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ROLE OF THE cAMP-DEPENDENT PROTEIN KINASE AS *THE* TRANSDUCER OF cAMP ACTION

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Substantial evidence, that has been accumulated over a decade and a half of intensive investigation, permits us to support, without reservation, the second messenger hypothesis of cAMP as elegantly propounded in 1960 by Sutherland and Rall [1]. Lexicographers would argue correctly that this should now be called a theory, not an hypothesis, since it no longer falls within the category of "a tentative assumption made in order to draw out and test its logical or empirical consequences" but is indeed a "set of facts." Thus, cAMP is a major, although importantly not the sole, intracellular mediator of a wide range of peptide hormones. In studies subsequent to Sutherland's original postulate, Walsh *et al.* [2], utilizing skeletal muscle glycogenolysis as a model system, discovered that a primary mechanism of cAMP action was through the activation of a (the) cAMP-dependent protein kinase. Experimentation spearheaded by Kuo and Greengard [3] demonstrated the presence of this enzyme in a broad, if not the total, spectrum of mammalian tissues. Although the hormonal regulation of glycogenolysis is a feature of essentially all eukaryotic cell types, the breadth of the postulate of cAMP action, as mediated by the activation of protein kinase, has since been supported by the identification of a wide range of phosphoproteins that serve as substrates for this enzyme. Physico-chemical studies elucidated that the cAMP-dependent protein kinase contained two types of subunits designated R (i.e. regulatory) and C (i.e. catalytic). Free C subunit exhibits full catalytic capacity to catalyze the phosphorylation of proteins. The R subunit is inhibitory when bound to C in the absence of cAMP, but this inhibition is released by the addition of the cyclic nucleotide, and R, with cAMP bound, can be separated from C by physicochemical techniques.

On the basis of these data, as provided by many laboratories, there has been derived the often quoted mechanism of cAMP action as presented in Fig. 1.

This commentary presents questions about this mechanism, queries which are designed to emphasize the current level of our knowledge of this system.

Question. In mammalian systems is the cAMP-dependent protein kinase *the* (i.e. only) transducer of cAMP action?

Commentary. cAMP is known to mediate a broad spectrum of physiological events which include, in a far from complete list, such diverse actions as the regulation of metabolism, contraction, secretion, macromolecular synthesis and degradation, ion transport, platelet aggregation, melanocyte dispersion, and the regulation of cell growth and the passage of cells through the cell cycle. The list of potential

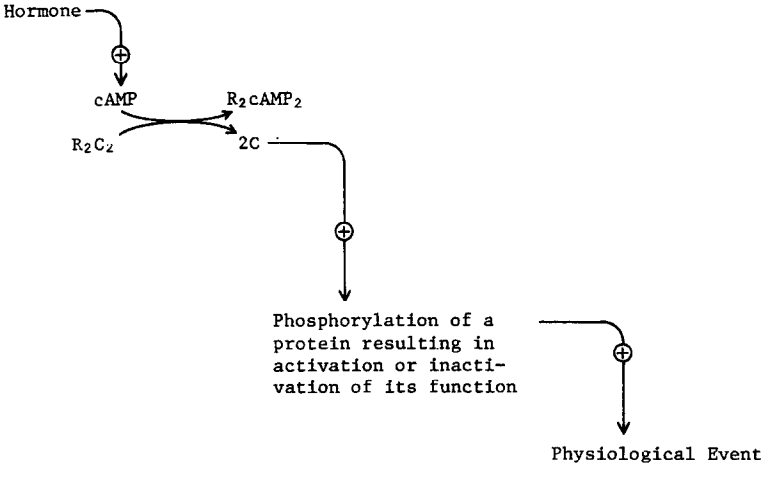

Fig. 1. General mechanism of cAMP action.

proteins that serve *in vitro* as substrates for the cAMP-dependent protein kinase is now substantial, and their phosphorylation *in vivo* may well account for many if not all of these physiological events regulated by cAMP. It should be recognized, however, that but few of these protein phosphorylations have as yet been shown to meet the, albeit stringent, criteria that have been defined in publications from this Department [4, 5] which should be required to establish that a physiological event is mediated by a cAMP-dependent protein phosphorylation. Thus, for many proteins for which covalent modification has been shown to result in altered function *in vitro*, neither altered function nor the corollary protein phosphorylation has been demonstrated in an intact cell system. Alternatively, for many other proteins, where the phosphorylation of a protein has been detected *in vivo* the function of such phosphorylation remains to be established. In consequence, it is not yet possible to be assured that the phosphorylation of specific proteins can account for all the myriad of physiological events that cAMP is known to mediate.

An alternate approach that can be used to determine whether the cAMP-dependent protein kinase is *the* transducer of cAMP action is to evaluate the potential diversity of cAMP-binding macromolecules. Such an approach is based solely upon the reasonable physicochemical assumption that for any mechanism of cAMP action there must exist a macromolecular cyclic nucleotide binding site. Unfortunately this approach is also fraught with difficulties. Detection of the binding of cAMP to potential receptor proteins is not simple. In 1970, shortly after the discovery of the protein kinase, Gilman [6] described the technique of determining the cAMP-binding component of the protein kinase based on the adsorption of the protein to Millipore membranes. This method, which has been used extensively because it is readily applicable for the determination of a large number of samples, is limited to the detection of only those cAMP-binding proteins which bind to nitrocellulose. This is a property of the cAMP-binding regulatory subunit, but there is no reason to assume that other cAMP-binding proteins would be similarly adsorbed. The Gilman procedure is also limited to the detection of only those binding species for which the rate of release of cAMP from the binding macromolecule is so slow that it permits the removal of unbound cyclic nucleotide ligand from the environment of the protein without significantly decreasing the amount of protein-bound ligand during the time period required for separation of bound and free ligand. The measurement of binding of cAMP to the protein kinase has a unique advantage in that the removal of C produces a system in which the reaction for the release of cAMP from the protein (i.e. reaction 1) is not the reverse of the reaction by which the cyclic nucleotide became bound (i.e. reaction 2).

$$RcAMP \rightarrow R + cAMP \qquad \text{(reaction 1)}$$
$$RcAMP + C \rightarrow RC + cAMP \qquad \text{(reaction 2)}$$

Procedures for measuring cAMP binding proteins other than the Gilman method include equilibrium dialysis or the Hummel and Dryer gel filtration technique. Each is superior to the Gilman method because it measures ligand binding under equilibrium conditions, but each is greatly limited by the time required to perform the experiment, thus resulting in substantial problems of phosphodiesterase-catalyzed degradation of cAMP.

Nevertheless, despite the drawbacks which have impeded investigations, three reports have been presented of cAMP-binding proteins which would appear to be distinct from the cAMP-dependent protein kinase. Yuh and Tao [7] and Ueland and Døskeland [8] reported the presence of a protein in rabbit erythrocyte and mouse liver, respectively, that bound cAMP with a dissociation constant (K_d) $= 3 \times 10^{-7}$ M, a value not incompatible with the physiological concentration range of cAMP. Each of these binding proteins was distinguished from the regulatory subunit of the cAMP-dependent protein kinase on the basis of a lack of effect of added catalytic subunit to promote release of bound-cAMP, a stringent criterion. Each of these proteins binds adenosine with a slightly higher affinity ($K_d \sim 1 \times 10^{-7}$ M) than cAMP but the relative affinities and physiological concentration ranges suggest that the cAMP-binding may well be of physiological consequence. Tsuzuki and Kiger [9] have described a cAMP-binding protein in early embryos of *Drosophila* which, although currently less well characterized, may be identical to those proteins from erythrocytes and liver. The function of the cAMP-adenosine-binding proteins is unknown, but credence of their existence would strongly infer that the cAMP-dependent protein kinase is not the *sole* transducer of cAMP action.

Question. What is the mechanism of cAMP activation of the protein kinase? Does a cAMP–R–C ternary complex exist and, if so, does it possess activity?

Commentary. Brostrom *et al.* [10] first presented evidence that the cAMP activation of the protein kinase should be classified as a "V" type system as defined by Monod, Wyman and Changeux, and it was proposed that dissociation of two distinct subunits led to activation of the enzyme. Subsequent extensive studies by many laboratories identified the two subunits and showed that, whereas the holoenzyme (RC) is inactive, free catalytic subunit C is active. However, as recently concluded by Ogez and Segel [11], it cannot be said with certainty whether cAMP binds to RC prior to dissociation of C (reaction 3) or whether cAMP binds only to free R thereby displacing a pre-existing equilibrium (reaction 4).

$$RC + cAMP \rightleftharpoons R \cdot cAMP \cdot C \rightleftharpoons RcAMP + C \qquad \text{(reaction 3)}$$

$$\left.\begin{matrix} RC \\ \\ cAMP \end{matrix}\right\} \begin{matrix} R \\ \\ \end{matrix} + C \rightleftharpoons R \cdot cAMP \qquad \text{(reaction 4)}$$

Determination of which of these mechanisms is correct is crucial to our full physiological understanding of the transducer role of cAMP. The mechanism of reaction 4 encompasses the concepts of cAMP action as currently generally envisaged, but

that of reaction 3 prompts consideration of whether an active ternary complex exists and if so whether dissociation of the protein kinase is a necessary physiological event. Sparked by the investigations of Corbin *et al.* [12], a number of investigators have used the index of

$$\left(\frac{\text{protein kinase activity assayed minus cAMP}}{\text{protein kinase activity assayed plus cAMP}}\right)$$

as a measure of cAMP action in intact cells. This determination *per se* is not a criterion of protein kinase dissociation *in vivo* although consideration of physico-chemical parameters heavily predisposes such an interpretation. The validity of such measurements resides heavily on the ability to maintain protein kinase during extraction in an "activity state" that is identical to that in the intact tissue and not reflective of cAMP levels in the extract, a process that in general has not been adequately controlled. It is of particular interest that recently Lincoln *et al.* [13] have characterized the cGMP-dependent protein kinase from lung and demonstrated that dissociation of subunits is not a characteristic of cyclic nucleotide activation. This enzyme has many characteristics that are similar to the cAMP-dependent protein kinase, prompting the suggestion of teleological identity [14]. The "activation state" of the cGMP-dependent enzyme more closely approximates the ternary complex proposed in reaction 3. The detailed presentation by Ogez and Segel [11] has provided a kinetic approach for the solution of which of the two activation mechanisms (reaction 3 or 4) for the cAMP-dependent protein kinase is correct.

Question. If dissociation of the cAMP-dependent protein kinase is required for activation, does this dissociation also serve some additional function?

Commentary. The classical allosteric type of activation of an enzyme is exquisitely sensitive to fluctuations in free ligand concentrations, and diminished levels of the activators result in virtually immediate cessation of enhanced activity. In contrast, if dissociation of subunits is essential for activation of the protein kinase, then inactivation will not occur immediately after a decrease in cAMP. The question is thus posed as to why this special mechanism has been of selective advantage. One possible explanation is that the system provides a mechanism for the continuation of the signal following triggering of the response independent of the duration of the initial stimulus. Alternatively, dissociation may provide for a separate action of both the subunits. Potentially RcAMP may serve as a regulator of cellular function independent of protein phosphorylation. An attractive possibility, for which, however, no support could be accumulated, is that RcAMP is analogous to the CAP protein that regulates mRNA synthesis in catabolite repression in prokaryotic cells. Dissociation of the protein kinase could also provide a mechanism for the transmission of the cAMP signal to within the subcellular organelles. The stimulation of hormonally sensitive adenyl cyclase, located on the cell membrane, elevates cytosolic cAMP levels. cAMP does not appear to migrate into the subcellular organelles, but some evidence has been presented from this and other laboratories that the catalytic subunit of the protein kinase may be so translocated. This mechanism would provide for a means by which polypeptide hormones could regulate such functions as nuclear genetic transcription.

Question. Within a single cell several substrates exist for the cAMP-dependent protein kinase. What controls the relative priority of substrate phosphorylation after a cAMP signal?

Commentary. Early in the investigation of cAMP-dependent protein kinase it was recognized that a wide spectrum of isozymes did not exist and thus it was reasonably concluded that within a single cell a single enzyme species catalyzed the phosphorylation of several different protein substrates. In general, two isozymes have been shown to exist in mammalian tissues, but the differences between the isozymes reside in the regulatory subunit whereas the catalytic specificity of the two isozymes is identical. It is possible that other isozymes, such as those known to be membrane bound, will be demonstrated. Nevertheless, there exists the situation that is rare, if not unique, of a single enzyme within a cell catalyzing multiple reactions. Recently, as an example, McCullough *et al.* (unpublished data) have demonstrated that the stimulation of a cardiac cell by epinephrine results in the phosphorylation of phosphorylase kinase, troponin and a sarcolemma membrane protein with $T_{\frac{1}{2}}$ values of 5, 8.5 and 20 sec respectively. Under these circumstances glycogen synthetase, an established substrate for the cAMP-dependent protein kinase, is not phosphorylated. What factors determine the substrate priority that exists, therefore, after a cAMP signal? The answer to this question is not known, but in addition to potential differences in kinetic parameters, other regulatory phenomena may well include the translocation and subcellular compartmentalization of the protein kinase catalytic subunit and the feedback inhibition of protein phosphorylation by components of the system regulated by such protein phosphorylation–dephosphorylation.

Question. What is an effective concentration of cAMP?

Commentary. A question that arose early in studies of cAMP-dependent protein kinase was why the apparent effective activation concentration of cAMP *in vitro* was an order of magnitude lower than basal cAMP concentrations within the cell. This problem has now been satisfactorily resolved when appropriate substrate and enzyme concentrations are considered [15]. However, it has been recognized recently that the concentration of cAMP during the proliferative phase of undifferentiated cells is lower than the basal concentration of the differentiated state, and this type of observation has led to the proposal that levels of cAMP are required to be low to permit cell duplication whereas elevated cAMP levels block cell proliferation and enhance differentiation. As now well illustrated for several cell types, fluctuations of cAMP occur during the cell cycle. The range of these fluctuations is modest when compared to those that are produced by the hormonal stimulation of a differentiated cell. A detailed comparison has yet to be made for a single cell

type, but it is a compatible and attractive hypothesis to consider that there are three effective concentration ranges of cAMP. The lowest of these, arbitrarily designated the "proliferative-basal," is that which is required for cellular proliferation and differentiation. Upon and during maturation of the cell, the cAMP level increases to the "differentiated-basal" level. This is a concentration that is sufficient to stop cellular proliferation but insufficient to promote the physiological events typical of the hormonal stimulation of the differentiated cell. The third level of cAMP may be called the "differentiated-activated." This is the concentration that promotes such events as the regulation of glycogen metabolism, secretion and contraction. This concept requires further experimental exploration prior to its validation, but its consideration provokes the question as to whether the processes regulated by cAMP fluctuations during proliferation (i.e. changes between "proliferative-basal" to "differentiated-basal") involve the same transduction mechanism as those regulated by fluctuations in a differentiated cell (i.e. changes between "differentiated-basal" to "differentiated-activated"). For the latter, we can be relatively confident that the regulation of protein phosphorylation is one, if not the sole, fundamental mechanism; however, if the concentration range is different for the two types of processes, then quite possibly a different mechanism should be envisaged by which cAMP regulates cellular growth. We have shown that many cells contain an inhibitor protein for the cAMP-dependent protein kinase sufficient to block approximately 20 per cent of the activity of the cAMP-dependent protein kinase in the cell [5]. The presence of this inhibitor could provide a means to establish a threshold of cAMP concentration below which modulation in cAMP would not effect protein phosphorylation. Fluctuation in cAMP beneath this threshold may thus be involved in the regulation of processes other than via protein phosphorylation and would be a suitable candidate for those occurring between "proliferative-basal" and "differentiated-basal," i.e. cellular growth.

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