if livers are obtained from adrenalectomized animals and perfused *in vitro* with glucagon, there is no increase in gluconeogenesis even though there is a normal rise in 3′,5′-AMP.[50] This result would argue that the lack of response is not due to the production of 3′,5′-AMP, but is due to a lack of the normal intracellular response to this nucleotide. It is possible that a redistribution of intracellular Ca^{++} is involved.

One of the difficulties in attempting to define the mechanism of action of the glucocorticoids has been the fact that these hormones exert effects upon a variety of apparently unrelated systems. Of particular interest to the present discussion, is the fact that glucocorticoids have striking effects upon calcium transport in the intestine[51] and probably bone, and equally striking effects upon gluconeo-

genesis in the liver.[52] It has been difficult to envision a common denominator which could account for these effects upon two such apparently unrelated systems. However, recent studies have shown that corticoid administration decreases the ability of rat liver mitochondria to accumulate and retain calcium *in vitro*,[53] and that in mitochondria from such animals, the pyruvate carboxylase activity is enhanced at any given level of external calcium between 2 and 50 μM.[54] Thus, corticoids apparently act upon intracellular membranes to alter their ability to handle calcium and the resultant redistribution of calcium can change the activity of key ion-sensitive enzymes, e.g. pyruvate carboxylase in the mitochondria and pyruvate kinase in the cytosol. These changes could lead to enhanced gluconeogenesis. They could also account for changes in the transcellular transport of calcium in another tissue, intestinal mucosa. Of particular relevance, the absence of corticoids would lead to a state in which calcium mobilization or redistribution by cyclic AMP would not be effective. Hence the enhanced production of 3′,5′-AMP brought about by glucagon in the liver of the adrenalectomized animal would be ineffective in stimulating gluconeogenesis. A similar mechanism could account for the lack of epinephrine-induced lipolysis in the adipose tissue from adrenalectomized rats.[48]

Equally well explained by this hypothesis are the effects of parathyroid hormone upon calcium transport in kidney, intestine, and bone. As first shown by Chase and Aurbach, parathyroid hormone increases the urinary excretion of 3′,5′-AMP when given to a parathyroidectomized animal.[7] We have extended these observations and have shown that dibutyryl 3′,5′-AMP mimics the effect of parathyroid hormone upon both kidney and bone[55] and that within one minute of infusing parathyroid hormone there is a dramatic increase in 3′5′-AMP in renal tissue (Table 2). Of particular importance, this latter change is associated

TABLE 2. *Effect of parathyroid hormone infusion upon renal 3′,5′-AMP, α-ketoglutarate, and isocitrate concentrations in parathyroidectomized rats.*

	3′,5′-AMP (10^{-11} moles/gm wet wt)	αKG (10^{-9} moles/gm wet wt)	ISC (10^{-9} moles/gm wet wt)	αKG/ISC
Control	10.5 ± 1.1	196 ± 5	23.1 ± 0.4	8.48
1 min after 5 μg PTH	28.2 ± 5.7	187 ± 6	30.0 ± 2.2	6.23
6 min after 5 μg PTH	17.0 ± 2.6	179 ± 7	32.2 ± 4.6	5.56

Parathyroidectomized rats were anesthesized with Dial with urethane and then given parathyroid hormone by intravenous infusion into the femoral vein. After the appropriate time interval, the left kidney was removed and quick-frozen. The Krebs cycle intermediates and 3′,5′-AMP were measured by fluorometric methods, using coupled enzyme systems upon suitable acid extracts of the renal tissue.[55]

with an equally prompt increase in intracellular ionized calcium as shown by a prompt, specific and significant inhibition of isocitric dehydrogenase (Table 2), an enzyme which is inhibited by calcium.[55] Hence in this situation there is a prompt rise in intracellular 3′,5′-AMP and ionized Ca^{++}. In this particular tissue, these changes are associated with an increased transcellular transport of Ca^{++} because of the polarity of these epithelial cells.

This thesis means that Ca^{++} is involved as an integrator of metabolic events in cells other than muscle,[42] nerve,[35] and those of secretory glands,[29] and that

3′,5′-AMP is an important regulator of the permeability of cellular membranes to Ca^{++} or of the binding of this ion to membranes. This is an extension of the thesis of Heilbrunn, who proposed that Ca^{++} plays an important regulatory role in cellular economy.[56]

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