THE ROLE OF CYCLIC AMP AND CALCIUM IN CELL ACTIVATION

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INTRODUCTION

Following its discovery as an intermediate in the action of epinephrine upon hepatic glycogenolysis (Sutherland and Rall, 1958, 1960), adenosine 3′:5′ cyclic monophosphate (cAMP) has been found in nearly all types of animal and bacterial cells (Robison, Butcher, and Sutherland, 1971). Its most widely recognized role is that of serving as a *second messenger* in the activation of many different cell types by specific first messengers, particularly peptide and amine hormones (Sutherland, ϕye, and Butcher, 1965). It may also play a role in other types of cell activation including synaptic transmission, exocrine secretion, and endocrine secretion (Robison, Butcher, and Sutherland, 1968; Robison et al., 1971).

Various aspects of this field have been reviewed extensively in the past several years (Robison et al., 1968; Robison et al., 1971; Garren, Gill, Masui, and Walton, 1971; Rasmussen, 1970a, 1971; Larner, Villar-Palasi, Goldberg, Bishop, Huijing, Wenger, Sasko, and Brown, 1968; Robison, Exton, Park, and Sutherland, 1967; Sutherland and Robison, 1966; Sutherland et al., 1968). This review will be confined to a consideration of the mode of action of cAMP in animal cells. Our discussion will be centered around an evaluation of the second messenger hypothesis since this hypothesis is the most widely held theory concerned with the role of cAMP in cell activation (Sutherland et al., 1965).

THE SECOND MESSENGER HYPOTHESIS

As originally proposed by Sutherland et al. (1965), the second messenger hypothesis can be stated as follows: when a specific first or extracellular messenger interacts with its specific receptor site on the cell surface, this messenger-receptor interaction leads to the activation of the membrane-bound enzyme, adenyl cyclase (Figure 1), resulting in an increase within the cell of the product of this reaction, cAMP. This serves in turn as a *second or intracellular messenger,* activating the cell to carry out its specific function(s). In subsequent discussions of this hypothesis, additional features have been added. In the first place, a general assumption has been made that the first messenger receptor site on the cell membrane is an integral part of, or closely coupled with, the

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adenyl cyclase enzyme. Secondly, in the most recent versions of this hypothesis, the primary proposed effect of cyclic AMP within the cell (Figure 1) is that of activating a class of enzymes known as protein kinases (Walsh, Perkins, and Krebs, 1968; Kuo and Greengard, 1969; Exton, Robison, Sutherland, and Park, 1971). These protein kinases have the general property of using proteins and ATP as substrate and catalyzing their conversion to ADP and a phosphoprotein.

An additional component of this control system is the enzyme phosphodiesterase (Sutherland and Rall, 1960), which catalyzes the hydrolysis of cyclic 3′5′ AMP to 5′ AMP (Figure 1). With this component and the observation that certain methyl xanthines (caffeine and theophylline in particular) inhibit its activity (Butcher and Sutherland, 1962), a set of criteria was developed (Sutherland et al., 1965; Sutherland and Robison, 1966) to be used as a guide for determining whether or not cyclic AMP served as a specific second messenger in the process of cell activation by particular first messengers.

These criteria included 1) an increase in the intracellular concentration of cAMP in response to a specific first messenger; 2) the ability of exogenous cyclic AMP, or its dibutyryl analog, to mimic the effects of the specific first messenger; 3) the ability of methyl xanthines either to mimic or potentiate the action of the first messenger; and 4) more recently, the presence of a cyclic AMP-dependent protein kinase in the tissue.

In applying these criteria and this model of cell activation, many investigators have made the following additional assumptions: 1) the only important biochemical or cellular effect of methyl xanthines is mediated by an inhibition of phosphodiesterase; 2) large concentrations of either cAMP or dibutyryl cAMP in the extracellular fluids are equivalent to small concentrations of internally synthesized cAMP; i.e., they truly do mimic the effects of first messenger; 3) protein kinase activation accounts for nearly all the effects of cAMP within the cell; 4) first messenger receptors and adenyl cyclase are synonymous; 5) cAMP is probably the sole second messenger when a cell is activated by its specific first messenger; 6) adenyl cyclase is located in the plasma membrane of the cell; and 7) when adenyl cyclase activation is blocked, all aspects of the physiological response are inhibited.

A survey of nearly any specific system in which cAMP has been implicated serves to demonstrate that many of these criteria and assumptions are not fulfilled. In many cases it can be shown that the addition of first messenger to an isolated system under a particular set of incubation conditions, e.g., lack of external calcium leads to an

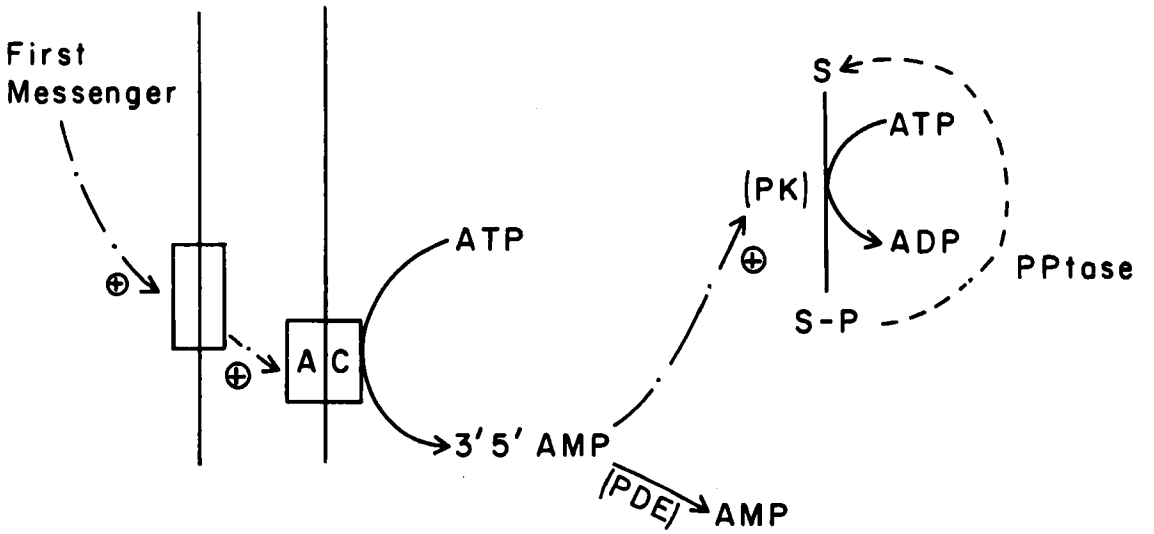


FIGURE 1. A schematic representation of the second messenger hypothesis in which first messenger interacts with the adenyl cyclase on the cell surface leading thereby to an increase in the concentration of the second messenger, cyclic AMP, within the cell. The second messenger brings about its physiological effect by activating one or more protein kinases. The concentration of cAMP is also controlled by its rate of hydrolysis, catalyzed by the enzyme phosphodiesterase (PDE). The concentration of the phosphorylated protein is determined both by protein kinase activity, and by the activity of phosphoprotein phosphatase (PPtase).

activation of adenyl cyclase, but, in spite of the increase in cAMP, no subsequent physiological response is observed (Rasmussen, 1970a, 1971). As a corollary, in many systems there is no simple relationship between the magnitude of the change in cAMP concentration and the magnitude of the observed physiological response (Schaeffer, Chenworth, and Dunn, 1969a,b; Miller, Exton, and Park, 1971; Kurokawa and Rasmussen, 1972).

There is clear evidence that exogenous cAMP and DBcAMP do not always produce the same response in the same tissue (Hilz and Tarnowski, 1970; Robison et al., 1971) and that they may have biphasic effects, stimulating a response at low concentration, but inhibiting it at higher concentrations (Swislocki, 1970; Wells and Lloyd, 1969). Also, it is clear that the addition of exogenous cAMP often does not mimic all the effects of the particular first messenger in a cellular system (Rasmussen and Nagata, 1970; Prince, Berridge, and Rasmussen, 1972).

A particular difficulty with interpreting the effects of dibutyryl cAMP upon cellular responses is that this analog does not activate isolated cAMP-dependent protein kinases (Miyamoto, Kuo, and Greengard, 1969). This being so, it is clear that addition of DBcAMP to an isolated system does not act directly as a more lipid-soluble cAMP, and, if protein kinases are major receptors for cAMP within the cell, then DBcAMP must influence their activity indirectly, possibly by inhibiting phosphodiesterase (Heersche, Fedak, and Aurbach, 1971).

Methyl xanthines induce significant changes in cellular metabolism which cannot be explained by an inhibition of phosphodiesterase (Bianchi, 1972; Corvallo, 1968; Bieck, Stock, and Westermann, 1968; DeWulf and Hers, 1968; Kakiuchi, Rall, and McIlvain, 1969). In particular, these drugs have been shown to alter calcium binding capacity of the isolated sarcoplasmic reticulum from both cardiac and skeletal muscle (Weber and Herz, 1968; Carvalho, 1968; Weber, 1968). This raises the distinct possibility that some of the effects of methyl xanthines in other tissues may be mediated by their effects upon cellular calcium metabolism rather than, or in addition to, effects upon phosphodiesterase.

It is generally accepted that adenyl cyclase is solely a component of the plasma membrane of the cell. This is most clearly established in the case of nucleated (Klainer, Chi, Friedberg, Rall, and Sutherland, 1962; Davoren and Sutherland, 1963) and nonnucleated (Shephard and Burghardt, 1969a,b) red cells and in liver parenchymal cells (Pohl, Birnbaumer, and Rodbell, 1969; Reik, Pelzold, Higgins, Greengard, and Barnett, 1970; Sweat and Hupka, 1971). However, there is increasing evidence that adenyl cyclase is present in the sarcoplasmic reticulum of cardiac and skeletal muscle (Rabinowitz, DeSalbs, Meisker, and Lorand, 1965; Entman, Levey, and Epstein, 1969a) and in the nuclear membrane of some cells (Soifer and Hechter, 1971; Liao, Lin, and Tymoczko, 1971). In all these cases, it is difficult to exclude completely the possibility that the respective preparations are contaminated with plasma membrane fragments.

These examples serve to illustrate some of the problems encountered when the second messenger hypothesis, as originally conceived, is applied to events in specific tissues. In addition to these problems, there are three other questions which require specific discussion. These are 1) is the first messenger receptor identical with adenyl cyclase; 2) is cAMP usually the sole second messenger; and 3) does protein kinase activation account for all the physiological effects of cAMP?

IDENTITY OF FIRST MESSENGER RECEPTOR AND ADENYL CYCLASE

There are two parts to this question: do first messengers, such as peptide and amine hormones, act solely on the cell surface; and, if so, do they act only by interacting with adenyl cyclase? Neither can be answered completely. Several peptide hormones, including insulin (Bessman, 1966) and parathyroid hormone (Rasmussen, Shirasu, Ogata, and Hawker, 1967; Goodman and Rasmussen, 1968), have been shown to have rather specific effects upon the function of isolated mitochondria. In each case, such evidence has, at one time, been interpreted in favor of the viewpoint that the respective hormone exerts some of its effects by altering the function of this subcellular membrane. However, the problem has been one of demonstrating that the particular hormone exerts the same effect upon this subcellular membrane when it is added to intact cells. The most convincing negative evidence is the study with sepharose-conjugated insulin. In this case, insulin which has been covalently linked to a large polymer, sepharose, still exerts its characteristic

biochemical effects on isolated cells (Cuatrecasas, 1969). The presumption is that the conjugated insulin does not enter the cell. However, even in this case, there has not been a complete analysis of the effect of this hormone-conjugate upon all metabolic parameters known to be influenced by insulin. Hence, it remains possible that certain metabolic effects of insulin will not be seen following sepharose-insulin administration. Until such time, however, it seems safer to conclude that the effects of insulin and possibly other peptide hormones are mediated by specific interactions of these hormones with receptor sites on the plasma membrane of responsive cells.

Even accepting this tentative conclusion does not help resolve the other aspect of this question: is the specific receptor a part of the adenyl cyclase molecule? Considerable progress has been made in partially purifying the membrane-bound adenyl cyclases from several tissues. A nearly universal experience is that hormonal responsiveness is lost before fluoride-stimulatable adenyl cyclase activity, and in at least one case, it has been possible to separate the hormone binding site from the catalytic portion of the adenyl cyclase (Lefkowitz, Roth, and Pastan, 1970). Also, a β-type cholamine receptor has been identified on the surface of human erythrocytes (Allen and Rasmussen, 1971, 1972), cells which possess neither a hormone nor a fluoride stimulated adenyl cyclase (Sheppard and Burghardt, 1969a,b). Thus, clearly either in nature or by chemical means, it is possible to show that adenyl cyclase and hormone receptor are not necessarily equivalent. However, this does not rule out the possibility that the receptor is a component of a regulatory subunit of the membrane-bound adenyl cyclase complex. In many soluble enzymes, it is possible to separate regulatory and catalytic subunits and show that the binding site for the enzyme modifier is on the regulatory subunit. The real question in the case of a membrane-bound enzyme like adenyl cyclase is whether the hormone receptor site is closely linked to the adenyl cyclase, or whether it occupies a physically distinct site on the membrane. In the latter case, the hormone receptor interaction would lead to a change in membrane structure which would lead, in turn, to a change in adenyl cyclase activity.

In the case of the receptor site existing as an integral part of the adenyl cyclase, the interaction between specific hormone and receptor would lead to a highly restricted change in the properties of the cell membrane. This is particularly evident when one considers that the total number of adenyl cyclase sites is probably quite small. On the other hand, in a model in which receptor site and cyclase are not intimately joined, a general change in membrane properties would be more likely with adenyl cyclase activation being only one consequence of this change.

Insufficient evidence is currently available to decide between these two alternative modes of cyclase activation with the possible exception of the data from mammalian erythrocyte. In this cell type, physiological concentrations of either catecholamines or prostaglandin E_2 lead to a change in the physical properties of their membrane as measured by a decrease in deformability, and an increase in the extent of hypotonic hemolysis (Allen and Rasmussen, 1971, 1972). The response to both hormones is similar whether one employs rat or human erythrocytes. Nevertheless, only the rat cells contain an adenyl cyclase and when these cells are activated by either hormone, there is an increase in intracellular cyclic AMP. No similar change is seen in the human cells.

These data are consistent with a model in which hormone-receptor interaction leads to a *general change in membrane state* which leads in turn to changes in the physical properties of the membranes of both human and rat cells, and to an activation of adenyl cyclase in the rat cells. In other words, if an adenyl cyclase is attached to this particular type of cell membrane, it will be activated as part of the general response of the cell to hormone addition, but the change in cell membrane structure will still occur following hormone-receptor interaction even if the adenyl cyclase catalytic unit is not part of the membrane.

This model is also supported by studies of the mode of action of insulin. Clear evidence has been obtained that insulin changes, apparently independently, a number of membrane properties including membrane potential (Zierler, 1966), sugar and amino acid transport (Levine, 1966), and adenyl cyclase activity (Butcher, Sneyd, Park, and Sutherland, 1966). In the case of the latter, insulin-receptor interaction leads to its inhibition, but it is clear that this inhibition cannot account for all the other effects of this hormone.

If this is a generally valid model, i.e., hormone-receptor interaction leads to a general change in membrane structure, then it follows that in most

instances one would expect that hormone-receptor interaction would lead to changes in membrane function other than just adenyl cyclase activation. This bears on the question of whether or not cyclic AMP is the sole intracellular second messenger following cell activation.

CYCLIC AMP AS SOLE SECOND MESSENGER

It has been known for some time that activation of many different cells by specific extracellular stimuli requires the presence of calcium in the external medium (Rasmussen, 1970a). This is particularly true of many secretory systems, of many hormonally-responsive cells, and of muscle. In the case of muscle and certain tissues involved in endocrine secretion, this requirement for Ca^{2+} has been shown to be associated with an uptake of calcium from the external medium when the cell is activated. Also, it has now become generally accepted that calcium ion is the coupling factor between excitation and contraction in all forms of muscle, as well as serving as coupling factor between excitation and secretion at nerve endings (Eccles, 1964; Rahamenoff, 1968), and a number of specific glands (Zor, Lowe, Block, and Field, 1970; Cuthbert, 1970; Douglas, 1968; Rasmussen, 1970a; Farese, 1971a,b,c; Curry, Bennett, and Grodsky, 1968a,b; Dean and Matthews, 1970a,b; Hales and Milner, 1968; Heisler, Fast, and Tenenhouse, 1972; Hermier and Justisz, 1969; Hertelendy, Todd, Peake, Machlin, Johnston, and Pounds, 1971; Hokin and Hokin, 1961; Justisz and de la Llose, 1970; Malaisse-Lagae and Malaisse, 1971; Rubin, 1971). The fact that calcium is involved in some fashion in the control of cell activation in many cells in which cyclic AMP has been implicated as a second messenger has led to an examination of their possible relationship (Namm, Mayer, and Maltebie, 1968; Rasmussen and Nagata, 1970b).

One possible way in which calcium and cyclic AMP could be related is that in the absence of external calcium, the specific first messenger would no longer activate its particular adenyl cyclase, i.e., cyclase activation is a calcium-dependent process. This does not appear to be generally true. In several different tissues, the mammalian salivary gland (Rasmussen and Tenenhouse, 1968), the mammalian heart (Namm et al., 1968), renal tubules (Nagata and Rasmussen, 1970 a,b), the thyroid gland (Zor, Lowe, Bloom, and Field, 1968), and the fly salivary gland (Prince, Berridge, and Rasmussen, 1972), the specific first messenger activates adenyl cyclase in the absence of external calcium, but no physiological response is observed. An exception to this rule is the case of ACTH, both in the adrenal cortex (Lefkowitz et al., 1970) and in adipose tissue (Bar and Hechter, 1969a,b). With this hormone, no cyclase activation is observed if calcium is removed from the medium. However, a low concentration (5×10^{-6} *M*) of calcium will restore cyclase responsiveness without supporting the physiological response (Lefkowitz et al., 1970).

In nearly all these same tissues, it can be shown that addition of the specific first messenger leads to an increase in the rate of entry of calcium into the cell (as measured by radio-calcium kinetics) and, in cases where it has been measured, to an increase in total cell calcium (measured chemically). It could be argued that this uptake of calcium is a secondary consequence of a rise in intracellular cAMP concentration. However, this does not seem likely in view of the fact that, in these same systems, the addition of endogenous cAMP, in a concentration sufficient to activate the particular cell type, does not lead to an increase in calcium uptake (Borle, 1972; Prince et al., 1972). Unless one assumes that intracellular cAMP can regulate calcium flux across the plasma membrane but that extracellular cAMP cannot, this evidence means that many first messengers, in addition to activating adenyl cyclase, increase the permeability of the cell membrane to Ca^{2+}. In accordance with the previously discussed model of the membrane changes resulting from first messenger-receptor interaction, a change in calcium permeability would represent an *independent consequence* of a general change in membrane structure. It would also suggest as a likely possibility that calcium ions, as well as cyclic AMP, serve a second messenger function in the activation of many cells by specific extracellular stimuli (Douglas, 1968; Rasmussen, 1970a). If this is true, then it immediately follows that there will be biological systems in which one of these two general second messengers is relatively more important than the other in the process of cell activation.

At present these two, cAMP and Ca^{2+}, are the most generally identified second messengers. However, it is probable that in many cases additional

messengers will be identified. For example, in some cells, hormone or first messenger addition leads to a change in membrane potential (Dean and Matthews, 1970a,b; Horowitz, Horowitz, and Smith, 1960; Mears, 1971; Friedmann, Somlyo, and Somlyo, 1971), signifying a shift in monovalent ion distribution across the membrane. It is possible that such shifts alter both the microenvironment of the cell membrane and the ionic environment within the cell cytoplasm, bringing about changes in both membrane function and metabolic activities.

One very significant consequence of altering transmembrane Na^+ distribution might be a movement of Ca^{2+} across the membrane. Hodgkin and Keynes (1955) and Caldwell, Hodkin, Keynes, and Shaw (1960) found that when Na^+ in the external medium bathing squid axon was replaced by Li, choline, or sugar, there was an increase in Na^+ efflux. Baker, Blaustein, Hodgkin, and Steinhardt (1969) and Baker (1970) found that the efflux was Ca^{2+} dependent, and that this Ca^{2+} dependent Na^+ efflux was much more sensitive to changes in internal Na concentration. They further demonstrated that this Na efflux is linked to Ca^{2+} influx, and that both Na^+ efflux and Ca^{2+} influx are markedly dependent on the internal Na^+ concentration. A reduction in external Na^+ has been shown to lead to an increase in Ca^{2+} entry in frog skeletal muscle (Cosmos and Harris, 1961), mammalian heart (Niedergerke, 1963), mammalian liver (Judah and Ahmed, 1964), and smooth muscle (Goodford, 1967). The physiological significance of this process is not known. It is obvious, however, that Ca^{2+} entry must be considered as a possible consequence of events which leads to a redistribution of Na^+, such as membrane depolarization.

PROTEIN KINASES AND PHYSIOLOGICAL RESPONSE

The next question to be answered involves a consideration of whether all the effects of cAMP can be accounted for in terms of protein kinase activation. To simplify this discussion, it is worthwhile considering what is known about the properties of this class of enzymes. The logical starting point is a consideration of phosphorylase b kinase kinase.

This enzyme has been isolated from skeletal muscle (Walsh, Perkins, and Krebs, 1968). It consists of two subunits, one of which possesses the catalytic site, the other the 3′5′ AMP binding site (Brostrom, Corbin, King, and Krebs, 1971). Cyclic AMP activates the complex by causing the dissociation of the regulatory from the catalytic subunit (Figure 2). A similar mechanism of activation has been described for several other cAMP-dependent protein kinases from other tissues (Gill and Garren, 1970; Tao, Salas, and Lipmann, 1970).

In skeletal and cardiac muscle, epinephrine administration causes a rise in cAMP concentration. Additionally, there is clear evidence that a substrate, phosphorylase b kinase, becomes phosphorylated with the purified protein kinase, phosphorylase b kinase kinase. As little as 1 x 10^{-8} *M* cAMP stimulates phosphorylation of substrate in vitro, and maximal activation is achieved by less than 10^{-7} *M* cAMP. The Km for cAMP activation is 1.7 x 10^{-8} *M* (Walsh et al., 1968), a concentration well within the physiological range.

These facts establish with a high degree of probability that, in muscle, cAMP acts as a modifier of phosphorylase b kinase kinase. Both the enzyme and its substrate have been identified and characterized. In addition, a second physiological substrate, glycogen synthetase, has been identified (Schlender, Wei, and Villar-Palasi, 1969; Soderling and Hickenbottom, 1970; and Reiman and Walsh, 1970). This enzyme, which is a component of the glycogen synthetase complex, is inactivated when it is phosphorylated. This arrangement makes considerable sense. When gly-

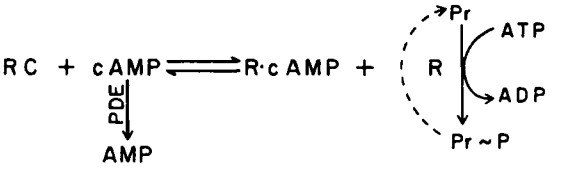


FIGURE 2. The mode of action of cyclic AMP upon protein kinases and the relationship to phosphodiesterase. When cAMP is complexed to the regulatory subunit of the protein kinase, it is resistant to hydrolysis by phosphodiesterase. R represents the regulatory subunit; C, the catalytic subunit; PK, protein kinase; PDE, phosphodiesterase; and Pr, and Pr ~ P, the nonphosphorylated substrate and phosphorylated product of the protein kinase reaction. Represented by the dotted line is the phosphatase which regulates the dephosphorylation of phosphorylated product. The means for controlling its activity is not known. This model is essentially that of Brostrom et al. (1971).

cogen breakdown is activated, glycogen synthesis is inhibited. Both effects are mediated by the activation of a single protein kinase by cAMP.

Since the initial discovery of the cAMP-dependent protein kinase in muscle, similar enzymes have been found in nearly all animal tissues, including the red blood cell, and in the tissue or cells from many different phyla (Kuo and Greengard, 1969). This has led to the conclusion that nearly all effects of cAMP are probably mediated via protein kinase activation (Kuo and Greengard, 1969; Exton et al., 1971). There are, however, several weaknesses in this conclusion. In the first place, glycogen synthesis and degradation are nearly universal attributes of all cells and tissues. This being so, the widespread occurrence of cAMP-dependent protein kinase may be nothing more than a reflection of the presence of phosphorylase and synthetase enzyme complexes in these tissues. On the other hand, it could be argued that phosphorylase b kinase kinase has a broad specificity in terms of its protein substrate (Walsh et al., 1968) and thus the presence of this enzyme would indicate the potential for a general control of cell metabolism; this control being achieved by having specifically different substrates in each cell type, i.e., there is only one protein kinase, but it has a different substrate or substrates in each specific responsive tissue. This possibility is supported, in a sense, by the fact that a variety of different proteins can serve as substrates for phosphorylase b kinase kinase, including casein, histone (Walsh et al., 1968), and neurotubulin (Goodman, Rasmussen, DiBella, and Guthrow, 1970). On the other hand, phosphorylase b kinase kinase is intimately associated with glycogen particles in the cytosol of muscle cells (Heilmeyer, Meyer, Haschke, and Fischer, 1970) and presumably in other tissue. This makes it most unlikely that this enzyme can normally phosphorylate proteins not associated with these particles, or those in other cell compartments.

At present, two very important kinds of information are generally lacking. One is whether there are cAMP-dependent protein kinases distinct from phosphorylase b kinase kinase in any or all tissues; the other is what are the natural substrates for these distinct kinases, if they do exist. The very fact that phosphorylase b kinase kinase has a broad specificity in terms of its protein substrates makes it extremely difficult to prove, on the one hand, that a particular protein kinase is, or is not, phosphorylase b kinase kinase, and, on the other, that a particular protein is a physiologically important substrate for this enzyme in any given tissue.

For example, it has been shown that highly purified microtubular protein from brain is an excellent substrate for a cAMP-dependent protein kinase from brain. When purified kinase and tubulin are added together, 10^{-7} *M* cAMP produces a seven- to tenfold increase in rate and extent of phosphorylation (Goodman et al., 1970). However, this enzyme will also catalyze the phosphorylation of histone and casein. The problem is to demonstrate that, under appropriate conditions in situ, this microtubular protein becomes phosphorylated, and that this phosphorylation is related to a particular cAMP-dependent change in nerve or brain function. To date this has not been achieved, although it has been shown that phosphotubulin has different physical properties from those of its nonphosphorylated precursor (Murray and Froscio, 1971).

In the case of liver histone, Langan has presented evidence to show that a specific histone, or more correctly a tryptic peptide derived from the histone fraction of liver nuclei, becomes phosphorylated by liver protein kinase in vitro after cAMP addition, and also becomes phosphorylated in situ after either glucagon or cyclic AMP administration (Langan, 1968, 1969, 1970). This histone fraction represents approximately 1% of the total nuclear histone. It is proposed that this change in histone structure leads to a change in histone-DNA interaction in the nucleus, and thus underlies a cAMP-induced increase in the rate of transcription of certain mRNA molecules. In support of this thesis is the fact that either glucagon or cyclic AMP administration does lead to an increase in the concentration of certain gluconeogenic enzymes in liver (Robison et al., 1971). However, until a better understanding of the physiological role of histones and histone phorylation is achieved, it is difficult to evaluate this hypothesis.

The data on histone phosphorylation are some of the strongest data in support of the concept that specific protein substrates other than phosphorylase kinase exist in particular cells, and become phosphorylated in situ when the cell is activated by a specific first messenger. However, even these data are not completely satisfactory. For example, there are a number of ways of

increasing the synthesis of gluconeogenic enzymes, some of which clearly do not involve cAMP. It would be of great interest to determine whether the same histone becomes phosphorylated under these conditions. Likewise, the explanation of this phosphorylation may not be an activation of a protein kinase, but merely reflect a change in the rate of turnover of either the inorganic phosphate pool or of an adenine nucleotide pool within the cell. A more suitable type of control experiment would be one in which gluconeogenesis was activated by two different means, and one of the livers prelabeled with ^{32}P and the other with ^{33}P. Then, upon mixing the extracts from the two organs, separating the proteins, and determining the $^{32}P/^{33}P$ ratio of each peak, it might be possible to demonstrate more clearly the phosphorylation of unique proteins.

Another system in which a protein kinase distinct from phosphorylase b kinase kinase has been found is in the ghost membrane of the erythrocyte (Guthrow, Allen, and Rasmussen, 1972). In this case, it has been shown that a cAMP-dependent kinase is a part of the membrane, and uses another membrane protein as substrate. At concentrations in the range of 10^{-7} *M*, cAMP stimulates a six- to sevenfold increase in the extent of phosphorylation. However, again in this case the physiological significance of this phosphorylation is not known. A particular difficulty stems from the fact that such a cAMP-dependent protein kinase has been identified as a component of the membrane of both human and rat erythrocytes, but only the latter possess a hormonally-stimulated adenyl cyclase.

In still other cases, the phosphorylation of isolated membranes by soluble cAMP- dependent protein kinases has been reported (Shlatz and Marinetti, 1971; Weller and Rodnight, 1970), but in these cases there is, as yet, no real clue as to their possible physiological significance.

The other major area of possible protein kinase involvement concerns ribosomal phosphorylation. There are a number of reports in which evidence is presented in support of the hypothesis that cAMP controls the rate of synthesis of one or more enzymes in hormonally-responsive tissues, e.g., cAMP or glucagon induce the synthesis of tyrosin transaminase (Greengard, 1969; Holt and Oliver, 1969) and serine dehydratase (Jost, Hsie, and Rickenberg, 1969; Jose and Sahib, 1971) in rat liver. There are other indications that cAMP may act by controlling enzyme synthesis at its translational level in several mammalian tissues. However, none of this evidence demonstrates that this change is a direct consequence of cAMP action, rather than being a secondary consequence of some primary alteration in cell metabolism. Nevertheless, the role of protein phosphorylation, and cAMP-dependent protein kinases in controlling ribosomal function has been investigated: the most detailed studies have been carried out in liver (Kabat, 1970; Eil and Wool, 1971; Lock and Blat, 1970; Blat and Lock, 1971) and adrenal cortex (Walton, Gill, Akrass, and Garren, 1971). In both cases, cAMP-dependent phosphorylation of proteins in membrane-bound ribosomes, or ribosomal subunits has been observed.

In the case of the liver, it has been shown that 1) ribosomal proteins can incorporate phosphate into serine residues in vivo or in vitro if a soluble protein kinase is added to the ribosomal fraction; 2) the in vitro rate of phosphorylation is stimulated by 10^{-8} to 10^{-6} *M* cAMP; 3) 30 minutes after glucagon administration in vivo with $H_3{}^{32}PO_4$, an increased incorporation of ^{32}P into ribosomal proteins is seen; 4) when rat liver homogenates are fractionated, two protein kinases can be prepared with very similar activities. Both will use either the 40S or 60S ribosomal subunit as substrate, and their activity is increased three- to fivefold by 10^{-5} *M* cAMP. However, there are discrepancies between the different reports concerning these phenomena. Particularly, in so far as Eil and Wool (1971) found that their crude supernatant fraction inhibited ribosomal phosphorylation, whereas Lock and Blat (1970) found this fraction contained cAMP-dependent phosphorylating activity.

None of the data have helped establish the possible role that this phosphorylation of ribosomal protein may play in ribosomal function. Eil and Wool (1971) found in their in vitro system that 3 proteins of the 40S subunit, and 9 of the 60S subunit became phosphorylated, a rather large number to be involved in a control mechanism. Furthermore, there is no evidence that the same proteins are phosphorylated in vivo and in vitro.

The studies on ^{32}P incorporation into phosphoproteins in vivo also suffer from a serious problem, that of the possibility that simple changes in the size of the inorganic phosphate pool within the cell, or the size and rate of turnover of

the nucleotide pool within the cell could be responsible for variations in protein labeling without a net increase or decrease in the total phosphoprotein existing within the cell or an organelle. These are extremely difficult experiments to control. At the very least, estimates of the specific activity of the ^{32}P in the ATP pool under different experimental conditions should be made in an effort to correct for any large changes in pool turnover and/or size.

Finally, if protein phosphorylation is to serve such a widespread regulatory role, another class of enzymes, the phosphoprotein phosphatases, must also be very prevalent and under strict metabolic control. Aside from the phosphorylase phosphatase of muscle (Haschke, Heilmeyer, Meyer, and Fischer, 1970) practically nothing has been done to locate these enzymes or to elucidate their control properties, but any complete understanding of these control systems will require such information.

Another facet of this subject is the discovery of both a *cAMP-stimulated* and a *cAMP-inhibited* protein kinase in the acellular slime mold *Physorum polycephalum* (Kuehn, 1971). In this instance, using casein as substrate, it was possible to identify two distinct kinases; the activity of one was completely inhibited by concentrations of cAMP as low as 10^{-9} *M*, the other was activated to half its maximal activity by 6×10^{-8} *M* cAMP. On the basis of preliminary evidence, it appears that the two enzymes exist in separate subcellular compartments. Unfortunately, the physiological functions of both enzymes are as yet unknown.

Another kind of experiment has been performed which indirectly tests the importance of cyclic AMP-dependent protein kinases as the final common pathway of first messenger action within the cell. Exton, Hardmann, Williams, Sutherland, and Park (1971) have examined the effects of cGMP upon the rates of glycogenolysis and gluconeogenesis in the perfused rat liver. They found this nucleotide to be one third to one half as potent as cAMP (on a molar basis) in stimulating these processes. However, in isolated tissue homogenates, or with partially purified protein kinases, a 100-fold greater concentration of cGMP is required to achieve the same activation as cAMP. Exton et al. (1971) showed that these effects of cGMP could not be accounted for by an increase in intracellular cAMP. However, they did find a greater uptake of cGMP than of cAMP by the perfused liver. Thus, it is possible that at the site of the appropriate protein kinase within the cell, a 100-fold greater concentration of cGMP than cAMP was achieved. Alternately, it is possible that cGMP acted by some means other than protein kinase activation. One possible means would be by changing the intracellular ionic environment. Both cGMP and cAMP (Exton et al., 1971; Friedmann and Park, 1968; Friedmann and Rasmussen, 1970; Friedmann, 1972) alter the flux of Ca^{2+}, Na^{+}, and K^{+} across the liver cell membrane; and it is possible that these ionic changes are a reflection of a significant change in intracellular ionic milieu.

Thus, there are data which lead to the conclusion that protein kinases are important sites of cAMP action, and other data which suggest that their activation cannot account for all the effects of cAMP. The evidence in favor of their being involved in the regulation of many cell functions is 1) their widespread occurrence; 2) the fact that they are activated by "physiological" concentration of cAMP; 3) that some at least are distinct from phosphorylase b kinase kinase; 4) the one completely established mechanism of action of cAMP involves the activation of specific protein kinase, phosphorylase b kinase kinase; and 5) protein phosphorylation appears to be a common way to control enzyme function. The difficulties with establishing the universal significance of this kind of control function for cAMP lie in the following: 1) the enzymes of glycogen metabolism must be widespread, hence detection of protein kinase activity in a cell may be no more than a reflection of this fact; 2) the specificities (in terms of protein substrate) of protein kinases are quite broad, so that mere phosphorylation in vitro is not a sufficient criterion on which to establish that a particular protein is a physiological substitute for this enzyme; 3) there is as yet no good method to demonstrate that phosphorylation is enhanced in situ following hormone action; and 4) there is physiological evidence which argues that not all the effects of cAMP can be accounted for by protein kinase activation.

CYCLIC AMP AND CELL CALCIUM

As noted previously, although a number of first messengers stimulate the uptake of calcium into the cell, cAMP does not mimic this first messenger

effect even though it induces a similar physiological response in the particular tissue (Borle, 1970; Rasmussen, 1970a, 1971; Farese,1971a,b,c; Prince et al., 1972; Leier and Jungmann, 1971; Parsons, Neer, and Potts, 1971; Parsons and Robinson, 1971). On the other hand, cAMP, as well as first messenger, does influence the rate of efflux of previously accumulated radiocalcium from a number of different cells, e.g., renal tubule (Borle, 1971), toad bladder (Schwartz and Walter, 1968), liver (Friedmann and Park, 1968), fly salivary gland (Prince et al., 1972) and the cellular slime mold *Dictyostelium discoideum* (Chi and Francis, 1971). A typical example is shown in Figure 3. In this case, fly salivary glands were incubated overnight in the presence of ^{45}Ca. They were then washed, resuspended, and the rate of appearance of radiocalcium determined. The addition of either first messenger, 5-hydroxytryptamine (5HT), or cAMP in concentrations sufficient to evoke a secretory response also evoked a marked increase in the rate of ^{45}Ca efflux from the glands. The questions to be answered are whether this change in calcium efflux is an important part of the physiological response of the particular tissue; what is the basis for the change in efflux; and how directly responsible is cAMP for this change in efflux?

In order to discuss these questions, it is first necessary to summarize briefly current knowledge of cellular calcium metabolism and homeostasis.

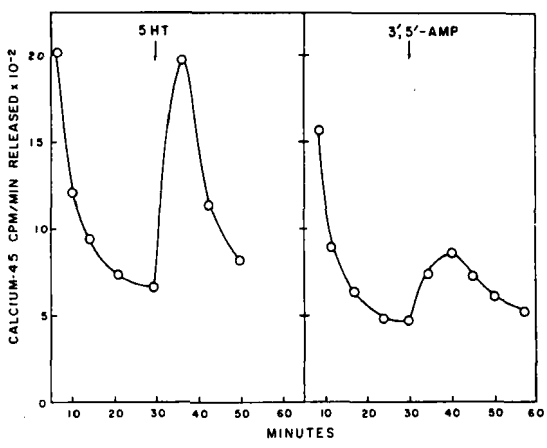


FIGURE 3. The effect of the addition of 10^{-8} *M* 5-hydroxytryptamine (5HT) or 10^{-2} *M* 3',5'-AMP upon the rate of release of ^{45}Ca from prelabeled fly salivary glands. Note that either first messenger, 5HT, or exogenous cAMP caused a significant increase in the rate of radiocalcium efflux.

CELLULAR CALCIUM METABOLISM

Any discussion of cellular calcium metabolism must begin with the striking fact that although the total calcium content of typical mammalian cells is in the range of 1 to 8 mM if soluble and evenly distributed throughout the cell H_2O, the estimated concentration in the cell cytosol is from 10^{-5} to 10^{-2} mM (Borle, 1967; Bianchi, 1968). This means that most of the cell calcium is concentrated in one or more subcellular compartments (Chance, 1965; Ebashi and Endo, 1968), and also that there is a gradient of calcium concentration across the plasma membrane of the cell, the extracellular fluids having a calcium ion concentration of approximately 1.0 mM. In cells other than muscle, the bulk of the intracellular calcium is within the mitochondrial matrix compartment (Lehninger, Carafoli, and Rossi, 1967) and in an organ like the kidney may be present at a presumptive concentration of 30 to 35 mM (Kimmich and Rasmussen, 1969). Clearly, not all of this calcium is soluble or in the ionic form (Rasmussen, 1966). Nearly all is probably in the form of an insoluble calcium phosphate complex, and in all likelihood the calcium ion concentration within the mitochondrial matrix space is less than that in the cell cytosol. In order to maintain this distribution of calcium within the cell and cell compartments, a constant supply of energy is necessary both to activate an externally directed calcium pump in the plasma membrane (Schatzmann, 1968) and an inwardly directed pump in the mitochondrial membrane. However, the most important feature of these pumps can only be understood when one considers the relationship of Ca^{2+} to HPO_4^{2-} and H^+ within the cell and cell compartments. There is an asymmetry in their distribution as well as that of Ca^{2+}. In the case of $[H^+]$, when the pH of the extracellular fluids is 7.4, that of the cytosol is approximately 6.8, and that within the mitochondrial matrix space is 7.8 (Figure 4). The distribution of phosphate within the cell is not well known. However, it is considerably higher in the cell than outside, and it is undoubtedly higher in the mitochondrial matrix space than in the cell cytosol. Furthermore, because of the differences in $[H^+]$ in the three compartments, the predominant ionic species of phosphate varies. The predominant extracellular form is $(HPO_4)^{2-}$, that of the cytosol, $(H_2PO_4)^-$, and that within the

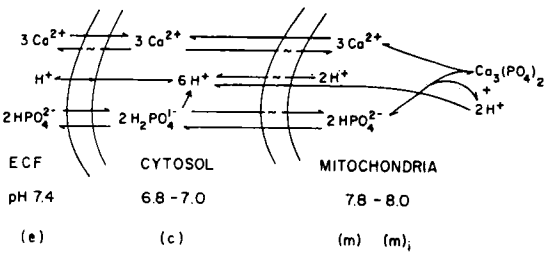


FIGURE 4. The interrelated nature of cellular calcium, phosphate, and hydrogen ion metabolism in a hypothetical animal cell. The major determinants of ion distribution are a primary H^+ ion pump in the mitochondrial membrane and a primary Na^+-K^+ pump (not shown) on the cell surface, and possibly a primary calcium pump as well. As a consequence, the pH of the ECF compartment (e) is 7.4; that of the cell cytosol (c) is 6.8 to 7.0; and that of the mitochondria (m) is 7.8 to 8.0. A large part of cellular calcium exists as an insoluble (m_i) phosphate complex in the mitochondrial matrix space. As such, it serves as a buffer system for maintaining the cytosol ion concentrations within a very narrow range.

mitochondria, $(PO_4)^{3-}$. The solubility of $Ca_3(PO_4)_2$ is extremely low, which means that nearly all the calcium and a significant part of the phosphate in the mitochondrial matrix space is in the insoluble, or nonionized form. Nonetheless, the calcium and phosphate in this nonionic mitochondrial pool is readily exchangeable with that in the cell cytosol. At least two basic (and probably three) ion pumps determine this distribution of ions: a primary Na^+ pump in the cell membrane; possibly a primary Ca^{2+} pump in the cell membrane; and a primary H^+ pump in the mitochondrial membrane (Mitchell, 1966). Because of the latter, both a hydrogen ion gradient and an electrical gradient exist across the mitochondrial membrane which act as driving forces for Ca^{2+} uptake and $OH^- \leftrightarrow HPO_4$ exchange (McGivan and Klingenberg, 1971). However, because of the high pH within the mitochondrial matrix space, much of the calcium and phosphate form insoluble $Ca_3(PO_4)_2$, or something akin to it, with a consequent release of H^+. Thus, the mitochondrial reservoir of calcium phosphate acts as a cellular buffer system, and the concentrations of Ca^{2+}, $H_2PO_4^-$, and H^+ within both the mitochondrial matrix space and the cell cytosol are intimately related. A change in the concentration of any one ion will change that of the other two, and, due to the events at the cell membrane, a change in Na^+ and H^+ distribution as well. In representative cells, the maximal rate of calcium flux across the cell membrane is 100 to 1,000 times less rapid than that across the mitochondrial membrane (Borle, 1967). This means that the mitochondrial system is the major one responsible for cell calcium homeostasis (Rasmussen, 1971) and also that a small change in the rate of calcium entry across the plasma membrane of the cell will produce very little sustained change in the calcium ion concentration within the cell cytosol. Clearly, if calcium is to play an important role as a second messenger in cell activation, as it apparently does in muscular contraction and glycogenolysis and in exocrine and endocrine secretion, there has to be a means of changing the calcium ion concentration in the cell cytosol sufficiently to induce an effective response. However, one of the characteristics of some calcium mediated responses, such as synaptic transmitter release (Rahaminoff, 1968) and muscle contraction, is that they respond as a function of the fourth power of the calcium ion concentration. Thus, a very small change is sufficient to induce a marked response. Nonetheless, a distinct change in concentration is a necessary prerequisite for activation.

There appear to be two basic ways in which a distinct change in cytosol calcium ion concentration might be achieved: either by regulating the flux of calcium across the mitochondrial membrane, or by amplifying the effect of a change in calcium permeability of the cell membrane. Two simple ways to achieve the latter would be to increase the calcium-binding capacity of the surface membrane or to increase the amount of intracellular membrane coupled electrically to the surface membrane.

To anticipate our discussion, it appears that both types of systems have evolved (Figure 5): ones in which control has been achieved by coupling events at the surface membrane with those at the mitochondrial membrane via cAMP, and ones in which control has been achieved by a marked increase in the amount of the surface-coupled membrane and/or its calcium-binding capacity. Examples of each type will be discussed in some detail because in both types cAMP may play a role. Those of the first type include the mammalian renal tubule and bone cells, the fly salivary gland, the mammalian adrenal cortex, and possibly the mammalian liver. Those of the second type include contraction and glycogenolysis in all forms of muscle, and possibly exocrine and endocrine secretion in mammalian glands. In addition

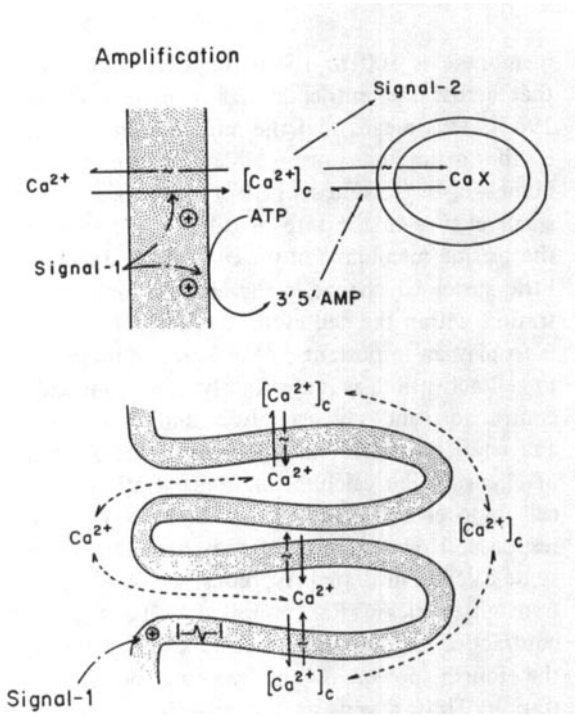


FIGURE 5. Two basic systems by which changes in cytosolic $[Ca^{2+}]$ can be amplified. Top, by coupling the events at the plasma membrane to those in the subcellular sites chemically *via* cAMP. Bottom, increasing the extent of cell surface and/or coupling events on the cell surface directly with events in a subcellular membrane as is seen in skeletal muscle.

to these, mitosis and cell growth will be discussed because both cAMP and Ca^{2+} have been shown to influence these phenomena, but insufficient data are available to define their exact relationship in the control of them.

Our discussion of specific systems will begin with a consideration of the relationship of calcium and cAMP to contraction and glycogenolysis in the several forms of muscle because these have been the most intensively studied systems and exemplify the range of variations in the role of calcium and cAMP in the control of the same basic process.

CALCIUM, cAMP, AND CARDIAC MUSCLE

The discussion of the calcium-cAMP relationship will focus upon the metabolic and mechanical responses of this organ to catecholamines. When epinephrine acts upon the heart, it brings about three important physiological changes: 1) an increase in heart rate; 2) an increase in the force of contraction; and 3) an increase in glycogenolysis (Sonnenblick and Stam, 1969; Langer, 1968; Katz, 1970; Caldwell, 1968; Robison et al., 1971). The mechanism underlying the first change is not well established. The remainder of this discussion will be concerned with the participation of Ca^{2+} and cAMP in the latter two effects.

Before beginning this discussion, the system of enzymes controlling glycogenolysis will be reviewed (Figure 6). A somewhat less complex set of enzymes controls the converse process, glycogen synthesis. These include a glycogen synthetase complex which exists in two forms, an activated or dephosphorylated form, and a nonactivated, phosphorylated form (Larner and Villar-Palasi, 1970). Phosphorylation is controlled by the same cAMP-dependent protein kinase, phosphorylase b kinase kinase, which stimulates the phosphorylation of phosphorylase b kinase. Thus, when cAMP concentration increases within the cell, there is a simultaneous conversion of phosphorylase b kinase from its nonactivated to its activated form, and of glycogen synthetase from its activated to nonactivated form.

In addition to these controls, several phosphoprotein phosphatases exist which catalyze the dephosphorylation of phosphorylase a, the activated form of phosphorylase b kinase, and the nonactivated form of glycogen synthetase. Much less is known about the factors which control their activities. Major investigative attention has been

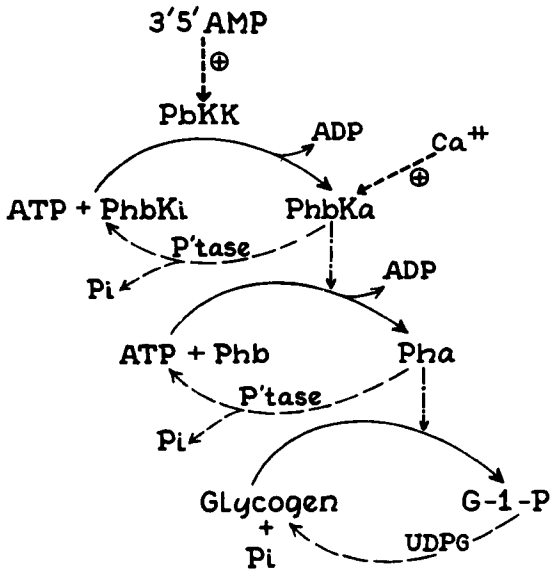


FIGURE 6. The cascade of enzymes involved in the control of glycogenolysis. See text for discussion.

devoted to the way in which cAMP and Ca^{2+} regulate the sequence of reactions from phosphorylase b kinase kinase to phosphorylase a. The system consists of a cascade of enzymes organized so that the initial enzyme in the sequence, phosphorylase b kinase kinase (PbKK), is activated by cyclic 3′5′ AMP. This enzyme is a specific protein kinase which catalyzes the conversion of the nonactivated form of phosphorylase b kinase (PbKi) to an activated form (PbKa) of this enzyme, activation being achieved by a phosphorylation of the inactive form. The activated form of phosphorylase b kinase is itself a protein kinase which catalyzes the phosphorylation of the inactive form of phosphorylase, phosphorylase b, to the active form, phosphorylase a. When epinephrine acts upon the heart, there is an increase in cAMP within the cell (Cheung and Williamson, 1965; Robison et al., 1971; Robison, Butcher, ϕye, Morgan, and Sutherland, 1965) whether Ca^{2+} is present or not (Namm et al., 1968). This rise in cAMP concentration within the cell leads to an activation of phosphorylase b kinase whether Ca^{2+} is present or not. In the presence of calcium this is followed by the conversion of phosphorylase b to a, resulting in an increase in glycogenolysis. In the absence of calcium, the phosphorylase b to a conversion does not take place. The reason for this lack of phosphorylase conversion is that the active, phosphorylated form of phosphorylase b kinase (the phospho-protein product of the cAMP-dependent phosphorylase b kinase kinase reaction) is activated by calcium (Ozawa, Hosoi, and Ebashi, 1967). Hence, in the absence of calcium, even though the enzyme has been converted to its active form, it is inactive. The question which is not completely resolved is whether an increase in $[Ca^{2+}]$ within the cell cytosol is a critically necessary part of the epinephrine-induced increase in the rate of glycogenolysis.

On the basis of these data, it could be argued that as long as sufficient Ca^{2+} is present, cAMP will act, and that phosphorylase conversion will take place even if the $[Ca^{2+}]$ does not increase. On the other hand, it is evident that the inotropic response to epinephrine ultimately involves calcium, i.e., epinephrine either increases the amount of calcium per beat, or the rate of its release, or both. Hence, it seems likely that epinephrine also influences calcium metabolism in these cells. This has been shown to be the case. Epinephrine stimulates Ca^{2+} influx in the heart (Nayler, 1967; Janke, Fleckenstein, and Jaedicke, 1970). However, the question is whether this is due to a direct effect of epinephrine upon membrane calcium transport, or is mediated by the effects of cAMP on one or more cellular membranes. There are data which support both possibilities.

In support of the concept of a direct effect of epinephrine upon calcium entry into the cell are the following: 1) epinephrine can produce a positive inotropic response under conditions where adenyl cyclase activation is blocked (Govier, 1968; ϕye and Langslet, 1972); 2) epinephrine at low concentrations will produce an inotropic response without altering cAMP concentrations in heart cells (ϕye and Langslet, 1972; Shanfeld, Frazer, and Hess, 1969; Langslet and ϕye, 1970); 3) perfusion of hearts with 10^{-4} *M* cAMP, in the presence of 3% dimethyl sulfoxide (DMSO) to make all the cell membrane more permeable to the cyclic nucleotide, leads to a significant activation of phosphorylase without a concomitant increase in force of muscle contraction (Kjekshus, Henry, and Sobel, 1971); 4) low doses of dibutyryl cAMP will enhance phosphorylase activity without a change in the force of muscle contraction (Robison et al., 1971); and 5) even though higher concentrations of dibutyryl cAMP will induce a positive inotropic effect, the time course of this effect is extremely slow, requiring 30 to 40 minutes, whereas the inotropic response to norepinephrine is maximal within two to four minutes (Skelton, Levey, and Epstein, 1970).

Other data support the notion that cAMP plays an important role in regulating cardiac calcium metabolism. These include: 1) larger doses of DBcAMP will provide a positive inotropic response of similar magnitude to that produced by catecholamines (Skelton, Levey, and Epstein, 1970); 2) the inotropic response to catecholamines is increased by theophylline (Rall and West, 1963); 3) propranolol depresses the contractile response to norepinephrine but not that induced by dibutyryl cAMP; 4) the microsomal fraction of canine myocardium contains catecholamine-sensitive adenyl cyclase (Entman, Levey, and Epstein, 1969a, b), and both norepinephrine and cyclic AMP cause an increase in calcium accumulation by this preparation; and 5) cyclic AMP in concentrations from 3.3×10^{-8} to 3.3×10^{-5} *M* will inhibit the Ca^{2+}-stimulated ATPase in both cardiac mitochondria and cardiac sarcolemma, but

enhance the activity of that in cardiac microsomes (Dietze and Hepp, 1972).

One of the unique features of the positive inotropic response to catecholamine is that it resembles the inotropic response associated with an increase in heart rate (Koch-Weser and Blinks, 1963). They are both characterized by an increase in the rate of rise of tension, a decrease in the time to peak tension development, a decrease in duration of systole, and an increase in rate of relaxation (Sonnenblick, 1962). A logical interpretation of these changes is that they reflect an increase in the size, rate of release, and rate of reaccumulation of the calcium pool in the cardiac sarcoplasmic reticulum. If so, the results of the studies of epinephrine and cAMP action can be incorporated into a model of events in which epinephrine may have at least three effects upon cellular calcium metabolism, at least two of which are mediated by cAMP (Figure 7).

The interaction of epinephrine with its receptor sites on the cell surface would lead to an increase in the rate of Ca^{2+} entry into the cell, and to an increase in adenyl cyclase activity. The subsequent rise in [cAMP] would lead to an inhibition of calcium efflux from the cytosolic compartment by inhibiting the calcium "pumps" both in the plasma membrane and in the mitochondria, but to a stimulation of calcium uptake by the microsomes or sarcoplasmic reticulum. The net result would be a shift of calcium to the sarcoplasmic reticulum and a means of increasing the rate of relaxation. The

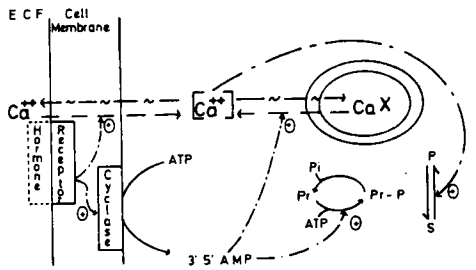


FIGURE 7. A model depicting the effect of epinephrine upon calcium metabolism in cardiac muscle. Note that epinephrine stimulates the entry of calcium across the surface membrane leading to an increase in the pool of trigger calcium, C_T, within the cell which, in turn, activates the release of bound calcium, CaX, from the sarcoplasmic reticulum (SR). Epinephrine also activates adenyl cyclase leading to a rise in the cAMP concentration. The cAMP acts upon several membranes to cause a shift of Ca^{2+} from the mitochondrial pool, CaX(M), to the sarcoplasmic pool. Calcium and cAMP also regulate glycogenolysis.

one component of the response which is important is the more rapid release of Ca^{2+} from this microsomal pool. It is possible that the activation of microsomal adenyl cyclase could play a role in this phenomenon, but insufficient data are available to make this judgment. On the other hand, the mammalian heart, like fast skeletal muscle, has a well developed T-system in which invaginations of the surface membrane are in close contact with the sarcoplasmic reticulum, and in which there is ionic (electrical) coupling between surface membrane depolarization and the release of calcium from the sarcoplasmic reticulum (Bianchi, 1972). Extracellular calcium plays a critical role in this coupling. When surface membrane depolarization takes place, a small amount of calcium (trigger calcium, C_T, Figure 7) enters from the extracellular fluids and/or is released from the surface membrane of the cell, and serves to initiate the release of calcium from the sarcoplasmic reticulum. Epinephrine may act directly to increase the magnitude of this signal, or the amount of this trigger calcium which enters the cell upon each depolarization.

The organization of this particular process has some interesting properties from an operational point of view. In particular is the fact that the trigger calcium, C_T, acts as a positive feedforward activator of calcium release from the sarcoplasmic reticulum. This being the case, it is possible to uncouple excitation from contraction by substituting Mg^{2+} for Ca^{2+} in the extracellular fluid. In other words, even though most of the calcium for contraction normally comes from the sarcoplasmic reticular pool, and the size of this pool is normal, contraction will not take place if the extracellular $[Ca^{2+}]$ is below a critical level. An analogous type of organization is seen in the response of bone cells to parathyroid hormone (see below).

Returning to the control of cardiac glycogenolysis, it is clear that the most important controlling factor is cAMP which operates by regulating phosphorylase b kinase kinase activity (Figure 8), but Ca^{2+} also probably plays a role in stimulating the phosphoprotein product of the cAMP-dependent protein kinase, i.e., the activated form of phosphorylase b kinase. In other words, in cardiac glycogenolysis, the two second messengers appear to act sequentially and in a concerted fashion.

The importance of calcium in the metabolic response to epinephrine is clearly demonstrated by

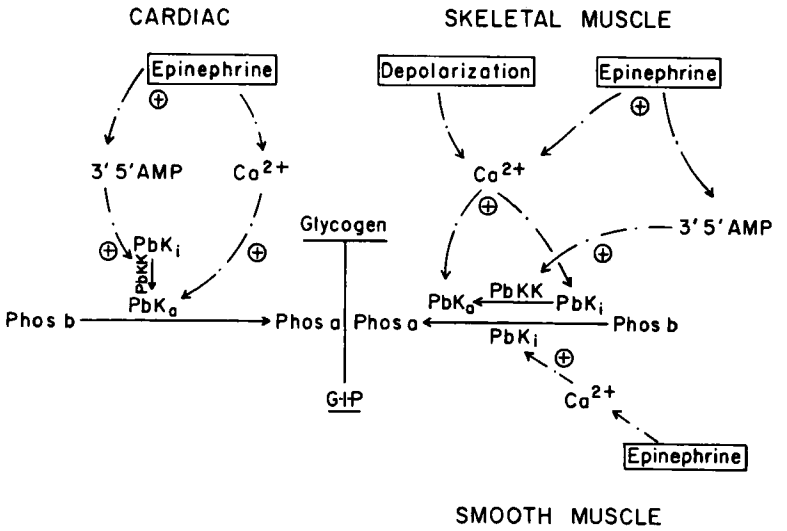


FIGURE 8. The control of glycogenolysis in three different forms of muscle. In cardiac muscle, cAMP and Ca^{2+} act sequentially and in a concerted fashion with cAMP being of relatively greater importance. In skeletal muscle, cAMP and Ca^{2+} concentrations are regulated by independent means, and these two second messengers act independently or in a concerted fashion with Ca^{2+} being of relatively greater importance. In arterial smooth muscle, the major and possibly sole intracellular mediator of glycogenolysis is Ca^{2+}.

The situation in skeletal muscle is of particular interest in the physiological sense because Ca^{2+}-mediated glycogenolysis is directly coupled to muscle excitation and serves primarily local metabolic needs, whereas cAMP-mediated glycogenolysis is primarily a hormonally-controlled response subserving the metabolic needs of the organism.

the alteration in response seen in the hearts of adrenalectomized animals (Miller, Exton, and Park, 1971). A comparison of the responses of hearts from these animals to those obtained from normal animals reveals that epinephrine causes the same increase in cAMP concentration, and the same increase in the concentration of activated form of phosphorylase b kinase, but both the inotropic response and the concentrations of phosphorylase a were less in the hearts from adrenalectomized animals. These changes are associated with an altered calcium metabolism (Hudgens, 1969; Rovetto and Hefer, 1970). In addition, Miller et al. (1971) found that a normal increase in phosphorylase a concentration after epinephrine was seen in the hearts from the adrenalectomized animals when the extracellular $[Ca^{2+}]$ was increased.

These results emphasize that both Ca^{2+} and cyclic AMP are necessary for an optimal response to epinephrine in the heart. Disorders of cellular calcium metabolism, such as occur in the hearts of adrenalectomized animals, lead to a partial inhibition of the physiological response without changing the initial rise in cellular cAMP concentration, or its effect upon protein kinase activation.

CYCLIC AMP, CALCIUM, AND SKELETAL MUSCLE

It is generally accepted that when skeletal muscle is stimulated to contract, calcium ions entering from the ECF or released from the sarcoplasmic reticulum are the coupling factors between excitation and contraction (Bianchi, 1972; Ebashi and Endo, 1968). Associated or occurring simultaneously with this contraction is a rapid activation of muscle glycogenolysis (Danforth, Helmreich, and Cori, 1962). Epinephrine also increases skeletal muscle glycogenolysis without causing contraction (Drummond, Harwood, and Powell, 1969; Stutt and Mayer, 1971). In the latter case, epinephrine has been shown to activate skeletal muscle adenyl cyclase and to increase the conversion of the nonactivated form of phosphorylase b kinase to the activated form with a resulting increase in phosphorylase b

to a conversion. This epinephrine-induced increase in phosphorylase a activity requires minutes. Electrical stimulation also induces phosphorylase a activity in skeletal muscle, but this response is much more rapid (seconds). Also, in the case of electrical stimulation, there is apparently no increase in the conversion of the nonactivated form of phosphorylase b kinase to the activated form, although there is a clear increase in the conversion of phosphorylase b to a (Drummond et al., 1969, Stull and Mayer, 1971; Posner, Stern, and Krebs, 1965). These data demonstrate that there are at least two independent means of activating glycogenolysis, one of which involves cAMP, while the other does not.

The mediator of the response to electrical stimulation is Ca^{2+}. Calcium is essential for the activity of phosphorylase b kinase in this tissue, and reversible stimulation of enzyme activity is achieved in the range of 10^{-7} to 10^{-6} *M* calcium ion (Meyer, Fischer, and Krebs, 1964; Ozawa, Hosoi, and Ebashi, 1967; Brostrom, Hunkeler and Krebs, 1971). Even more direct evidence for the importance of Ca^{2+} in the control of this reaction has been obtained by Heilmeyer, Meyer, Haschke, and Fischer (1970) in their studies of glycogen particles from skeletal muscle. These particles contain phosphorylase, phosphorylase b kinase, and phosphorylase phosphatase. Heilmeyer et al. (1970) have shown that Ca^{2+} stimulates either the nonactivated or activated form of phosphorylase b kinase, and, in conjunction with ATP, controls the activity of the phosphatase as well.

These results show that in skeletal muscle either the nonactivated or activated forms of phosphorylase b kinase is stimulated by changes in $[Ca^{2+}]$ within the cell. The only difference is that the Km for stimulation by $[Ca^{2+}]$ is approximately 10^{-6} *M* for the nonactivated form of the enzyme and 10^{-7} *M* for the activated form (Brostrom, Hunkeler, and Krebs, 1971). In the case of electrical stimulation, the release of calcium is sufficient to stimulate the nonactivated form of the enzyme and bring about a change in the phosphorylase b to a conversion independent of cAMP (Figure 8).

The situation after catecholamine stimulation appears to involve primarily cAMP, but Ca^{2+} may be involved as well. In a careful study of this phenomena, Stull and Mayer (1971) have identified three phases of phosphorylase formation in muscle stimulated by catecholamines. At a concentration of 4×10^{-12} *M*, *l*-isoproterenol phosphorylase a activity increased without a change in the concentration of cAMP within the cell, or an increase in the conversion of the nonactivated to the activated form of phosphorylase b kinase. Between 4×10^{-10} and 4×10^{-9} *M*, *l*-isoproterenol phosphorylase a activity increased further and was associated with an increase in cAMP and an increase in the concentration of the activated form of phosphorylase b kinase. However, the increase in phosphorylase a concentration preceded the rise in activated phosphorylase b kinase and cAMP concentrations. At higher concentrations of *l*-isoproterenol, there was a further stimulation of phosphorylase b to a conversion with a further increase in cAMP, but without any greater increase in the concentration of the activated form of phosphorylase b kinase.

The second phase is the classically accepted sequence of adenyl cyclase activation, phosphorylase b kinase kinase stimulation, an activation (phosphorylation) of phosphorylase b kinase, and, finally, an increase in phosphorylase b to a conversion (Figure 8). However, Stull and Mayer (1971) suggest that there are other components to even this response, and that catecholamine-induced release of membrane calcium may be an important aspect of these hormones' action in controlling glycogenolysis in this tissue. This possibility is supported by the fact that in skeletal muscle catecholamines stimulate K^+ efflux and membrane hyperpolarization (Goffat and Perry, 1951), a response similar to that seen in other tissues and thought to be associated with changes in intracellular Ca^{2+}.

There are two difficulties with this hypothesis. The first is that catecholamines do not stimulate muscular contraction in this tissue, yet one might support they should do so if they cause an increase in calcium entry into the cell. However, the calcium which normally enters the cell across the cell membrane is insufficient to raise the concentration of Ca^{2+} at the myofibrils sufficiently to stimulate contraction. A release of Ca^{2+} by the sarcoplasmic reticulum is necessary (Bianchi, 1968, 1972). Thus it is possible that a sufficient change in calcium concentration could take place in the vicinity of the glycogen particles without a change occurring at the myofibril sufficient to induce contraction.

The second problem is that the concentration of cAMP within these cells is approximately 2 x

10^{-8} *M*, which is greater than the Km for the activation of isolated phosphorylase b kinase kinase (protein kinase). This must mean that a considerable portion of the cAMP is normally bound or sequestered in some compartment. If so, it is possible that the initial effect of the catecholamine is to cause a release of bound cAMP without an actual change in its concentration. However, even if this were the case, one would have to argue that cAMP has a direct effect upon phosphorylase b to a conversion not mediated through phosphorylase b kinase kinase, but Heilmeyer et al. (1970) found no effect of cAMP upon phosphorylase b to a conversion in the isolated glycogen particles. Thus, at present, the most likely conclusion is that calcium is the sole coupling factor between excitation and glycogenolysis and participates as a coupling factor in catecholamine-stimulated glycogenolysis (Figure 8).

CYCLIC AMP, CALCIUM, AND SMOOTH MUSCLE

Two general types of smooth muscle can be identified in terms of their responses to catecholamines. In one type, exemplified by intestinal smooth muscle, catecholamines cause relaxation; in the other, exemplified by arterial smooth muscle, these agents cause contraction.

In the case of arterial smooth muscle (Hudgens, 1969; Namm, 1971), contraction is induced by high concentrations of catecholamine (5×10^{-4} *M*) as well as by histamine (10^{-5} *M*), phenylephrine (5×10^{-6} *M*), or angiotensin (10^{-6} *M*). In the case of all of these activators, there is also a simultaneous increase in phosphorylase activity. The effect of isoproterenol is blocked by α-adrenergic but not by β-adrenergic blocking agents, and neither cAMP nor DBcAMP caused either contraction or phosphorylase activation. The magnitude of both the drug-induced increase in muscle tension and phosphorylase a activity was a function of the $[Ca^{2+}]$ in the extracellular fluid, and depletion of tissue calcium led to a marked reduction in response. Namm (1971) has suggested that Ca^{2+} is the common mediator of both the mechanical and metabolic events in this tissue (Figure 8). There is, at present, no evidence that cAMP plays any role in the regulation of glycogenolysis in this tissue.

The situation with regard to intestinal smooth muscle is even more unique. In this tissue, epinephrine causes relaxation (Levine, Carrerata, and McNelly, 1967; Bubring and Tomita, 1969) rather than contraction. This relaxation appears to be mediated by cAMP. If it is assumed, as appears likely, that the contractile state of the muscle is regulated by the $[Ca^{2+}]$ of the myoplasm, then it follows that cAMP must in some way control this $[Ca^{2+}]$. The important point concerning the Ca^{2+}-cAMP relationship in this tissue can be summarized as follows (Anderson, Lundholm, Mohme-Lundholm, and Nilsson, 1972; Andersson and Mohme-Lundholm, 1969, 1970; Andersson, Lundholm, and Mohme-Lundholm, 1971): 1) epinephrine activates adenyl cyclase (Butcher, Ho, Meng, and Sutherland, 1965; Bueding, Butcher, Hawkins, Timms, and Sutherland, 1966); 2) the increase in cAMP precedes relaxation; 3) the magnitude of the rise in [cAMP] is directly proportional to the degree of relaxation; 4) cyclic AMP itself will induce relaxation; 5) cAMP stimulates the uptake of calcium by a microsomal or plasma membrane fraction of smooth muscle; 6) Ca^{2+} inhibits phosphodiesterase in this tissue; 7) a decrease in the external $[Ca^{2+}]$ reduces the cAMP content of this muscle; and 8) contractile agents such as carbacholine increase the [cAMP] but only in the presence of Ca^{2+}. A model depicting these relationships is shown in Figure 9.

There are several important points to be made about this control system. First, in this situation cAMP acts to cause an uptake of calcium from

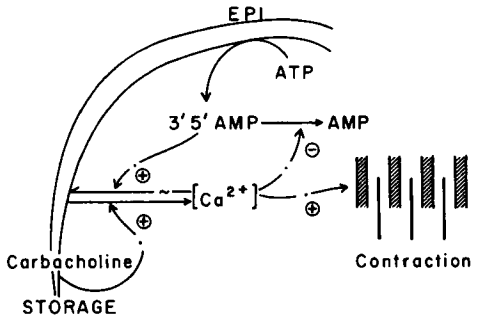


FIGURE 9. The model of events in smooth muscle when stimulated to contract by acetylcholine or to relax by epinephrine. Note particularly that the relationship between calcium and cyclic AMP in this system is a mirror image of that depicted in Figure 7. Calcium regulates cAMP concentration by inhibiting phosphodiesterase activity and thereby inhibits cAMP hydrolysis. Conversely, cAMP stimulates the uptake of calcium by membrane pools, thus decreasing cytosolic calcium concentration.

cytosol into a membrane-bound form in contrast to the case in heart muscle. Second, the two intracellular messengers Ca^{2+} and cAMP influence each other's contractions because of a feedback relationship between them, but the situation in intestinal smooth muscle is a mirror image of that in heart in the sense that, in smooth muscle, an increase in [Ca^{2+}] leads to an increase in [cAMP] whereas in the heart an increase in [Ca^{2+}] leads to a decrease in [cAMP]. Conversely, a rise in [cAMP] leads to a decrease in [Ca^{2+}] in smooth muscle but to a rise in [Ca^{2+}] in the heart. However, from a cybernetic point of view, the control system is organized in a similar manner with the two control elements each a factor in controlling the concentration of the other element.

Of considerable importance is the fact that a rise in cAMP concentration is seen whether the smooth muscle is stimulated to contract by carbacholine, or to relax with epinephrine. The classical second messenger hypothesis is incapable of explaining this apparent paradox, but the model depicted in Figure 9 readily accounts for it. Another situation has been described in which two hormones with opposing physiological effects upon a particular tissue both increase the cAMP content of that tissue. The particular case of calcitonin, parathyroid hormone, and bone will be discussed subsequently, but it is worth pointing out the fact that a rise in cAMP may occur in the same cell type under two very different physiological states because intracellular control systems operate as an extensive network of interrelated factors.

CYCLIC AMP, CALCIUM, AND MUSCLE GLYCOGENOLYSIS

A survey of the several different forms of muscle reveals that in the control of glycogenolysis both Ca^{2+} and cAMP participate as second messengers. However, their relationship and importance vary from system to system (Figure 8). In heart, cAMP is particularly important and the effect of Ca^{2+} is directly dependent upon the cAMP-mediated step; in skeletal muscle both are important; but Ca^{2+} can induce a cAMP-independent activation of phosphorylase. In vascular smooth muscle Ca^{2+} is the principal mediator.

In considering the relationship of cAMP and Ca^{2+} to contraction, Ca^{2+} is clearly the coupling factor between excitation and contraction in all forms of muscle. In contrast, cAMP plays a subsidiary role mediated by its effects upon subcellular membranes, particularly the sarcoplasmic reticulum. In skeletal muscle, there is no clear evidence of an effect of cAMP; in cardiac muscle it enhances contraction by controlling the intracellular distribution of calcium, but in intestinal smooth muscle it is of major importance in reducing the [Ca^{2+}] of the cell cytosol, and inducing, thereby, relaxation.

These contrasting relationships between two second messengers emphasize the great plasticity of control achievable by a cellular control system containing two rather than a single control element. The data in cardiac and intestinal smooth muscle indicate that cAMP can control the accumulation of calcium by subcellular membranes, and thereby control the [Ca^{2+}] of the cell cytosol.

Clearly then, in different forms of muscle, cAMP has a significant influence upon calcium distribution and binding. The effects are mediated by an action upon one or more subcellular membranes. The question to be answered is whether the effects are direct in the sense that cAMP interacts with membrane sites and by its binding causes a change in membrane transport function, or whether they are mediated indirectly by catalyzing the activity of a membrane-bound protein kinase which stimulates, in turn, the phosphorylation of membrane components involved in calcium transport. Further work is necessary before a choice between these two alternatives is possible.

CYCLIC AMP, CALCIUM, AND SECRETION

Secretion of stored material in response to specific stimuli is a process common to a number of mammalian organs and tissues. The secretory response is believed to be mediated by cAMP in a number of these systems (Robison et al., 1971). These include both exocrine and endocrine glands as well as synaptic transmission. The stimuli or first messengers range from specific hormones to high K^+ and action potentials, and to drugs such as tolbutamide. Not all of these separate systems will be reviewed. We will restrict our discussion to those systems in which the secretory process is

well enough understood to allow a realistic evaluation of the participation of cAMP in the response.

MECHANISM OF SECRETION

The synthesis, storage, and release of secretory protein have been extensively studied in the exocrine pancreas (Siekevitz and Palade, 1960; Redman, Siekevitz, and Palade, 1966; Caro and Palade, 1964; Jamieson and Palade, 1967a, b, 1971a, b; McCuskey and Chapman, 1969), the β cells of the endocrine pancreas (Lacey, 1970; Coore and Randle, 1964; Milner and Hales, 1968; Malaisse, Malaisse-Lagae, and Mayhew, 1967; Curry, 1970; Dean and Matthews, 1970), and the anterior pituitary (Farquhar, 1962; Fleischer, Donald, and Butcher, 1969; Hedge, 1971; Justisz and Paloma de la Llosa, 1970; Steiner, Peake, Utiger, Karl, and Kipnis, 1970; Meyer and Knobel, 1966; LaBella, 1964; Hertelendy, Todd, Peake, Mochlin, Johnston, and Rounds, 1971). The important features of the secretory process in all three systems were found to be similar.

Secretion from the exocrine pancreas is the best understood, primarily as a result of the work of Palade, Jamieson, and their associates (Siekevitz and Palade, 1960; Redman et al., 1966; Caro and Palade, 1964; Jamieson and Palade, 1967a, b; Jamieson and Palade, 1971a, b). Five phases of the secretory cycle can be distinguished: 1) synthesis of protein on the rough endoplasmic reticulum and its release into the lumen of the endoplasmic reticulum; 2) transport of the protein to the Golgi zone of the cell; 3) packaging of the protein in membrane-bound vesicles; 4) maturation of these vesicles and their migration to the cell periphery; and 5) fusion of the vesicle membrane with the plasma membrane of the cell. At the point of fusion, the membranes rupture and the contents of the vesicle are discharged into the extracellular space. The granule membrane is retained and, in time, dissociates from the plasma membrane to reappear as an empty vesicle within the cell.

It is implicit in this scheme that all secretory proteins are contained within membrane-bound vesicles and that stimulation of secretion leads to a release only of protein contained within these vesicles. However, Rothman (1970) found that the ratio of different enzymes secreted in response to stimulation can differ from the ratio of the same enzymes contained within similar cells. He suggests that either the zymogen granules are heterogeneous with respect to their content of enzymes or that a pool of enzyme is secreted which is not contained in vesicles. Using the technique developed by Jamieson and Palade (1967a) to label zymogen (vesicle) protein with radioactive amino acid, Heisler, Fast, and Tenenhouse (1972) have obtained evidence in support of this latter suggestion. They found that, in the absence of added secretagogue, there is a calcium-evoked spontaneous release of amylase activity from slides of exocrine pancreas incubated in vitro, but there was no similar calcium-evoked secretion of ^{3}H-labeled protein in the same slices (Figure 10.) In contrast, when carbachol was added, the secretion of both radioactive protein and amylase activity increased in a parallel fashion dependent upon calcium concentration. These results raise

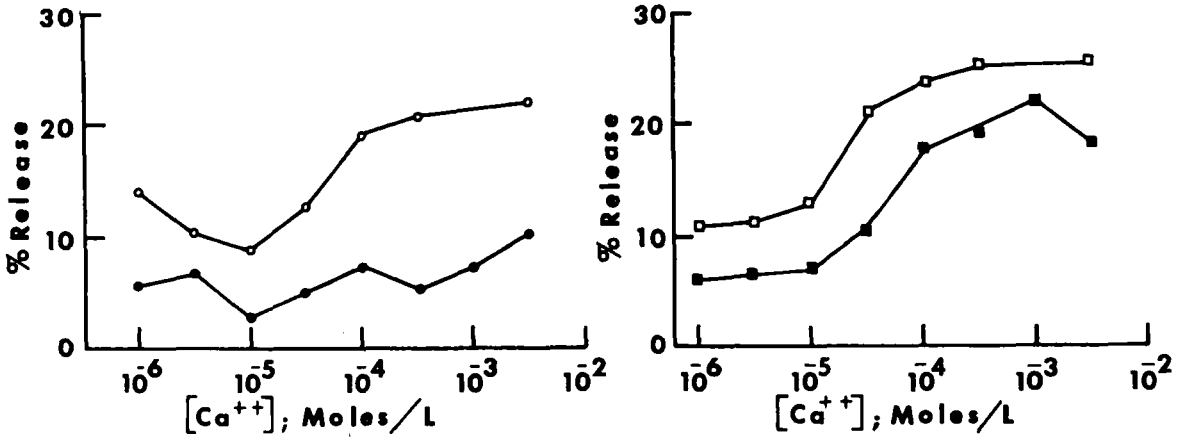


FIGURE 10. (Left) The effect of calcium concentration on the release of amylase activity (○ – ○) and ^{3}H-leucine labeled protein (● – ●) in the absence of added secretagogue. (Right) The effect of calcium concentration on the release of amylase activity (□ – □) and ^{3}H-leucine labeled protein (■ – ■) in the presence of 10^{-5} *M* carbachol.

the possibility that there is more than one pool of secretory protein and that more than one type of secretory mechanism may exist. A similar possibility has been discussed in other systems. Curry, Bennett, and Grodsky (1968a, b) have presented evidence to support the concept of two distinct insulin pools in the β cells, one secreted in response to stimulation by either glucose or tolbutamide, and the other by glucose alone. They suggest that the secretory mechanisms for the two pools may differ, although they share at least one common characteristic, a Ca^{2+} requirement. Similarly, Kracier and Milligan (1971) have shown that the release of ACTH induced by high external K^+ is inhibited by colchicine, whereas release induced by hypothalamic extracts is not.

It is obvious that if multiple mechanisms of secretion exist within the same cell, then it is difficult to interpret experimental data concerning the role of a particular intracellular intermediate, such as cyclic AMP, in the secretory process. In the ensuing discussion, we will limit ourselves to three questions concerning the role of cAMP in secretion: 1) Is a change in cAMP concentration essential for secretion to occur? 2) If an increase in cAMP does occur, is it an obligatory intermediate in the sequence of events which links stimulation to secretion? 3) What other factors are involved in stimulus-secretion coupling and how are they related to changes in cAMP metabolism?

ENZYME SECRETION IN THE EXOCRINE PANCREAS

Both acetylcholine, or its analogs, and pancreozymin stimulate the secretion of digestive enzymes by the exocrine pancreas (Kulka and Yalonsky, 1966; Dickman and Morril, 1957; Hokin and Hokin, 1962; Rothman; 1967). Kulka and Sternlicht (1968) reported that cAMP, DBcAMP, theophylline, and monobutyryl cAMP would all enhance the rate of protein secretion from slices of mouse pancreas in vitro. They also found that secretion induced by the agents carbamycholine (an acetylcholine analog) or pancreozymin was blocked by 3′AMP. On the basis of these findings, they proposed that cyclic AMP was an intermediate in this secretory process.

The results obtained with 3′AMP were interpreted as indicating an inhibition of a cAMP-mediated response of this tissue by competing for 3′5′AMP receptors within the tissue. However, the experience of others with 3′AMP (Robison et al., 1971) shows that its effects are not highly specific.

In confirmation of the results of Kulka and Sternlicht, others have reported a secretory response to cAMP in exocrine pancreas. Ridderstap and Bonting (1969) found an increase in the rate of both total protein and amylase secretion, but the percentage change in total secretion was always greater than that of amylase secretion in response to pancreozymin, acetylcholine, theophylline, and cAMP. Theophylline was found to augment the effect of pancreozymin on protein secretion but not on amylase secretion. These data illustrate the difficulty in interpreting the effects of cAMP. If, as suggested by Ridderstap and Bonting, the differential effects of these secretagogues upon total protein vs. amylase secretion support the multiple pool hypothesis of Rothman (1970), then it must be concluded that the cyclic nucleotides effect secretion from the different pools differently.

Other workers (Knodell, Toskes, Reber, and Brooks, 1970; Case, Raundy, and Scratcherd, 1969; Baudoin, Rochus, Vincent, and Dumon, 1971) have reported conflicting results. Both DBcAMP and theophylline have been reported to stimulate the secretion of both trypsin and chymotrypsin from rabbit pancreas (Knodell et al., 1970). However, the increase in rate of secretion was small compared to the effects of carbachol, and a more appropriate statement is that in the presence of these agents secretion was maintained for longer periods of time. In contrast, no effect of either DBcAMP or theophylline could be demonstrated on amylase secretion in the cat pancreas (Case et al., 1969). Extremely high concentrations of cAMP (33 mM) were found necessary to provoke chymotrypsinogen secretion in rat pancreas, but 0.1 mM DBcAMP was found to be effective (Baudoin et al., 1971). Theophylline was found to be ineffective alone, but to potentiate the effects of DBcAMP. Of particular interest, Baudoin et al. (1971) found that secretory doses of carbamycholine inhibited the incorporation of ^{14}C-glucose into protein and stimulated ^{32}P incorporation into phospholipids, but the DBcAMP at doses sufficient to evoke secretion had no effect upon either of these metabolic parameters.

Heisler et al. (1972) have examined the effects of these agents more fully on protein secretion in slices of rat pancreas. They also found that cAMP itself did not stimulate secretion but that DBcAMP

or theophylline did. However, the maximal secretory rate observed in the presence of optimal concentrations of the latter two agents was only approximately 30% of that seen with optimal concentrations of carbachol. Most significantly, the rate of secretion in the presence of optimal concentrations of both carbachol and DBcAMP was significantly greater than with carbachol alone. Their effects were synergistic. Theophylline, on the other hand, did not enhance the secretory effect of carbachol.

The failure of cAMP to exert any effect upon secretion does not rule out the possibility of its involvement in the secretory response. Exogenous cyclic AMP is relatively ineffective in a number of systems in which it has been proposed as a second messenger (Robison et al., 1971). The usual explanation for this ineffectiveness is that this nucleotide penetrates poorly into cells. The dibutyryl analog is thought to enter cells more rapidly, and this could account for its effectiveness in the pancreas. As discussed previously, the situation is much more complex than this, and we really know very little about the relative penetration rates of these nucleotides into most cells.

It is not easy to interpret the effects of DBcAMP and theophylline on the pancreas in terms of the second messenger hypothesis. The ability of both to stimulate secretion could be taken as evidence for the normal participation of cAMP in the secretory process. However, theophylline does not enhance the effect of carbachol. Also, it is difficult to account simply for the effects of DBcAMP in the presence of maximal doses of carbachol. The fact that DBcAMP increases secretion in this circumstance can be interpreted in one of several ways: 1) cAMP is involved in the sequence of events between stimulus and secretion; and the level of cAMP limits response; 2) some other element is rate limiting and DBcAMP can, in some way, overcome this limitation; or 3) DBcAMP causes secretion by a pathway independent of that controlled by carbachol. If the first were true then theophylline should have produced a response similar to that seen with DBcAMP. In view of the fact that theophylline did not have this effect but that it did, by itself, induce some secretion, could then be interpreted to mean that cAMP is involved but, under conditions of maximal carbachol stimulation, is no longer rate limiting. If, as suggested in the third alternative, DBcAMP was acting via an independent secretory pathway, the effects of DBcAMP plus carbachol should have been simply additive. However, DBcAMP exerted a synergistic effect on the action of carbachol. The likeliest explanation thus appears to be that DBcAMP in some way increases the availability or improves the efficiency of utilization of an independent element in the secretory process. The possible nature of this element will be considered below after a discussion of the information concerning changes in cyclic AMP concentration in pancreatic tissue after stimulation with carbachol and pancreozymin.

The only published report of the measurement of cAMP concentrations in the presence of secretagogues is that of Johnson, Sherratt, Case, and Scratcherd (1970). They found that either pancreozymin or acetylcholine caused a rapid, but transient, increase in the cAMP content of cat pancreas. The maximal values were observed within 30 seconds after drug addition, and the cAMP content had returned to control values within one minute even though secretion continued for many minutes. They also found that pretreatment of the animal with atropine blocked the secretory response to acetylcholine but had no effect upon the changes in acetylcholine-induced cAMP concentrations.

Heisler et al.(1972) have made similar observations using carbachol to evoke secretion. In addition, they investigated the effect of carbachol upon cAMP accumulation as a function of the Ca^{2+} concentration in the incubation medium. When cAMP content was measured two minutes after carbachol addition, it was found to increase when the $[Ca^{2+}]$ was 10^{-4} *M* or lower, but to actually decrease if the $[Ca^{2+}]$ was 5×10^{-4} *M* or greater (Figure 12). When external $[Ca^{2+}]$ was 5×10^{-6} *M*, the content of cAMP was nearly 15 times greater (Figure 11) in the presence than in the absence of carbachol. These results are to be contrasted to the effect of changing medium $[Ca^{2+}]$ upon the rate of carbachol-induced protein secretion (Figure 11). Below 10^{-5} *M* Ca^{2+}, carbachol had no effect upon secretion, and its maximal effect required between 10^{-4} and 5×10^{-4} *M* Ca^{2+}, a concentration at which the cyclic AMP content was changed very little by carbachol addition. Thus there was, under these circumstances, an inverse relationship between cAMP concentration and the rate of protein secretion. In further work (Heisler et al., 1972), it was found

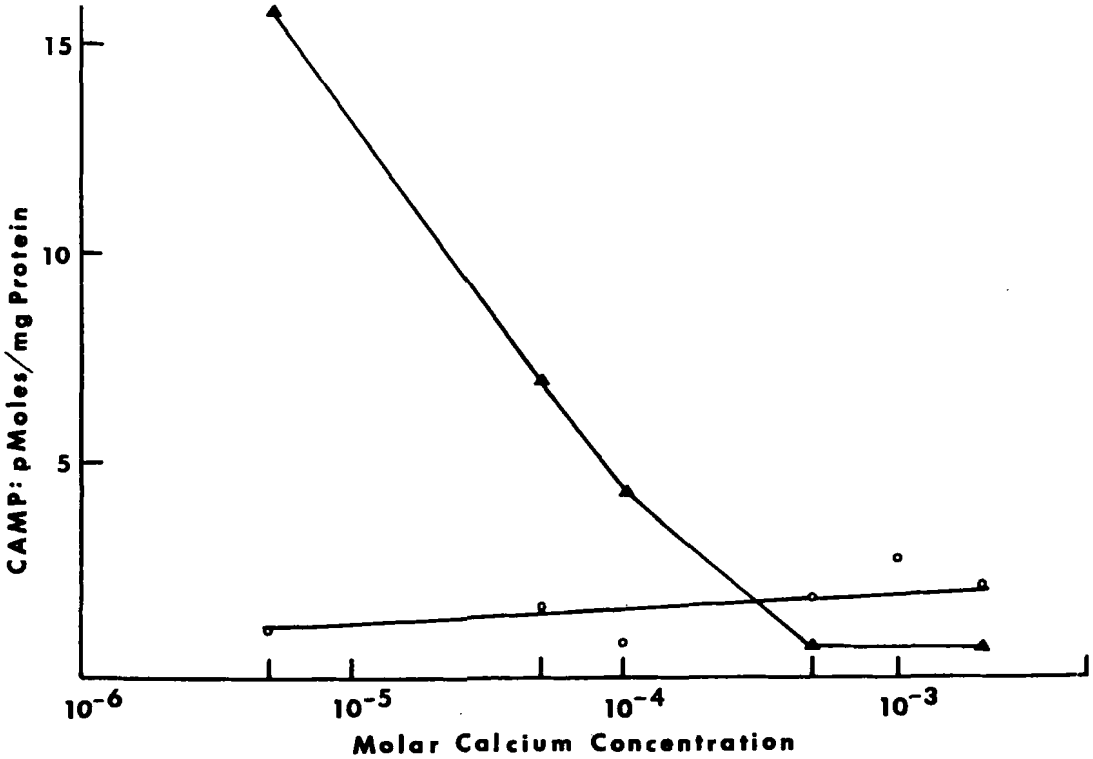


FIGURE 11. Effect of carbachol on cAMP concentration in rat pancreas in vitro as a function of $[Ca^{2+}]$. Incubation time 2 min.

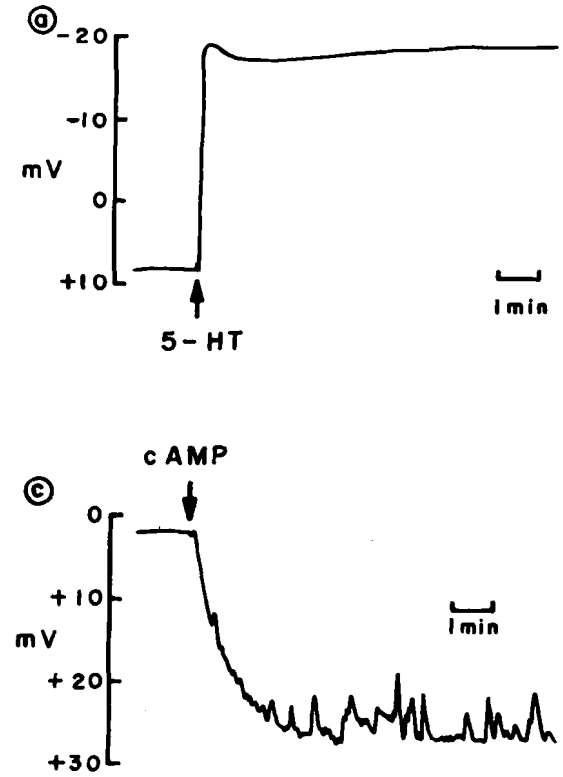


FIGURE 12. The effect of the addition of either 10^{-8} *M* HT (above) or 10^{-2} *M* cAMP (below) upon the transepithelial potential of the isolated fly salivary gland. (See Prince et al., 1972 for details.) Note that even though both agents, at these concentrations, induce a maximal increase in fluid secretion, their effects upon transepithelial potential are exactly the opposite.

that the effects of DBcAMP upon secretion were not blocked by the addition of Mn^{2+} to, or the removal of Ca^{2+} from, the medium. The addition of Mn^{2+} blocked carbachol-induced secretion, and it is thought to act by inhibiting Ca^{2+} entry (Sabatini-Smith and Holland, 1969; Bulbring and Tomita, 1969; Hagiwara and Nakagima, 1966; Dean and Matthews, 1970a,b). Thus, in contrast to carbachol-induced secretion, DBcAMP-induced secretion apparently did not require external calcium. However, if the pancreatic slices were incubated in EGTA, then DBcAMP-induced secretion was blocked. This suggests that DBcAMP mobilized calcium from an intracellular pool, and brought about its effects on secretion by a primary effect on calcium mobilization.

One interpretation of these data is that as calcium enters these cells under the influence of carbachol (Hokin and Hokin, 1961) and the $[Ca^{2+}]$ within the cytosol increases, then the adenyl cyclase which has been activated by carbachol is inhibited and/or the phosphodiesterase is activated. This would mean that as extracellular $[Ca^{2+}]$ was lowered, the rate of calcium entry would be slower, and the cAMP concentration would fall less. If this is a reasonable model of the events in secretion, it suggests immediately that the key second messenger in secretion is Ca^{2+} and not cAMP. The model would account for the ability of DBcAMP to enhance carbachol secretion, if one postulates that DBcAMP can promote the movement of calcium out of subcellular stores into the cytosol in this tissue as it does in others. However, it is still not readily apparent from this model why theophylline does not potentiate the effect of carbachol unless a calcium-activated phosphodiesterase is no longer inhibited by theophylline.

We must, therefore, conclude that the only apparent role of cAMP in the control of the secretory response of the exocrine pancreas is that of guaranteeing the availability of calcium for stimulus-secretion coupling.

ENZYME SECRETION IN THE PAROTID GLAND

The secretion of amylase by the parotid gland in response to epinephrine takes place by a mechanism which is thought to be morphologically similar to that in the exocrine pancreas (Amsterdam, Ohad, and Schramm, 1969). However, a major stimulus for secretion is epinephrine and the addition of this agent to parotid slices leads to an immediate and sustained increase in cAMP concentration within the tissue. Thus, the situation differs in this regard from that seen in the pancreas. Schramm and his colleagues have presented considerable evidence in support of the concept that cAMP is an obligatory intermediate in this response (Bdolah and Schramm, 1965; Babad, Ben-Zvi, Bdolah, and Schramm, 1967; Schramm and Naim, 1970). Although cAMP itself will not evoke secretion, DBcAMP or caffeine will. Caffeine or theophylline will enhance the effect of a submaximal dose of epinephrine. Salmon and Schramm (1970) have described a specific cAMP binding site in isolated microsomes from these glands. No suggestion was offered as to how cAMP binding was translated into secretion. One possibility is that in this gland, as in the pancreas, cAMP functions to liberate calcium for stimulus-secretion coupling. It is known that in many organs, including the parotid gland (Selinger, Naim, and Lasser, 1970), the microsomal fraction has the capacity to take up and store large quantities of calcium. It is also known that Ca^{2+} is required for either epinephrine or DBcAMP-induced secretion (Rasmussen and Tenenhouse, 1968; Selinger and Naim, 1970). It thus seems possible that cAMP binding is the first step in a Ca^{2+} release process. If so, the situation in the parotid is similar to that in the exocrine pancreas except that the relative importance of two major elements in the control system, Ca^{2+} and cAMP, are reversed in the two glands, i.e., in the parotid the direct uptake of Ca^{2+} by the gland in response to epinephrine is insufficient to evoke high rates of secretion, but cAMP-mediated calcium release from internal stores supplies a significant proportion of the required calcium for stimulus-secretion coupling.

Other evidence makes it clear that DBcAMP does not mimic all the effects of epinephrine on the parotid (Batzri, Amsterdam, Selinger, Ohad, and Schramm, 1971). When epinephrine acts, in addition to protein secretion, there is a rapid release of K^+ and the formation of numerous intracellular vesicles. Both responses require the presence of Ca^{2+}. When monobutyryl cAMP is used to induce secretion, there is no associated K^+ release or vacuole formation. Thus, as in other tissues, cAMP or its analog does not completely mimic the effects of the first messenger.

The situation in secretory cells may be similar to the activation of glycogenolysis in different types of muscle as discussed above (Figure 8) in terms of different possible relationships between Ca^{2+} and cAMP in coupling cell activation to cell response. In addition, the source of calcium for coupling stimulus to secretion may vary as it does in the case of excitation-contraction coupling in different forms of muscle (Bianchi, 1972) being extracellular in some cases, intracellular in others, and both in many others.

The above view of the function of cyclic AMP in secretion does not preclude its functioning in additional ways. One suggested way is that of altering microtubular function (Rasmussen, 1970; Goodman et al., 1970), but at the present no good information is available to judge this possibility. There is, in addition, the problem that following the initial secretory response, a necessary component of secretion is the translation of vesicles deep within the cell body toward the cell surface. Very little is known about the control of this process, but it is possible that cAMP is involved. This possibility remains to be seriously examined.

INSULIN SECRETION

The most thoroughly studied example of the control of endocrine secretion is that of insulin secretion from the β-cells of pancreatic islets. In general, it is felt that the basic process is similar to that observed in the exocrine pancreas (Lacey, 1970; Mayhew, Wright, and Ashmore, 1969; Frohman, 1969). The situation is complicated by the fact that a number of different agents will stimulate insulin release, including hexoses, pentoses, amino acids, peptide hormones, tolbutamide and related drugs, and DBcAMP. How these separate controls operate is not known, but it seems possible that they all do not act through a single mechanism. It is generally agreed that the single most important first messenger is glucose. An increase in glucose concentration is associated with an increase in insulin release. On the other hand, under most test conditions, the other secretagogues are either ineffective or minimally effective in the absence of glucose (Malaisse, Malaisse-Lagae, and Mayhew, 1967; Buchanan, Vance, and Williams, 1969; Curry, 1970). To complicate matters further, there is considerable evidence which suggests that it is not glucose itself but a glycolytic intermediate which triggers insulin release (Malaisse et al., 1967; Hug and Schubert, 1967; Kuzuya, Kanazawa, and Kosaba, 1966) Thus, the first requirement for initiating insulin release is the capacity to generate this, as yet unidentified, glycolytic intermediate. It is immediately apparent that many of the secondary secretagogues could act in the presence of glucose to alter the rate of synthesis or degradation of this intermediate and thereby influence insulin secretion.

The second essential element in the secretory process is calcium. Extracellular calcium ion is essential for secretion regardless of the secretion-provoking agent (Grodsky and Bennett, 1966; Milner and Hales, 1968; Hales and Milner, 1968; Curry, Bennett, and Grodsky, 1968a,b; Malaisse-Lagae and Malaisse, 1971; Dean and Matthews, 1970a,b). There is an uptake of calcium associated with a calcium current (Dean and Matthews, 1970a,b). High extracellular Mg^{2+} blocks this uptake of calcium and also blocks secretion. Within a range of $[Ca^{2+}]$ from 0.05 to 2.0 mM there is a linear increase in the rate of insulin secretion in the presence of an optimal amount of extracellular glucose. However, when tolbutamide is used to stimulate secretion, there is a linear increase in secretion rate between 0.1 and 6.0 mM extracellular Ca^{2+}. In addition to Ca^{2+}, sodium ions are required (Milner and Hales, 1968; Hales and Milner, 1968). It is not yet clear whether Na^+ actually participates directly, or whether the relationship between Na^+ and Ca^{2+} in this tissue is similar to that in nerve and other tissues (see above) in which the gradient and fluxes of Na^+ determine in large part the distribution and fluxes of Ca^{2+}. One of the most general conclusions concerning secretory processes is that they all require Ca^{2+} as a key factor in stimulus-secretion coupling (Douglas, 1968; Rubin, 1970; Douglas and Poisner, 1964).

The possibility that cAMP might be involved in the process of insulin release came initially from a study of the action of epinephrine on the endocrine pancreas. Coore and Randle (1964) found that epinephrine inhibited insulin secretion. Porte (1967a,b) found that the addition of α-adrenergic blocking drugs, such as phentolamine, reversed the effect of epinephrine. In the presence of α-blockers, epinephrine was found to stimulate rather than inhibit insulin secretion. This stimulation could be inhibited by the addition of

β-adrenergic blocking agents. It was then found that theophylline would stimulate secretion and would lead to an increase in tissue cAMP content. This rise in cAMP content could be blocked by epinephrine (Turtle and Kipnis, 1967; Turtle, Littleton, and Kipnis, 1967), but if phentolamine was added, then secretion was stimulated and cAMP content increased. There was a positive correlation between insulin secretion and cAMP content.

The finding that glucagon and ACTH, hormones which stimulate adenyl cyclase in other tissues (Makman and Sutherland, 1965; Robison, Exton, Park, and Sutherland, 1967; Exton and Park, 1968; Murad and Vaughn, 1969; Butcher, Baird, and Sutherland, 1968; Grahme-Smith, Butcher, Ney, and Sutherland, 1967), stimulated insulin release lent further support to the concept that cAMP was a key second messenger in the control of insulin release. The effect of glucagon on insulin secretion was enhanced by methyl-xanthines (Lambert, Jeanrenaud, and Renold, 1967; Lacey, Young, and Fink, 1968; Allan and Tepperman, 1969), and so was the effect of ACTH (Lebovitz and Pooler, 1967). In addition, Turtle and Kipnis (1967) found that glucagon induced an increase in the cAMP content of islet tissue. Much of this data involving the effect of methyl xanthine upon secretion has been interpreted as supporting evidence that cAMP is involved in these processes. However, in view of the effects of these drugs upon calcium movements in muscle (see above) and the important role of calcium in secretory processes, it is not possible to exclude an effect of these drugs upon cellular calcium metabolism as the basis for their effects upon secretion.

All of these data support the concept of a key and obligatory role of cAMP in insulin secretion. However, one very striking piece of negative information argues against this conclusion. There is no evidence that glucose, in concentrations which stimulate insulin release, causes any change in the cAMP content of islet tissue. Montague and Cook (1970), in failing to demonstrate a change in cAMP content, were led to conclude that this cyclic nucleotide was not involved in glucose-induced insulin secretion. Nevertheless, as discussed above, there is considerable evidence that cAMP and agents which alter its metabolism can exert an important influence on the insulin release process.

One explanation for the role of cAMP, first offered by Malaisse et al. (1967), is based on the premise that a rate limiting factor in insulin release is the as yet unidentified glycolytic intermediate. In this case, cAMP could act by altering the concentration of this key intermediate by an effect upon glycolysis (Samols, Marri, and Marks, 1966). However, there is an alternative explanation, i.e., a direct effect of cAMP upon calcium metabolism within the β cells. In their most recent studies, Malaisse-Lagae and Malaisse (1971) have shown that regardless of which agent is used to induce insulin secretion, there is an associated uptake of ^{45}Ca. Conversely, when insulin secretion was inhibited, ^{45}Ca uptake was also inhibited. They were led to conclude that Ca^{2+} is the primary coupling factor between stimulus and secretion in this tissue, and that other agents acted by changing the calcium uptake into these cells. They favored the view that cAMP and those agents which appear to act via cyclic AMP did so by regulating the content of the postulated critical glycolytic intermediate which, in turn, controls cellular calcium uptake and, thereby, secretion. However, none of their data rule out the alternative possibility that cAMP acts by altering intracellular Ca^{2+} distribution rather than glycolysis as a means of controlling insulin secretion, and in more recent studies they (Brisson, Malaisse-Lagae, and Malaisse, 1972) have concluded that the effect of cAMP upon insulin secretion could be due to a cAMP-induced translocation of calcium within the cell from an organelle-bound pool to a cytosolic ionized calcium pool. This conclusion was based upon the facts that: 1) neither glucose nor leucine could promote insulin release in the absence of external calcium by DBcAMP nor could theophylline partially restore responsiveness; and 2) theophylline was shown to increase ^{45}Ca efflux from prelabeled glands even in the absence of glucose. These results are similar in many respects to those discussed in relationship to exocrine secretion in the pancreas.

CYCLIC AMP AND SECRETION IN PERSPECTIVE

In conclusion, both exocrine and endocrine secretion in the pancreas represent well-studied examples in which the second messenger hypothesis, as generally stated, is inadequate to

account for the available information. The evidence in both cases favors the view that the primary second messenger is calcium, and that cAMP plays a subsidiary role. However, in view of the range of possible relationships between Ca^{2+} and cAMP in cell activation, as already discussed in this review, it is possible that in other secretory systems, e.g., the parotid, the relative importance of these two second messengers is reversed.

There are many other secretory systems, including nerve endings, in which there is a great deal of evidence that both of these second messengers are involved in some fashion (Douglas, 1968; Rubin, 1970; Robinson et al., 1971; Rasmussen, 1970). However, as yet the evidence is even less complete and more contradictory than in the systems already examined. No attempt will be made to review this mass of data. It is our belief that calcium will be found to be a critically important component of all of these processes (Douglas, 1968), and that the role of cyclic AMP will be found to vary over the same range of involvement as seen in the systems already discussed.

A particular feature of these secretory systems which is yet to be established is the source of the intracellular calcium which appears to be mobilized by cAMP. It is possible that this calcium represents either that stored in mitochondria, or in the cell microsomes; that, in other words, secretory systems of this kind have an intracellular organelle analogous to the sarcoplasmic reticulum of muscle.

CYCLIC AMP, CALCIUM, AND HORMONE ACTION

As noted previously, there are other systems in which mitochondrial calcium appears to play a significant role, and in which cAMP regulates cytosolic $[Ca^{2+}]$ by regulating calcium flux across the mitochondrial membrane. Not all systems of this type will be discussed, but several of the more thoroughly studied will be examined. Of these, two are secretory systems: the fly salivary gland and the mammalian adrenal cortex. Both differ in a fundamental way from the previously discussed secretory systems in that in neither is there any evidence of a quantal release of a product stored in intracellular vesicles. In the fly salivary gland, the secretion is a clear fluid, nearly equivalent to isotonic KCl; and in the case of the mammalian adrenal cortex, steroid hormones are secreted without evidence of intracellular packaging.

Serotonin and the Fly Salivary Gland

The abdominal portion of the salivary gland of the blowfly *(Calliphora erythrocephala)* has been used to study the mode of action of 5-hydroxytryptamine (Berridge, 1970; Berridge et al., 1963; Berridge and Prince, 1971, 1972; Oschman and Berridge, 1970; Prince, Berridge, and Rasmussen, 1972). When the abdominal portion of this gland is exposed to 10^{-8} *M* or higher concentrations of 5-hydroxytryptamine (5HT), there is a marked increase in the rate of fluid secretion. A similar increase in rate is observed following the addition of 10^{-2} *M* cAMP. Two important similarities in the response of 5 HT and cAMP are observed: the maximal rates of secretion induced by maximal doses of the two agents are the same, and the composition of the fluid secreted by the gland in response to cAMP is the same as that of the fluid secreted in response to 5HT, being an isotonic solution of potassium and chloride with minor amounts of other ions. The logical conclusion is that cAMP mimics the physiological effect of 5HT in this organ, thus fulfilling one of the major criteria used to establish that cAMP is a second messenger in the response of this gland to 5HT. The other criteria are fulfilled as well: cAMP concentrations increase in response to 5HT, and theophylline potentiates the effect of submaximal doses of 5HT. However, if the change in transepithelial membrane potential following addition of cAMP is compared to that seen after 5HT addition, a completely different response is seen (Figure 12). The addition of *5HT* leads to an immediate and sustained *increase* in potential, i.e., a more negative potential of the luminal fluid as compared to the serosal fluid, while *cAMP* addition leads to a less rapidly induced decrease in transepithelial potential, i.e., a more positive potential of the luminal fluid as compared to the serosal fluid.

The importance of these results lies in the fact that when examined at one level of function, i.e., fluid secretion, the response of the salivary gland to cAMP appears to mimic the effect of the first messenger, 5HT, but when examined at a different level of function, there is a distinct difference between the effects of these two stimulants. The difficulty in applying the criteria established for validating the second messenger hypothesis is

immediately evident. Does one look at the potential response and argue that because 5HT and cAMP act differently, cAMP is not a second messenger, or does one argue that the integrated cellular response, namely fluid secretion, is similar and, therefore, that cAMP is a second messenger? Obviously, neither alternative is a useful one. The point is that the highly differentiated cells of this gland are capable of only one response, fluid secretion, regardless of the initiating stimulus. This means that the metabolic and structural components of these cells are organized as a net, rather than as a linear, sequence. The closest analogy, in a well characterized biochemical control system, is that involved in blood clotting. This system responds in a very stereotyped fashion to a number of different initial stimuli, clearly acting at different focal points within the metabolic net. However, precisely because the system operates as a net with multiple positive and negative feedback loops, the final common event, the clotting reaction, is always the same.

If, as seems most likely, the metabolic reactions controlling fluid secretion in the fly salivary gland operate in a similar net-like fashion, then the difference between the effects of 5HT and cAMP upon transepithelial potential and their similarities on fluid secretion can be accounted for simply by assuming that they activate the net at different points, or, alternately, that 5HT has several simultaneous and integrated effects upon the net, only one of which is mediated by the second messenger cAMP.

Experimental evidence supports this latter alternative. Studies of the role of calcium in the action of 5HT upon the blowfly salivary gland demonstrate that both cAMP and Ca^{2+} are involved as second messengers in the activation of secretion in these cells (Prince et al., 1972). This conclusion is based upon the following facts: 1) when either 10^{-8} *M* 5HT or 10^{-2} *M* cAMP is added to a medium in which the abdominal portion of the salivary gland of the blowfly is incubated, both evoke maximal secretion of fluid of identical composition; 2) if the glands are preincubated in ^{45}Ca and the effects of the two agents upon radiocalcium efflux are examined, both increase the rate of ^{45}Ca efflux (Figure 3); 3) when calcium is removed from the medium, both agents induce a submaximal increase in the rate of secretion which is not sustained, but maximal rates of secretion are reestablished upon the addition of calcium (Figure 13); 4) the time course of the secretory response induced by 5HT in a calcium-free medium is the same as the time course of increase in ^{45}Ca efflux under similar conditions of incubation (Figure 13); 5) 5HT causes a rise in cAMP concentration within the cell whether calcium is present or absent; 6) 5HT, but not cAMP, causes an increase in the uptake of radiocalcium by this gland; 7) 5HT causes the transepithelial potential to increase, but cAMP causes it to decrease (Figure 12); and 8) if Ca^{2+} is removed from the medium, then both cAMP and 5HT cause a decrease in potential.

It is possible to construct a model of the events in these cells (see Berridge and Prince, 1972). In this model (Figure 14), both Ca^{2+} and cAMP serve as second messengers following 5HT action. The cytosolic $[Ca^{2+}]$ is controlled both by a direct effect of 5HT upon calcium uptake, and by an effect of cAMP on the intracellular distribution of Ca^{2+}. However, it seems clear that cAMP must exert an additional effect, largely independent of calcium, which may be a direct activation of a K^+-pump on the luminal membrane of the cell. On the other hand, Ca^{2+} must also exert an effect, independent of cAMP, to account for the calcium-dependent differences between the 5HT and cAMP-induced responses (Figure 12). This effect of Ca^{2+} may well be that of controlling the chloride permeability of the luminal membrane. These points of action of Ca^{2+} and cAMP are not established but are consistent with presently available information. It remains possible that calcium is a necessary component of the cAMP-controlled system in the luminal membrane.

The one aspect of this system which has yet to be investigated is the role of protein kinases. It is an attractive possibility that cAMP controls the activity of the K^+-pump by controlling the activity of a membrane-bound protein kinase. However, there are no data at present concerning this point.

The other aspect of this response which is of particular interest is the increase in radiocalcium efflux seen after either 5HT or cAMP addition (Figure 3). As shown in Figure 13, the time course of this increase in efflux, and the time course of response of the gland to 5HT addition in the absence of external calcium, are quite similar. The most likely interpretation of this relationship is that secretion is maintained at higher than normal, although constantly declining, rates as calcium is mobilized from an intracellular pool under the

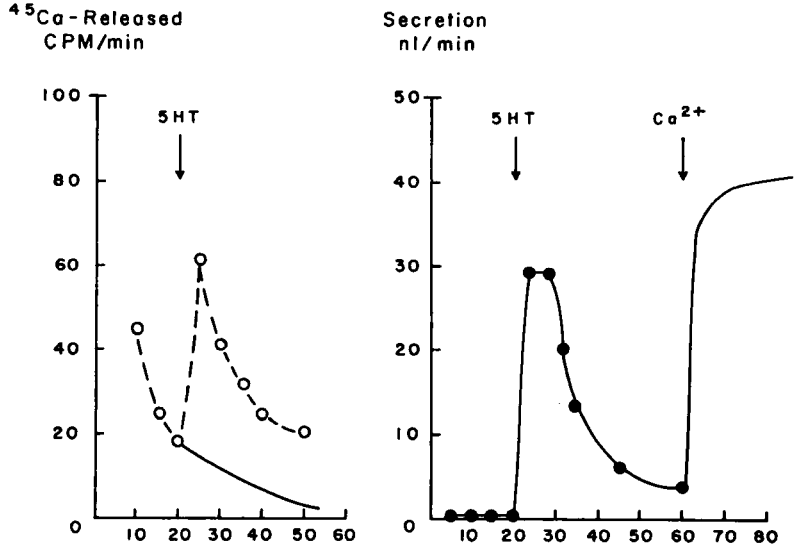


FIGURE 13. The effects of the addition of 10^{-8} *M* 5HT to isolated fly salivary glands. On the left is shown the effect of 5HT addition upon radiocalcium efflux in glands prelabeled with ^{45}Ca and then incubated in a calcium-free Ringer solution containing 5 mM EGTA. The dotted line (o – o) represents glands to which 5HT was added, the solid line (–) the efflux from untreated control glands. On the right is shown the effect of 5HT upon glands preincubated in a Ringer solution containing calcium and then placed in a calcium-free Ringer solution containing 5 mM EGTA at time 0. Twenty minutes later, 10^{-8} *M* 5Ht addition led to an initial increase in rate of fluid secretion which was not sustained. Readdition of calcium to the Ringer solution restored fluid secretion to the maximally expected rate. Note particularly that the time courses of radiocalcium efflux and that of fluid secretion after 5HT are nearly the same.

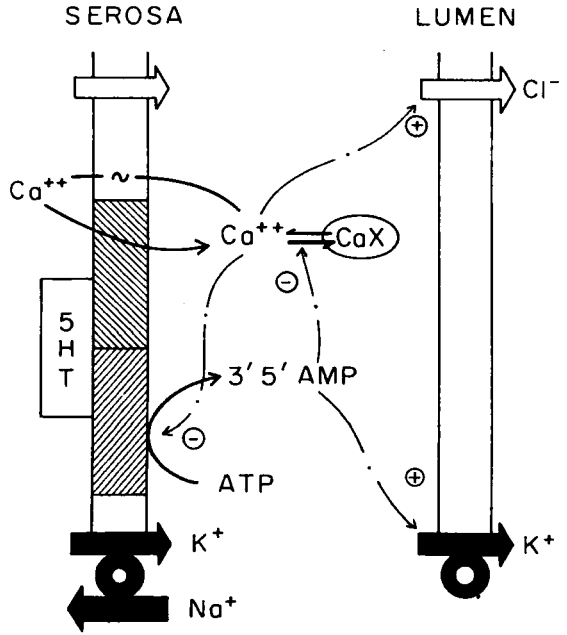


FIGURE 14. A model of the events in the fly salivary gland following 5HT addition. The first messenger, 5HT, increases the entry of Ca^{2+} across the serosal membrane and activates membrane-bound adenyl cyclase. The resulting increase in 3'5' AMP concentration causes a shift of bound calcium, CaS, from a subcellular (probably mitochondrial) compartment to the cytosol and also exerts a direct effect upon the transport functions of the luminal membrane, possibly an activation of a primary potassium pump. The calcium concentration in the cell cytosol increases as a consequence of both the direct effect of 5HT upon calcium entry, and the effect of cAMP upon the subcellular redistribution of calcium. The rise in cytosol Ca^{2+} concentration exerts an independent effect upon luminal membrane function, possibly that of altering chloride permeability. In addition, Ca^{2+} in the cytosol serves as a feedback inhibitor of adenyl cyclase.

influence of cAMP. Readdition of external calcium at any point reestablishes secretion by repleting this cellular calcium pool. The subcellular location of this pool remains to be established. However, as will be discussed more fully in our consideration of parathyroid hormone action, the most likely site is the calcium pool in the mitochondrial matrix space.

The two important conclusions to be drawn from these studies on the fly salivary gland are 1) just as in the mammalian heart, cAMP regulates cellular calcium metabolism and this is a critically important component of the action of this cyclic nucleotide; 2) first messenger exerts effects upon cell function in addition to an activation of adenyl cyclase, one of which appears to be that of altering the permeability of the surface membrane to calcium; and 3) both cAMP and Ca^{2+} serve a second messenger function in this gland.

ACTH AND ADRENAL STEROIDOGENESIS

The control of steroid synthesis in the adrenal cortex by the tropic hormone adrenocorticotropin, ACTH, will be discussed as a model of tropic hormone action because more is known about this system than about the control of ovarian or testicular steroid synthesis. However, what is known about the latter suggests that the general features are similar to those in the adrenal (Robison et al., 1971). When ACTH acts, the following changes in adrenal metabolism are seen (Sayers and Travis, 1970; Garren, Gill, Masui, and Walton, 1971; Robison et al., 1971): 1) an increase in the steroid synthesis, primarily corticosterone and cortisol; 2) an increase in intracellular cyclic AMP; 3) an increase in glycogenolysis; 4) a depletion of ascorbic acid; 5) an increase in protein synthesis; 6) an increased uptake of calcium; and 7) an increase in the conversion of cholesterol esters to cholesterol. Steroid synthesis can also be increased by high concentrations of exogenous cAMP. When cAMP stimulates steroidogenesis, there is no increase in Ca^{2+} uptake, but a requirement for Ca^{2+} is still apparent (Farese, 1971a,b; Leier and Jungman, 1970; Garen et al., 1971). The addition of cAMP also enhances protein synthesis, glycogenolysis, and the conversion of cholesterol esters to cholesterol. It is not clear whether glycogenolysis and cholesterol ester hydrolysis require calcium, but both the cAMP-induced increase in steroid synthesis and protein synthesis require calcium. The same is true when ACTH invokes a response. However, the problem is complicated in the case of ACTH because when $[Ca^{2+}]$ is decreased markedly, the ACTH-activation of adrenyl adenyl cyclase is diminished (Lefkowitz et al., 1970).

It is generally agreed that in order for either ACTH or exogenous cAMP to induce steroid secretion, continual protein synthesis is required (Ferguson, 1963). If protein synthesis is inhibited, then steroid synthesis is inhibited. These findings have led to the proposal that steroid synthesis is controlled by an adrenal protein which turns over with a very rapid halflife (ca. 15 minutes). However, inhibitors of protein synthesis do not block either the conversion of cholesterol esters to cholesterol or the breakdown of glycogen (Garren et al., 1971). Hence it appears that ACTH has at least three independent intracellular effects which are normally coordinated to insure optimal and continued steroid synthesis. From the point of view of our present discussion, the most interesting finding is that relating enhanced protein synthesis to changes in $[Ca^{2+}]$. When adrenal sections are first depleted of calcium and then treated with either ACTH or cAMP and calcium re-added in various concentrations, good correlations are observed between the rate of steroid production and the rate of protein synthesis (Farese, 1971c).

It is of interest that extracellular K^+ has also been shown to be required both for ACTH-induced protein synthesis and steroidogenesis, but no studies have been carried out to determine K^+ and/or Na^+ flux during ACTH-induced steroid synthesis.

It is not yet completely clear how ACTH or cAMP brings about an increase in protein synthesis. It has been shown that following ACTH administration there was an increase in amino acid incorporating activity in the 15,000 g supernatant of adrenal homogenates. When this supernatant was further fractionated into a 105,000 g supernatant and a microsomal fraction, both fractions showed enhanced activity compared to similar fractions obtained from control cells. The increased activity in the 105,000 g supernatant was due to a macromolecular factor which enhanced the transfer of amino acid from acyl ~ transfer RNA to protein, the so-called transfer enzyme. When 5×10^{-8} *M* calcium was added to this

transfer enzyme fraction prepared from a homogenate of control glands, the incorporation of ^{14}C-glycine into protein was stimulated (1700 to 2300 cpm). In contrast, when the transfer enzyme fraction was obtained from ACTH-treated glands, this activity was already high (2500 cpm) and rose very little (2700 cpm) upon addition of 5×10^{-8} *M* Ca^{2+} (Farese, 1971c). In contrast, when similar fractions were treated with 10^{-5} *M* EGTA (a relatively specific chelator of calcium), there was little change in the activity of the transfer enzyme fraction from control glands, but there was a significant inhibition of activity when this fraction from ACTH-treated glands was employed.

These results are consistent with a model of cell activation in which ACTH causes an increase in cytosolic [Ca^{2+}] which, in turn, activates transfer enzyme function and stimulates protein synthesis. However, there is an additional effect of ACTH upon microsomal function which is apparently not mediated by calcium. This may be due to the activity of cAMP-dependent protein kinase. Such a protein kinase has been identified in the adrenal microsomes (Walton, Gill, Abrass, and Garren, 1971). It has been found to catalyze the phosphorylation of ribosomes and to be stimulated by 10^{-7} *M* cAMP. The problem, as noted earlier, is that of determining the specificity and, therefore, the physiological significance of this phosphorylation, and what function it may serve at the level of the ribosome. Assuming it is specific and related to control of adrenal cell function, a tentative model emerges in which both calcium and cAMP serve as intracellular messengers following ACTH action, and each acts upon different but sequential steps in protein synthesis to control, in a concerted fashion, the synthesis of a specific protein(s) required for steroid synthesis. This would, of course, be similar, operationally, to the situation in the heart where the two messengers Ca^{2+} and cAMP act in a concerted fashion to enhance glycogenolysis. In the case of the adrenal, it is not yet clear whether the other effects of ACTH on glycogenolysis and cholesterol ester hydrolysis are mediated by cAMP, or Ca^{2+}, or both. Additionally, there is no clear evidence yet available that cAMP alters radiocalcium efflux from adrenal cells, or that an increase in intracellular [Ca^{2+}] regulates the activity of either adenyl cyclase or phosphodiesterase. This information is needed before it can be concluded that the Ca^{2+}-cAMP relationship is fundamental to the control of adrenal steroidogenesis. On the other hand, none of the presently available information is incompatible with this supposition, and much of it strongly supports this concept.

GLUCAGON AND HEPATIC GLUCONEOGENESIS

When glucagon is perfused into the isolated liver from a fasted animal, it increases the rate of glucose synthesis from pyruvate, lactate, or alanine and other amino acids. This increase in the rate of gluconeogenesis is preceded by a rise in cAMP concentration and a release of cAMP into the perfusate. The infusion of cAMP will mimic the effect of glucagon (Exton, Mallette, Jefferson, Wong, Friedmann, Miller, and Park, 1970). In addition, the removal of Ca^{2+} from the perfusate does not alter the effects of either glucagon or cAMP (Friedmann and Rasmussen, 1970; Exton et al., 1971). Additionally, neither glucagon nor cAMP increases the uptake of calcium into the liver cells. Here, then, appears to be an exception to the general rule that Ca^{2+} and cAMP are interrelated second messengers in cell activation. Clearly, cAMP is the only well identified second messenger in glucagon action. However, even in this system, the infusion of first messenger or cAMP induces an increase in the rate of calcium efflux qualitatively similar to the efflux changes seen in the fly salivary gland (Friedmann and Park, 1968). This change in Ca^{2+} efflux precedes the increase in glucose synthesis. Also, after the peak of increased calcium efflux, there is an increase in K^+ and Na^+ efflux and a decrease in perfusate pH (Exton et al., 1971, Friedmann, 1972) associated with a hyperpolarization of the plasma membrane of the cell (Friedmann, Somlyo, and Somlyo, 1971). Similar changes in electrolyte fluxes are seen when cyclic GMP is employed even though present evidence indicates that cGMP does not activate cAMP-dependent protein kinases (Exton et al., 1971).

It is difficult to assess the importance of these ionic changes in the control of hepatic gluconeogenesis. On the one hand, if the liver is perfused with the local anesthetic tetracaine before either glucagon or cyclic AMP is infused, neither of the latter agents stimulates gluconeogenesis or alters ion fluxes even though the basal rate of O_2 consumption and glucose production are not greatly altered (Friedmann and

Rasmussen, 1970). Thus, it would seem that by stabilizing some cellular or intracellular membrane(s) with tetracaine, the effect of cAMP on gluconeogenesis is blocked. However, in the case of livers from adrenalectomized animals, the infusion of glucagon leads to a rise in cAMP concentration, and a normal change in ion fluxes, but very little increase in glucose formation (Friedmann, Exton, and Park, 1967; Friedmann and Park, 1968; Exton et al., 1970). So, under a different set of conditions, changes in ion flux occur but gluconeogenesis is not enhanced.

In view of the interrelationships between Ca^{2+} HPO_4^{2-} and H^+ depicted in Figure 4, and their coupling to Na^+ and K^+ exchange across the cell membrane, these ionic changes in the liver could all be a consequence of a primary inhibitory effect of cAMP upon mitochondrial calcium uptake with a consequent redistribution of subcellular calcium. Because of these interrelationships, whenever there is a significant change in the flux of one ion, e.g., Ca^{2+}, across cell or subcellular membrane, the distribution of all other ions is changed; the entire ionic milieu within the various subcellular compartments is altered, leading to an amplification of the original change and serving to integrate the response of the metabolic systems. Thus, it seems quite possible that these ionic changes are of considerable importance in the hepatic response to glucagon.

Studies of this hepatic system also reveal another of the difficulties in applying the original second messenger criteria to a particular problem. By the established criteria, cyclic AMP is a second messenger in the action of glucagon on the liver. One of the reasons for reaching this conclusion is that the infusion of cyclic AMP will produce an increase in gluconeogenesis as does glucagon infusion. However, the infusion of 10^{-6} *M* manganese will also produce an increase in the rate of gluconeogenesis without producing any change in the rate of cyclic AMP formation or its concentration (Friedmann and Rasmussen, 1970). The question which arises is should we consider Mn^{2+} as a second messenger in the action of glucagon? Unfortunately, it is not possible to measure changes in the intracellular or cytosolic concentrations of Mn^{2+}, so it is not possible, at present, to rule in or out the possibility that such changes are involved in the response of the liver cell to glucagon. However, the difficulty is technical rather than conceptual.

The major difficulty in further defining the role of Ca^{2+} and cAMP in hepatic gluconeogenesis is that, in spite of intensive study, the exact step or steps in the gluconeogenic pathway which is controlled by cAMP has not been identified. The most likely candidates are the mitochondrial enzyme pyruvate carboxylase and the cytosolic enzyme PEP carboxykinase. However, to date no one has demonstrated a direct effect of cAMP on the activity of either isolated enzyme. There is also no evidence that either enzyme is activated or inactivated by phosphorylation or dephosphorylation. While it has not been shown that calcium regulates the activity of PEP carboxykinase, it has been demonstrated that Ca^{2+} is an inhibitor of pyruvate carboxylase (Kimmick and Rasmussen, 1963) and of the cytosolic enzyme pyruvate kinase (Bygrave, 1966). One model of control is one in which a shift in calcium from mitochondrial matrix space to the cell cytosol simultaneously activates (deinhibits) pyruvate carboxylase and inhibits pyruvate kinase. If this were the mechanism, or an important part of the mechanism underlying glucagon action, it would then suggest that just as in the other cells, so too in the liver, cAMP controls the intracellular distribution of calcium. At this stage of our knowledge, it is clear that in the case of hepatic gluconeogenesis neither the calcium model nor the protein kinase activation model has been shown to be operative in the immediate control of this metabolic sequence. The data which are available indicate that cAMP probably does influence the distribution of calcium within liver cells, but it is not clear whether, or how, this is related to changes in the rate of gluconeogenesis.

RENAL GLUCONEOGENESIS

In contrast to the situation in the liver, gluconeogenesis in the renal cortex is controlled by both the extracellular $[Ca^{2+}]$ and $[H^+]$ as well as by cyclic AMP and the first messenger, parathyroid hormone (Nagata and Rasmussen, 1970a,b; Rasmussen and Nagata, 1970; Kurokawa, Ohno, and Rasmussen, 1972). From studies with both tissue culture cells derived from mammalian kidney (Borle, 1970a,b, 1972) and isolated rat renal cortical tubules, the following have been established: 1) PTH activates adenyl cyclase and stimulates the uptake of calcium; 2) the effect of either PTH or cyclic AMP on renal gluconeogenesis

requires calcium; 3) the activation of adenyl cyclase by PTH does not require calcium; 4) both PTH and cyclic AMP stimulate Ca^{2+} efflux; 5) in the absence of Ca^{2+}, H^+ still stimulates gluconeogenesis; 6) even though cAMP and PTH stimulate glucose formation from the same substrates, the degree of stimulation is not the same, e.g., both stimulate glucose formation from malate to the same degree, but cAMP is a much more effective stimulus of gluconeogenesis from pyruvate; 7) an increase in $[Ca^{2+}]$ in the medium, or the addition of either PTH or cAMP in the presence of calcium, appears to regulate the same steps in the overall process, suggesting that they all operate by altering the $[Ca^{2+}]$ in the cell cytosol; 8) increasing intracellular $[Ca^{2+}]$ decreases [cAMP], probably by inhibiting adenyl cyclase, although possible effects of Ca^{2+} upon phosphodiesterase activity have not been examined; 9) a cAMP-dependent protein kinase is present in these tubules (Kuo and Greengard, 1969; Winickoff and Aurbach, 1970), but its function is not known; it probably does not act as a phosphorylase b kinase kinase since cortical nephrons store very little glycogen; and 10) an increase in the phosphate concentration in the medium increases the uptake of calcium by the cell, inhibits calcium-dependent gluconeogenesis, inhibits PTH-stimulated gluconeogenesis, but at the same time increases the cAMP concentration (Kurokawa and Rasmussen, 1972).

Two features of this PTH, Ca^{2+}, cAMP relationship are of particular interest. In comparing the results of Borle concerning the effects of changing the calcium concentration in the extracellular fluids, in the presence and absence of PTH, upon the size of the intracellular calcium pool to the results of Nagata and Rasmussen concerning the effects of these same variables upon glucose formation from pyruvate, a striking similarity is observed (Figure 15). When one compares the rate of gluconeogenesis and the size of the total pool of intracellular calcium, an increase in the concentration of extracellular calcium from 0 to 2.5 mM leads to an increase, but higher concentrations of extracellular calcium have no further effect. Likewise, when PTH is added at any finite extracellular calcium concentration between 0.05 and 2.5 mM, it increases both pool size and glucose formation, but has no effect upon either intracellular calcium pool size or glucose formation when the extracellular calcium concentration is above 2.5 mM (Figure 15).

This is the strongest evidence in support of the view that the rate of renal gluconeogenesis is some function of intracellular calcium pool. However, when phosphate is added, intracellular calcium pool size increases, but gluconeogensis decreases so that there is not always a direct correspondence between the two.

The other feature is that either PTH or cAMP induces an increase in radiocalcium efflux from isolated kidney cells prelabeled with ^{45}Ca. When the effect of cAMP upon radiocalcium efflux from fly salivary glands is compared to its effects upon mammalian kidney cells, there is a striking similarity in response (Figure 16) which is also similar to that seen in the perfused liver (Friedmann and

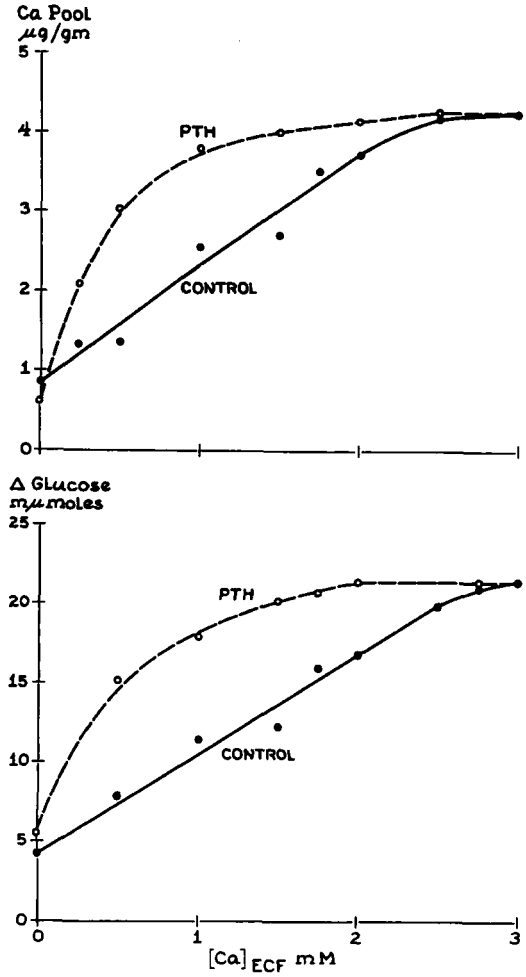
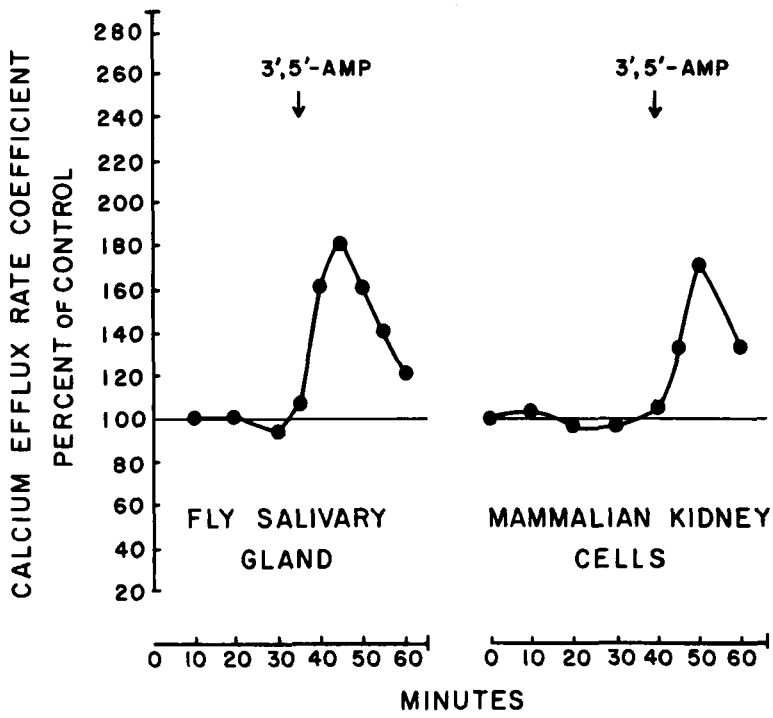


FIGURE 15. The size of the pool of intracellular calcium in kidney cells (top) and the rate of gluconeogenesis from pyruvate in renal tubules (bottom) as a function of the extracellular calcium concentration in the presence (o – o) and absence (• – •) of PTH. The data in the upper part of the graph are from Borle (1970b), and in the bottom from Nagata and Rasmussen (1970b).



FIUGRE 16. The effect of the addition of cAMP upon rate coefficient for calcium efflux in fly salivary gland (from the data of Prince et al., 1972); and the mammalian kidney cell (from Borle, 1972).

Park, 1968) or the amphibian urinary bladder (Schwartz and Walter, 1968). These results argue that in both types of cells, cAMP increases the efflux of calcium from the intracellular pool. The problem is that of identifying this pool. From morphological and biochemical criteria alone, the most logical site in these two organs is the mitochondrial matrix space. This conclusion is supported by the work of Borle upon isolated kidney cells (Borle, 1972) and of Friedmann and Rasmussen (1970) in the perfused liver.

The information bearing on effects of cAMP upon intracellular distribution of calcium concerns its effects upon calcium efflux from isolated kidney cells grown in tissue culture (Borle, 1972). In these cells, a detailed kinetic analysis of calcium uptake and exchange has been performed. Borle (1972) has identified two intracellular calcium pools-compartments with half times of exchange of 20 to 300 minutes, respectively. The estimated sizes of these two intracellular calcium pools are 1.56 and 2.32 nmol/mg protein when the extracellular [Ca^{2+}] was 0.6 mM and the phosphate 1.0 mM. An increase in the extracellular [Ca^{2+}] to 1.3 mM led to an increase in the two pools to 1.83 and 2.54 nmol/mg protein. On the other hand, an increase in phosphate concentration to 2 mM led to an increase in the two pools to 3.19 and 10.3 nmol/mg protein, respectively. Thus, a doubling of phosphate concentration led to a doubling of the size of the smaller pools and to nearly a fivefold increase in the size of the larger. When the phosphate concentration was raised to 5 mM with a [Ca^{2+}] of 1.3 mM, the size of the first pool was 6.0 and that of the second, 470 nmol/mg cell protein. Inhibition of oxidative phosphorylation, by either warfarin or antimycin, inhibited the uptake of calcium into the second pool without greatly changing the size of the first. When cAMP or PTH was added to the cells prelabeled with calcium, either agent increased the rate of Ca^{2+} efflux and the rate of turnover of the second, large, phosphate-dependent intracellular calcium (Borle, 1971, 1972).

These data are consistent with the concept that cAMP alters the rate of calcium efflux from mitochondria to cytosol. It is still not possible to define a mechanism. Cyclic AMP could alter either the rate of energy-dependent calcium uptake or the rate of passive calcium efflux. Either effect could be direct, or mediated indirectly through some additional intracellular mediator. Also, it is

possible that cAMP acts by altering the equilibrium within the mitochondrial matrix space between ionized calcium and the complexed calcium within this subcellular compartment. However, the recent data of Dietze and Hepp (1972) in the heart, showing that cAMP inhibits the Ca^{2+}-stimulated ATPase in the mitochondrial cell fraction, would be consistent with an inhibitory effect of cAMP upon calcium uptake by mitochondria.

All of these facts can be incorporated into a general model of cell activation similar to that seen in the fly salivary gland (see Figure 14). The first messenger, PTH, brings about at least two initial effects: an activation of adenyl cyclase and an increase in the calcium permeability of the cell membrane. The increase in cAMP, in turn, alters the distribution of calcium within the cell, favoring an increase in cytosolic calcium. It is the change in cytosolic calcium resulting from both the direct effect of PTH on the plasma membrane and of cAMP on the subcellular formation. The rise in $[Ca^{2+}]$ also leads to a decrease in cAMP concentration, probably by an inhibition of adenyl cyclase or possibly an activation of phosphodiesterase. The effect of phosphate is best accounted for by assuming that it causes a net uptake of calcium by the cell but also a shift of calcium from cytosol to mitochondrial matrix space wherein the calcium is deposited as a calcium phosphate salt. This would be consistent with the known effects of phosphate on calcium uptake by isolated mitochondria (Rasmussen, Chance, and Ogata, 1965; Lehninger, Carafoli, and Rossi, 1968). This effect of phosphate would counteract the effect of cAMP within the cell, cause a lowering of cytosolic $[Ca^{2+}]$, relieve the inhibition of the adenyl cyclase, and lead to an increase in cAMP concentration. When a new steady state is reestablished, it should be one in which the concentration of cytosolic cAMP is higher and cytosolic $[Ca^{2+}]$, lower.

In the more general case involving these two second messengers, as long as they are both involved in coordinate regulation of a metabolic sequence, as for example in cardiac glycogenolysis, a change in their relative concentrations might have very little effect upon the overall rate of the pathway involved. However, if changes in either Ca^{2+} or cAMP concentration influenced reactions within the cell which were not influenced by the other second messenger, then a change in their concentration ratio by a third factor, such as phosphate concentration, would alter the ultimate physiological response of the cell. There is, in other words, a rational basis for the fact that changes in the ionic environment of cells change their response to hormones, even those thought to involve cAMP as a second messenger, i.e., there is considerable plasticity of cellular response. In addition to phosphate, changes in $[H^+]$ within the cell, by altering mitochondrial calcium accumulation and the chelation of calcium (Figure 4), can also lead to relative shifts in second messenger concentrations, allowing for an even greater plasticity of cellular response.

It is worth emphasizing that the renal tubule is another instance, similar to the situation in smooth muscle or in the heart from adrenalectomized animals, in which there is no simple and direct correlation between cAMP concentration and the physiological response of the cell under many physiological conditions.

A particular feature of the Ca^{2+}-cAMP relationship exemplified by these studies involves the effect of the Ca^{2+} upon the activities of adenyl cyclase and phosphodiesterase (Kakiuchi and Yamasaki, 1970a,b; Kakiuchi, Yamasaki, and Teshima, 1971). When the calcium concentration in the external medium is varied from 0.05 to 3.0 mM, the initial increase in cAMP concentration in isolated renal tubule in response to parathyroid hormone is nearly the same at all calcium concentrations (Rasmussen, 1970a). However, the cAMP concentration falls with time, even in the continued presence of PTH. If cAMP concentrations are measured after 15 minutes of incubation, the concentration is found to vary inversely with the calcium concentration. This has been construed as evidence that intracellular $[Ca^{2+}]$, but not extracellular $[Ca^{2+}]$ (except insofar as it influences intracellular $[Ca^{2+}]$), regulates adenyl cyclase activity. This hypothesis is supported by the fact that when cell membranes are disrupted, the adenyl cyclase activity is inhibited by Ca^{2+} (Marcus and Aurbach, 1971; Robison et al, 1971) and activated by EGTA-relatively specific calcium chelator.

In other systems, calcium ions also influence the activity of a phosphodiesterase, e.g., the phosphodiesterase in the 100,000 g supernatant of brain homogenates (Kakiuchi and Yamasaki, 1970a,b; Kakiuchi et al., 1971). The brain phosphodiesterase is activated by Ca^{2+}, whereas that in

intestinal smooth muscle is inhibited. In the presence of a protein, the phosphodiesterase activating factor, addition of as little as 10^{-7} *M* Ca^{2+} leads to an activation of the brain enzyme with maximal activation (four- to sixfold) achieved at 1.9×10^{-6} *M* Ca^{2+}. The effect of Ca^{2+} is greater at higher cAMP concentrations. A similar activation of heart muscle phosphodiesterase by Ca^{2+} has been described (Kakiuchi, 1972). Thus, there is evidence that an increase in intracellular [Ca^{2+}] can regulate cAMP concentration both by inhibiting adenyl cyclase, i.e., decreasing its rate of synthesis, and activating phosphodiesterase, i.e., increasing its rate of destruction.

Having presented this model in which Ca^{2+} rather than cAMP appears to be the second messenger of major importance in renal cells, it is important to add that stimulation of renal gluconeogenesis is not the major physiological effect of parathyroid hormone. Its major physiological effect is to alter electrolyte excretion, particularly phosphate, Ca^{2+}, K^+, and H^+, and often Na^+ and Mg^{2+} as well (Rasmussen, 1968). It remains to be demonstrated that the general features of cellular control discussed above can account for all these changes. In all likelihood, as our understanding increases, it will be found that the changes in [cAMP], as well as the changes in [Ca^{2+}], influence one or more aspects of renal cell function.

It is not yet possible to correlate completely this in vitro model of cell activation with the physiological effects of parathyroid hormone, but it has been possible to extend it to a discussion of the mode of action of this hormone on bone. With its use, it has been possible to explain readily a number of previously unexplained and perplexing observations. It is worth doing this because any biochemical model of cell activation should be interpretable in terms of physiological events if valid, and should help explain perplexing facts derived from physiologic inquiry.

In order to discuss the situation in bone, it is first necessary to review briefly the relevant physiology (Rasmussen, 1970b, 1971). The control of bone resorption is achieved by altering the activity of two kinds of bone cells, osteocytes and osteoclasts. The former appear to be involved in the minute-to-minute control of calcium homeostasis, and the latter are, under optimal circumstances, primarily concerned with skeletal remodeling or homeostasis. However, under adverse circumstances, e.g., calcium deficiency, the osteoclasts increase in activity and number and probably become of paramount importance in controlling mineral removal from bone. The activity of both types of cells is controlled by both parathyroid hormone and calcitonin. Vitamin D, or more correctly one or more of its active metabolites, is also involved in this process, but this involvement is complex and not directly related to the adenyl cyclase control system although it undoubtedly influences the process of cell activation. Parathyroid hormone increases both osteocytic and osteoclastic bone resorption, as well as increasing the number of osteoclasts. These effects lead to a resorption of bone, both matrix and mineral (a complex salt of calcium and phosphate), and a net movement of calcium and phosphate from insoluble bone mineral to soluble, ionized, or complexed mineral ions in the bodily extracellular fluids. Calcitonin inhibits the resorptive activity of both classes of cells, and decreases the number of osteoclasts. These changes inhibit the removal of bone mineral to extracellular fluids, and thereby lead to a fall in concentration of both calcium and phosphate in the extracellular fluids. Both hormones also influence the activity of bone forming cells, osteoblasts, and the cells in other organs, kidney, and intestine, but it is not necessary to consider these effects in the context of the present discussion.

A summary of the effects of calcitonin (CT) and parathyroid hormone (PTH) upon bone cell function is presented in Table 1. The most striking thing about this summary is that in most regards their effects are inverse. Thus PTH decreases the membrane potential of osteoclast, and CT increases it (Mears, 1971). PTH increases resorption, CT decreases it, etc. However, there is one striking exception to this generalization: both PTH and CT increase the concentration of cAMP in bone cells (Chase and Aurbach, 1970; Murad, Brewer, and Vaughan, 1970). This has led to the conclusion that these two hormones both activate adenyl cyclase in receptive cells which must mean, in turn, that they act upon different groups of bone cells, i.e., CT blocks resorption by acting on one group of cells and PTH stimulates resorption by acting upon a different group. This is a logical explanation in the context of the second messenger hypothesis, but it ignores the mass of biochemical, histological, and physiological evidence that these two hormones affect the physiological function of the same bone cells. The

TABLE 1

A Comparison of the Effects of Parathyroid Hormone and Calcitonin on Bone

CELL RESPONSE	PTH	CT
Calcium uptake	↑	–
Calcium efflux	↑	↑ then ↓
Osteocytic osteolysis	↑	↓
Osteoclast number	↑	↓
Membrane potential	↓	↑
Cyclic 3′5′ AMP	↑	↑

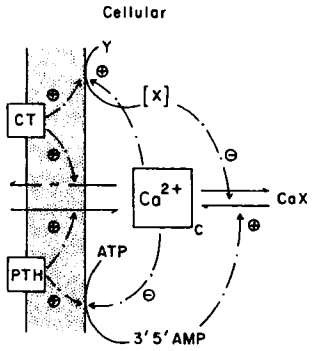


FIGURE 17. A model to depict the control of bone cell cytosolic calcium by parathyroid hormone (PTH) and calcitonin, CT. Y and X represent hypothetical compounds, Y being the precursor of X. X is postulated to function in a manner opposite to that of cyclic AMP and catalyze a shift of calcium from cytosol to intracellular stores. In this model, both hormones have at least two simultaneous effects upon cytosolic calcium, one mediated by a change in cell membrane activity and the other by the generation of an intracellular mediator which regulates the intracellular distribution of calcium. In the case of PTH, this mediator is cAMP.

most logical conclusion from all present evidence is that PTH and CT affect the activity of all bone cells, or nearly all. If this conclusion is correct, we are then faced with the apparent paradox that two hormones which exert opposite effects in the physiological sense apparently do so by producing an increase in the concentration of cAMP within the same cells of the responsive tissue. However, we have already seen in at least two other circumstances, smooth muscle and kidney cells, how a rise in cAMP can be observed under circumstances where the converse of the expected physiological response is observed. The key to the understanding of both of these latter circumstances was the recognition that there was an additional second messenger, calcium ion, and that there existed a complex feedback relationship between the two (Ca^{2+} and cAMP) so that a change in the concentration of the one affected the concentration of the other.

Viewed in this context, the PTH-CT relationship, and the effect of these two hormones upon bone cell function, can be accounted for in a logical manner (Figure 17). The effect of PTH upon bone cells is similar to its effects upon kidney cells, an activation of adenyl cyclase and an increased uptake of calcium into the cell leading thereby to an increase in the calcium ion concentration $[Ca]_c$ in the cell cytosol. CT, on the other hand, would act to stimulate calcium efflux from cells by a direct effect upon the plasma membrane calcium pump (Parkinson and Radde, 1970). The resulting fall in $[Ca]_c$ would relieve the inhibition of adenyl cyclase (and possibly reverse as well an activation of phosphodiesterase) by $[Ca]_c$ resulting in a rise in cAMP. In addition, however, in order to account for the late effect of CT, that of inhibiting calcium efflux from bone or from isolated cells (Borle, 1969), it is necessary to postulate that CT stimulates the production of an unidentified second messenger which inhibits or counteracts the effect of cAMP upon the mitochondrial calcium transport system. The nature of this messenger is not known. It could be a marked increase in cytosolic phosphate either due to phosphate uptake by the cell, or the hydrolysis of organic phosphates within the cell, or it could be a completely unknown agent.

If this postulated new second messenger does exist, it is in all likelihood not confined to this function in bone cells, but will probably be found to carry out a similar kind of function in other tissues, possibly in response to other first messengers. If so, it can be predicted that other cases will be found in which two hormones with opposite physiological effects upon a particular tissue will both be found to cause an increase in cAMP under appropriate conditions.

Regardless of the presence of this additional presumed second messenger, the model of bone cell activation is of interest because it makes

certain predictions which can be verified experimentally. As noted above, PTH causes an increase in the net removal of calcium and phosphate from bone to blood, yet, according to the model depicted in Figure 17, it should stimulate the uptake of calcium by bone cells which one might more logically think would be associated with a decrease in the removal of calcium from bone. If the model is correct, it predicts that the initial effect of PTH would be to lower the plasma calcium concentration by stimulating the uptake of calcium by bone cells. This prediction has been verified experimentally (Copp, 1965; Parson, Neer, and Potts, 1971; Rasmussen, 1971). PTH stimulates the uptake of calcium from blood into bone (presumably bone cells) before there is a net removal of calcium and phosphate from bone. In other words, an increase in $[Ca]_c$ appears to be one of the important signals in activating these cells to increase bone resorption.

This type of control system can be depicted schematically as shown in Figure 18. Two important features of this system are that there are at least three important factors which control $[Ca]_c$, the concentration of calcium in the extracellular fluids $[Ca]_e$, and the concentrations of CT and PTH. Under conditions of low calcium intake and, thus, a lower than normal plasma calcium concentration, the two major determinants of $[Ca]_c$ would be [PTH] and $[Ca]_e$. The second feature is that $[Ca]_c$ would be positive feedforward activator of bone resorption. These two features predict that under some conditions where $[Ca]_e$ is low, $[Ca]_c$ will never increase sufficiently to activate bone resorption even though there is within bone enough calcium to completely supply 10^5 times the amount of calcium needed to restore the $[Ca]_e$ to normal. This situation is analogous to that previously discussed in the mammalian heart (Figure 7). In a sense, the extracellular calcium of the bone cells serves as a source for the trigger calcium necessary to activate the bone cell to carry out its calcium-mobilizing function.

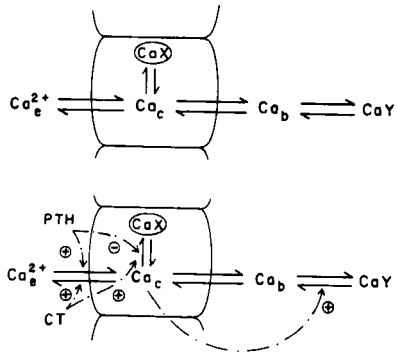


FIGURE 18. A hypothetical model concerning the relationship between extracellular calcium, Ca_e; intracellular cytosolic calcium, Ca_c; intracellular stored calcium, Ca_x; bone extracellular fluid calcium, Ca_b; and bone mineral calcium, Ca_y. The most important feature of this proposed relationship is that the concentration of Ca_c, calcium in bone cell cytosol, controls, in a positive and direct sense, the rate of mobilization of Ca_y to Ca_b, and eventually to Ca_e. This represents a positive feed-forward loop in this control system and is analogous to a similar positive loop in the calcium controlled cardiac contraction depicted in Figure 7.

Two circumstances in which this model appears to account for experimental facts are vitamin D deficiency with severe hypocalcemia in humans, and milk fever in cattle.

As noted in Table 1, an increase in [PTH] normally leads to an increase in the number of osteoclasts in bone. However, in vitamin D-deficient humans with a low plasma calcium concentration, in spite of very high concentrations of PTH in blood there is no increase in osteoclast number (Bordier, unpublished). However, when these patients receive a calcium infusion, the concentration of calcium in blood returns toward a normal value, the concentration of PTH in blood *falls*, but the number of osteoclasts in bone *simultaneously increases*, i.e., an increase in extracellular calcium concentration served as the signal to stimulate the conversion of preosteoclasts to osteoclasts, increasing, thereby, the rate of bone mineral mobilization.

Milk fever is a disease of parturient cattle who, at the time of their rapid synthesis of milk after calving, develop a severe and fatal hypocalcemia. The concentration of PTH in their blood is increased but obviously ineffective, and the administration of exogenous PTH is without effect (Letterdike, Whipp, and Schroeder, 1969). However, the infusion of calcium in sufficient amounts to raise the plasma calcium concentration near normal restores the responsiveness to PTH. Usually one single massive injection of calcium is sufficient even though the cow continues to produce large amounts of milk, and to secrete and excrete much more calcium than that originally administered. Again, an increase in $[CA]_e$ has served as the

signal to reestablish the normal operation of this control system.

The purpose for going this far afield from the topic of cAMP is to point out that a model of cell activation which accounts in general terms for the hormonal control of fluid secretion in the fly salivary gland (Figure 11) also serves as an apparently valid model for explaining paradoxical physiological observations concerning mammalian extracellular calcium homeostasis. This correlation implies that cAMP is an important factor in those systems which regulate intracellular calcium homeostasis in a wide variety of animal cells, and that mammalian extracellular calcium homeostasis has been achieved by an adaptation and extension of the basic mechanisms underlying intracellular calcium homeostasis. Thus, aspects of mammalian calcium homeostasis which appear illogical at first glance are readily explicable in terms of systems which developed early in evolution to control intracellular calcium homeostasis.

In summary, there is considerable evidence that many first messengers influence the uptake of calcium by cells independently of their effect upon adenyl cyclase, that cyclic AMP alters the distribution of calcium within the cell, and conversely, that intracellular calcium concentration regulates cAMP concentration (Rasmussen, 1970, 1971). What remains to be established is that the effect of cAMP upon calcium metabolism is a direct one, and not one that is mediated indirectly; and that this effect does not involve a cAMP-dependent protein kinase. Insufficient evidence is available to decide either of these questions.

It is not possible, in this review, to consider many other systems in which an interrelationship between Ca^{2+} and cAMP in cell activation is apparent (see Rasmussen, 1970a, 1971). However, there is one additional general cellular function in which both appear to play an important role. This is the process of cell division and cell differentiation.

CYCLIC AMP, CELL DIVISION, CELL GROWTH, AND DIFFERENTIATION

The final general cellular functions which will be discussed in relation to cAMP action concern cell division, growth, and differentiation. In these cases, only a bare beginning has been made. It has been shown that cAMP influences these processes. The physiological importance of this influence is more difficult to assess. In some cases, such as the cellular slime mold, the case for a normal physiological role in cell differentiation is quite strong. In others, such as growth and transformation of animal cells, the pharmacological fact is indisputable, the physiological significance, undetermined.

Three major systems will be considered: 1) cAMP and mitosis in animal cells – particularly thymocytes; 2) cAMP and growth rates of mammalian cells grown in tissue culture; and 3) the phenomena of aggregation and differentiation in the cellular slime mold *Dictyostelium discoideum*.

cAMP AND MITOSIS

One of the most interesting, but in a way most confusing, sets of observations concerning cAMP in cell function is the study of its role in regulating the mitotic rate in bone marrow and thymocytes. These studies have been carried out largely by MacManus, Perris, Whitfield, and their associates (MacManus, Perris, Whitfield, and Rixon, 1970; MacManus, Whitfield, and Youdale, 1971; MacManus, Youdale, Whitfield, and Franks, 1972; Perris, MacManus, Whitfield, and Weiss, 1971; Whitfield, MacManus, and Gillan, 1970; Whitfield, MacManus, and Rixon, 1970; Whitfield and Perris, and Youdale, 1969; Whitfield, Rixon, Perris, and Youdale, 1969). The major part of the work has concerned the study of the rate at which isolated lymphocytes prepared from the rat thymus enter metaphase. They were arrested at metaphase by the presence of 0.06 mM colchicine in the incubation medium. Of major importance is the fact that only 10 to 20% of the total cell population are lymphoblasts which are either proliferating or are able to enter the S phase (DNA synthetic phase) of the mitotic cycle after exposure to a number of specific agents. The other 80 to 90% of the cells are small mature lymphocytes which do not normally undergo further cell divisions.

The most thoroughly studied stimulant is parathyroid hormone (MacManus et al., 1972). When this hormone is added to a washed preparation of thymocytes, the cellular content of ^{3}H-cAMP derived from ^{3}H-ATP is increased within the first few minutes; the incorporation of ^{3}H-thymidine into DNA is increased in the first hour; and the number of cells flowing into mitosis is increased in four to six hours. None of these

changes are seen if $[Ca^{2+}]$ is removed from the medium. Also, these changes in DNA synthesis and mitosis are blocked by the addition of calcitonin to the medium. There is, however, no report of what CT does to cAMP concentrations in these cells. An increase in extracellular $[Ca^{2+}]$ from 0.6 to 2.4 mM will induce the same set of changes as that induced by PTH in the presence of 0.6 mM Ca^{2+}. These results are fairly similar to those described when PTH acts on the isolated renal tubule (see above), but there are two important differences: in thymocytes, PTH does not increase [cAMP] in the absence of Ca^{2+}, but in renal cells PTH increases [cAMP] in the absence of Ca^{2+}; and in thymocytes an increase in extracellular $[Ca^{2+}]$ leads to an increase in [cAMP], but in renal cells it does not. Also, the adenyl cyclase prepared from renal cells is stimulated by PTH (Marcus and Aurbach, 1971), but one prepared from thymocytes is not. These facts have led to the proposal (MacManus et al., 1972) that in thymocytes the sequence of events is 1) a PTH-stimulated increase in the uptake of Ca^{2+} by the cell; 2) the increase in intracellular $[Ca^{2+}]$ leads to an increase in [cAMP] by inhibiting phosphodiesterase; 3) cAMP in some manner, possibly by histone phosphorylation, increases DNA synthesis and thereby the flow of cells into metaphase.

In support of this model, it has been shown that 10^{-9} to 10^{-7} *M* cAMP will stimulate DNA synthesis and mitotic rate in the absence of Ca^{2+}, but that Ca^{2+} enhances the effects of low concentrations of exogenous cAMP upon these events, presumably by inhibiting its destruction (MacManus et al., 1972).

However, in another set of experiments, the same authors (MacManus, Whitfield, and Braceland, 1971) attempted to determine whether cAMP entered these cells. They concluded it did not, and it probably acted upon the cell surface. These experiments can be challenged on technical grounds, so it is difficult to assess their importance.

Further support for their basic argument comes from the observation that epinephrine will also stimulate mitosis, and increase cAMP (MacManus et al., 1971). However, it produces its effects in the absence of Ca^{2+} and has been shown to increase the activity of a particulate adenyl cyclase from these cells. Thus, it is thought to act in a more conventional manner, i.e., adenyl cyclase activation, but to bring about the same result, an increase in [cAMP].

In addition, the following agents have been shown to increase mitotic rate: Mg^{2+}, bradykinin, vasopressin, growth hormone, and oxytocin (MacManus et al., 1972). All are thought to act via cyclic AMP. The activity of all (except Mg^{2+}, which has not been examined), including PTH, is enhanced by caffeine and inhibited by imidazole, drugs which are thought to act respectively by an inhibition and activation of phosphodiesterase. The surprising feature is that Ca^{2+} and caffeine apparently act synergistically even though both are thought to act by inhibiting PDE.

A weak point in the model is that the PDE from these cells is inhibited significantly only by high concentrations of calcium (0.5 mM) (MacManus et al., 1972), and it is unlikely that the intracellular calcium concentration ever reaches this value. Also, studies with isolated liver (Burgoyne, Wagar, and Atkinson, 1970a) and thymic nuclei (Burgoyne, Waqar, and Atkinson, 1970b) have shown that DNA synthesis is markedly stimulated by Ca^{2+} with half maximal activation at 30 mM. The addition of cAMP over a wide concentration range with or without calcium had no effect. Unfortunately, the effect of cAMP was not examined after the addition of protein kinase. Nor were its effects on RNA synthesis examined. However, in isolated nuclei from liver cells, 10^{-4} *M* DBcAMP has been shown to increase the activity of RNA polymerase activity (Jost and Sahib, 1971). It seems unlikely that this effect has any important relationship to the effects of 10^{-7} *M* cAMP added extracellularly to isolated thymocytes.

Finally, it has been shown that the removal of the parathyroid glands from rats leads to atrophy of the thymus gland in vivo (Perris, Weiss, and Whitfield, 1970). An increase in the mitotic rate in the cells of such glands can be produced by the injection of PTH, Ca^{2+}, or cAMP into these animals (Perris, Whitfield, and Rixon, 1967). Similar changes in the mitotic rate of bone marrow cells have also been seen in vivo. This is taken as evidence for this effect being of physiological significance.

It is difficult to evaluate all of these results. Only approximately 15% of the total cell population is capable of undergoing the change in DNA synthesis and mitotic rate, yet all the biochemical measurements of cAMP production and uptake

were done on the entire cell population. There is no evidence that only the particularly large cells responded to hormone with a change in [cAMP]. Thus the change in [cAMP] may not be related in any way to the other events. This weakness is partially answered by the fact that 10^{-7} *M* cAMP does stimulate mitosis itself.

The amazing feature of these data is that, if correct and interpreted correctly, they show that, in addition to the two ways already outlined, e.g., epinephrine in mammalian heart and epinephrine in intestinal smooth muscle, cAMP and Ca^{2+} can be related in a third fashion in which Ca^{2+} serves as the initial second messenger and, by inhibiting phosphodiesterase, increases [cAMP] within the responsive cell. If correct, the logical means by which CT should act in these cells is to increase Ca^{2+} efflux, lower [Ca^{2+}] in the cell, and thereby lower [cAMP]. Thus, it becomes of importance to measure [cAMP] within these cells in the presence of CT.

Before finally accepting this third model of Ca^{2+}-cAMP coupling in cell activation, it would seem necessary to attempt to separate the 10 to 20% of responsive cells from the bulk of the unresponsive cells in order to carry out the proper biochemical analyses. This is particularly true because it has been shown that in small lymphocytes (similar to those comprising the majority of the thymocyte population) the addition of phytohemagglutinin (PHA) leads to an activation of adenyl cyclase and this is followed in approximately 24 hours by an increase in mitotic rate (Smith, Steiner, Newberry, and Parker, 1971). If PTH is acting on all the cells in the population, it then becomes of interest to know whether it also stimulates cell division in the smaller cells. If not, and at present there is no evidence that it does, then one is faced with the problem of two agents both increasing [cAMP] by slightly different mechanisms in the same cell population and bringing about different physiological responses.

Believing as we do that all cellular control systems are modulated by feedback circuits, it is not apparent in this third model, as in the other two models relating Ca^{2+} and cAMP, what these circuits are. Whether or not cAMP influences calcium fluxes in these cells becomes of considerable interest. Regardless of the eventual nature of their relationship, it is clear that calcium and cAMP are intimately related in the control of mitotic activity in these cells.

CELL GROWTH, CELL MORPHOLOGY, AND cAMP

The possible role of the calcium-cAMP relationship in mitogenesis may not be confined to thymocytes. Balk (1971) has shown that the proliferation in vitro of recently isolated chicken fibroblasts depends directly on the calcium ion concentration, being higher in the presence of calcium than in its absence when these cells are incubated in a medium containing heat inactivated plasma. In contrast, the rate of proliferation of similar fibroblasts after transformation by Rous sarcoma virus was insensitive to changes in the extracellular calcium concentration when grown in media containing heat-inactivated plasma. The addition of heat-inactivated serum instead of plasma to the culture medium resulted in a five- to sixfold increase in the rate of proliferation of both types of cells. Under these circumstances, changes in calcium concentration directly altered proliferative rates in both normal and transformed cells, but at any given calcium concentration the growth rate of the transformed cells was approximately two- to threefold greater than the normal cells. Balk did not relate these changes to possible changes in adenyl cyclase activity of cAMP content, but it has been shown by others that the adenyl cyclase activity of certain liver and mammary tumor cells is considerably higher than normal. On this basis, MacManus and Whitfield (1971) suggested that calcium was probably controlling the proliferative rate by controlling cAMP concentrations in analogy to the thymocyte system.

Otten, Johnson, and Pasten (1971) have measured the cAMP concentration in both transformed and untransformed fibroblasts. When a variety of cell types were compared during their logarithmic growth phase, there was an *inverse*, rather than direct, correlation between growth rate and cAMP concentration. In addition, in untransformed cells, the cAMP concentration increased at confluency, suggesting that cAMP, in addition to influencing growth rate, in some way mediated contact inhibition of growth in these cultured cells. Makman (1971), however, has found an inverse relationship between adenyl cyclase and rate of cell growth in transformed cells. He has also found that hormone-sensitive adenyl cyclase activity increases with increasing cell density, and has proposed that the activity of this enzyme may

be enhanced by cell-to-surface, or cell-to-cell, contact; and that this may represent a means by which a tissue controls its responsiveness to hormones. In contrast to untransformed cells, transformed cells, which do not normally exhibit contact inhibition, did not have an increase in cAMP concentration at confluency.

The addition of exogenous cAMP or DBcAMP to transformed cells alters their morphological appearance toward normalcy and slows their growth rate. In the presence of cAMP, these cells display some of the characteristics of contact inhibition (Johnson, Friedman, and Pasten, 1971). Reversion to the transformed state occurs as soon as the cyclic nucleotide is removed from the culture medium. In similar studies, Hsie, Jones, and Puck (1971) have shown that testosterone potentiates the effect of DBcAMP. The two agents together lead to the conversion of Chinese hamster ovary cells to a fibroblastic form which exhibits contact inhibition, the ability to synthesize collagen, and altered responses to plant agglutinins. Other steroid hormones will not replace testosterone in this regard. Since it requires 0.1 to 1.0 mM DBcAMP or 3mM cAMP to bring about these morphological changes, it is difficult to know whether these represent physiological or pharmacological effects of cAMP. That they might represent a physiologically important role of cAMP is suggested by the fact that the addition of prostaglandin E_1 to certain transformed cells leads to their reversion to more normal morphology and growth characteristics. These changes are associated with an increase in intracellular cAMP. Certain cell lines which do not contain a PGE-sensitive adenyl cyclase do not undergo reversion upon addition of this agent (Peery, Johnson, and Pastan, 1971).

The relationship between these changes in cell morphology and cell growth to either protein kinases or cellular calcium has not been established. However, it is of interest that several recent discussions of the control of mitosis and of contact inhibition have suggested that membrane permeability and membrane potential may be key determinants in these processes (Burton, 1971; Cone and Tangier, 1971).

AGGREGATION IN THE CELLULAR SLIME MOLD

One of the most fascinating systems in which cyclic AMP has been found to participate is the phenomenon of aggregation in the cellular slime mold *Dictyostelium discoideum*. To understand the role of cyclic AMP in this process, it is first necessary to briefly review the life cycle of this organism (Bonner, 1967, 1971; Gerisch, 1968). Spores of this organism germinate under appropriate environmental conditions of nutrition and humidity. Upon release, the amoebae exist as separate cells and begin feeding on suitable bacteria. These amoebae will grow and divide as long as food is available. The bacteria upon which they feed are found by chemotaxis, and the presence of these bacterial chemotactic agents prevents the amoebae from entering into their further development. When the food disappears, the amoebae continue to move about for approximately one half hour. During this period of time, they exhibit no polarity in the sense that pseudopod production can occur at any point on their surface. The amoebae then become stationary for a period of six to eight hours (interphase). At the end of interphase, motion is resumed, but the amoebae are now polar, moving only by pseudopod formation from one end. In this phase, certain amoebae become so-called founder cells, forming centers towards which adjacent amoebae migrate. Once this aggregative phase has been initiated, anywhere from 100 to 200,000 amoebae will stream toward this center and, in the process, form cell-to-cell contacts. Eventually a multicellular organism, the slug, is formed which migrates a short distance from its original site of origin. This slug undergoes a complex series of internal reorganizations leading to the formation of a new spore. During this process, nearly all of the cells in the slug die in forming the stalk and spore sac; only a few viable organisms are preserved within the spore proper, ready to repeat the life cycle. Even as early as the stage of slug formation, it is evident that a differentiation of the cells has taken place in the sense that those destined to become the base plate, stalk, and spore sac can be identified by their position in the slug.

In terms of cyclic AMP action, the most important time in the life cycle, or at least the one most intensively studied, is the phase of aggregation. However, it appears likely that a major chemotactic substance produced by the bacteria is cyclic AMP and that as long as its level in the culture medium is high, it acts to inhibit the

development of the multicellular stage of the life cycle. During this free-swimming, unicellular phase, the amoeba makes little or no cyclic AMP (Bonner, 1971). However, after interphase the amoebae begin to make and release into the medium both cyclic AMP and a phosphodiesterase (Konijn, Barkley, Chang, and Bonner, 1968; Bonner, Barkley, Hall, Konijn, Mason, O'Keefe, and Wolfe, 1969; Barkley, 1969; Chang, 1968). During the process of aggregation, the flow of amoebae is not continuous but discontinuous. The amoebae move in a pulsatile fashion toward the center, and this appears as an outwardly propagated wave moving from center to periphery. Cohen and Robertson (1971) have constructed a mathematical model based on an earlier, more general phase-shift model for spatial and temporal organization in developing systems (Goodwin and Cohen, 1969) to account for this wave propagation during the early stages of aggregation. Their basic notion is that cyclic AMP is released from the center cells, diffuses to neighboring cells where it initiates motion, and, with a time delay, triggers these cells to release cyclic AMP. These cells then go through a refractory period during which they do not respond to cyclic AMP. The extracellular phosphodiesterase released by the amoebae hydrolyzes the cyclic AMP. Thus, the concentration of cyclic AMP builds up and decays rapidly.

This model is particularly interesting because it represents a system in which cyclic AMP apparently acts, in a sense, as its own first messenger and induces the synthesis and/or release of cyclic AMP from a second cell.

The action of cyclic AMP on sensitive cells appears to involve at least two separate effects, an initiation of motion, and, after a time delay, a release of cyclic AMP. On the basis of the nearly universal involvement of Ca^{2+} in biological contractile systems, Chi and Francis (1970) investigated the effect of cyclic AMP upon ion fluxes in slime mold. They could find no effect of this cyclic nucleotide on calcium uptake or upon Na^{+} efflux or influx, but they found that cyclic AMP induced a very marked efflux of radiocalcium (^{45}Ca) from prelabeled amoebae. This change in rate of efflux was similar to that seen when cyclic AMP or first messenger is added to different mammalian cell types (see above). From these data, Chi and Francis (1970) suggested that a release of calcium within the cells was the event that triggered movement in susceptible amoebae. However, the one weakness in their experiments was the fact that they employed high concentrations (10^{-4} to 10^{-3} *M*) of cyclic AMP compared to the amounts which are necessary to induce movement and aggregation, 10^{-8} *M* or less. Mason, Rasmussen, and DiBella (1971) have presented evidence which supports the likelihood that calcium is involved in the aggregation phenomena. They found that if the calcium ion concentration was reduced below 10^{-6} *M*, then aggregation was inhibited although cyclic AMP production was not. They also showed that the local anesthetic tetracaine blocked aggregation without inhibiting cyclic AMP production. However, these facts do not identify the role which calcium plays nor the precise relationship between cyclic AMP and calcium in these cells.

Perhaps the most novel experiments, and ones which most clearly establish the basic premise of the Robertson-Cohen model, are the experiments in which aggregation in a culture of *Dictyostelium discoideum* was controlled by externally applied pulses of cyclic AMP (Robertson, Drage, and Cohen, 1972). By applying external pulses of cyclic AMP at precisely timed intervals, it was possible to induce aggregation. The pulses of cyclic AMP were sufficiently small (6×10^{11} molecules/pulse) that without amplification by the sequence of events outlined above, there would not have been sufficient cyclic AMP present to serve as attractant for the peripheral cells which eventually became part of the aggregate.

The participation of cyclic AMP at later stages in the phenomena of differentiation is likely (Bonner, 1971), but so little is currently known of this phase of the process that it is not useful to discuss it. What the available evidence does show is that cyclic AMP is a critical component in the process of aggregation, and thus may be an important controlling element in the process of cell differentiation in these organisms. The unique feature of this situation is that cyclic AMP acts as an extracellular rather than intracellular messenger. However, before concluding, it is worth pointing out that the other cellular slime molds which aggregate in a similar manner do so in response to some other chemical messenger. Cyclic AMP is not a universal acrasin (Bonner, 1967, 1971).

A GENERAL MODEL OF CELL ACTIVATION

On the basis of the foregoing facts, a general model of cell activation which involves both Ca^{2+} and cAMP as second messengers has been proposed (Rasmussen, 1970a, 1971). In this model of activation (Figure 19), the interaction of first messenger with its receptor site on the cell surface leads to at least two simultaneous events, an activation of adenyl cyclase and an increase in the calcium permeability of the cell membrane. The resulting increase in intracellular cAMP has at least two effects, that of activating one or more protein kinases, and that of altering the subcellular distribution of calcium so as to lead to an increase in the calcium ion concentration in the cell cytosol. Thus, the calcium ion content of the cell cytosol increases both because of a primary effect of the first messenger on the cell membrane, and a secondary effect mediated by cyclic AMP on one or more intracellular membranes. The resulting increase in $[Ca^{2+}]$ is responsible for several changes within the cell: an inhibition of adenyl cyclase and/or an activation of phosphodiesterase; an activation of enzymes, some of which are the phosphoprotein products of cAMP-dependent protein kinases; and an increase in the permeability of the cell membrane to monovalent cations. These relationships between cAMP, Ca^{2+}, and the first messenger are summarized in Table 2.

Perhaps the most important features of this model (Figure 19) which should be stressed are that both Ca^{2+} and cAMP are envisioned as second messengers, and their respective concentrations are reciprocally controlled by the other messenger. From the cybernetic point of view, this arrangement makes considerable sense. Quenching of a signal is equally as important as its generation.

There is an important difference, in the control sense, in the respective roles of the two second messengers in different cell types. In some, calcium acts as a negative feedback inhibitor controlling cAMP concentration by either inhibiting cAMP synthesis or stimulating cAMP hydrolysis, or both; whereas cAMP acts as a positive feedforward effector increasing the $[Ca^{2+}]$ of the cell cytosol. In others, the converse arrangement appears to operate. One can see immediately that the magnitude of change (induced by the first messenger) in the permeability of the cell membrane to calcium will play a critical role in defining the new steady state concentrations of both cAMP and Ca^{2+} in the cell

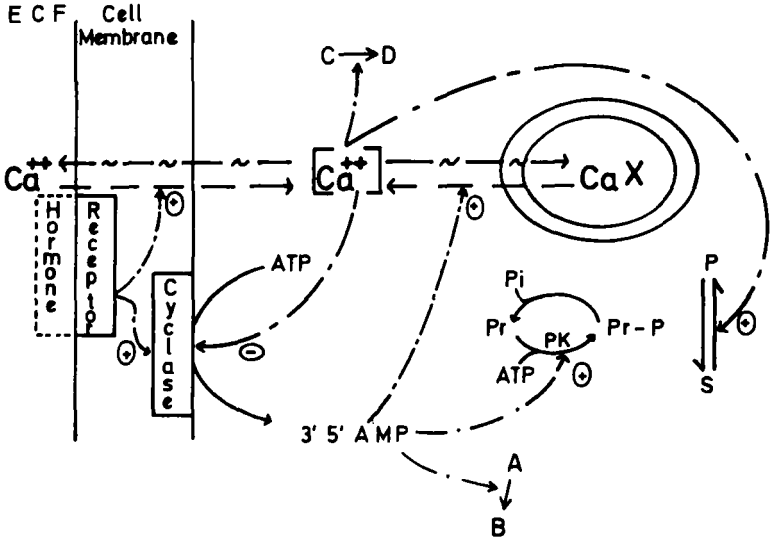


FIGURE 19. A general model of cell activation in which cyclic 3',5' AMP and Ca^{2+} both serve as second or intracellular messengers, and each regulates the cytosolic concentration of the other. In addition, cAMP controls a group of enzymes, protein kinases, PK, and the phosphoprotein products, some of which are regulated by Ca^{2+}. Each of these intracellular messengers may also exert additional independent effects upon cell function (A → B or C → D). Note also that the model of events thought to operate in smooth muscle, as depicted in Figure 9, is a mirror image of this model.

TABLE 2

Relationship Between Cyclic AMP, Calcium, and First Messenger in Cell Activation

A. Role of first messenger
1. Increased uptake of calcium by cell
2. Increased activity of adenyl cyclase
3. Increased radiocalcium efflux from prelabeled cells (probably via 3'5' AMP) due to a mobilization of calcium from an intracellular pool
4. Membrane depolarization

B. Cyclic AMP
1. No effect on calcium uptake
2. Increased radiocalcium efflux from prelabeled cells
3. High exogenous concentrations produce a physiological response
4. Activation of protein kinases
5. Possible gene activation – yet to be established in mammalian cells
6. Inhibition or stimulation of calcium accumulation by subcellular membranes

C. Role of Calcium
1. Physiological response requires presence
2. Activation of adenyl cyclase occurs in its absence
3. Inhibits adenyl cyclase and/or activates phosphodiesterase
4. Activates some phosphoprotein products of cyclic AMP-dependent protein kinases
5. Alteration of cell membrane permeability to K^+ and Na^+
6. Activation and inhibition of enzymes
7. Activation of mitosis and stimulation of nuclear DNA synthesis
8. Changes in its flux coupled to changes in fluxes of other ions

cytosol. If calcium permeability increases greatly, very little change in cAMP concentration would take place. If, however, there were very little increase in calcium uptake after cell activation, cAMP concentrations would rise to a much greater extent. Hence, in different cell types, even though both cAMP and Ca^{2+} concentrations would increase to new steady state levels after activation, there would be considerable plasticity of response in the sense that the $[Ca^{2+}]/[cAMP]$ would vary considerably from cell type to cell type. If, in addition to controlling certain metabolic pathways jointly, e.g., sequentially as in the case of cardiac glycogenolysis, each of these two second messengers also controlled other cell functions separately, a great plasticity of cell response could be achieved.

CONCLUDING REMARKS

In the foregoing review, we have made no attempt to be comprehensive. We have attempted to discuss the role of cyclic AMP in the animal cells in the context of the second messenger hypothesis. This discussion has concentrated particularly on those aspects of cyclic AMP physiology which are not readily accounted for by this hypothesis as it is generally stated. Of most importance, we believe, is the evidence of a close relationship between cyclic AMP and calcium in cell activation, a relationship not dealt with in the second messenger hypothesis. However, it should be clear from the foregoing that many aspects of this calcium-cyclic AMP relationship remain to be established before its true range and importance in the control of cellular responses is apparent. Incorporating this relationship into a general model of cell activation has helped us to understand in better perspective the role of cyclic AMP in cell activation. A comparison of a model of this type (Figure 19) with the second messenger model (Figure 1) reveals that it is not a refutation but only an elaboration and extension of this latter model. The value of this newer model appears to be that of helping to understand from an evolutionary perspective how cellular control systems have evolved. The evidence discussed in the preceding review indicates to us that the same basic elements operate in the control of the

activation of many different cell types by extracellular messages. However, the organization of these basic control elements into the cell net which determines an integrated cellular response exhibits great variability, and an understanding of these separate variations on a theme can be more readily understood if the basic theme has been identified. We make no claim that this theme has been completely identified, but we believe that at least two of its elements are calcium and cyclic AMP.

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