Effects of Phosphodiesterase Inhibitors on the Activity of Rat Myocardial Adenyl Cyclase

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EFFECTS OF PHOSPHODIESTERASE INHIBITORS ON THE ACTIVITY OF RAT MYOCARDIAL ADENYL CYCLASE

by
Garry Lee DeGraaf

A Thesis Submitted to the Faculty of The Graduate College in partial fulfillment of the Degree of Master of Arts

Western Michigan University Kalamazoo, Michigan August, 1972
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Special thanks to my wife, Linda, whose patience and understanding have made it possible to complete this paper.

Garry Lee DeGraaf
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INTRODUCTION

Adenosine-3',5'-monophosphate (C-AMP), (Figure 1), is recognized as an important metabolic regulator and mediator of hormone action.

The discovery of this compound by Sutherland and his associates in 1958 and the determination of the structure and molecular weight by Lipkin, et al. (1959) was initiated by the observations made in earlier work on the effects of glucagon and epinephrine on liver glycogenolysis. Over several years of investigation it was learned that a heat stable substance was responsible for activation of the liver phosphorylase system which, when stimulated by glucagon or epinephrine, was directly linked to a decrease in liver glycogen and an increase in free glucose (Sutherland, 1950; McChesney, et al. 1949). Additional efforts which led to the discovery of C-AMP have been summarized by Robison, et al. (1971). Subsequent investigations led Sutherland and Rall, (1960) to postulate the "second messenger theory". This hypothesis contends that a hormone or other agent, the first messenger, stimulates a membrane bound enzyme which catalyzes the formation of C-AMP from adenosine-5'-triphosphate (ATP) in the presence of magnesium ion (Mg^{++}), (Figure 1). The C-AMP formed functions as an intracellular or second messenger, regulating various metabolic processes.
FORMATION AND DEGRADATION OF ADENOSINE-3',5'-MONOPHOSPHATE

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ADENOSINE-5'-TRIPHOSPHATE (ATP) \[ \rightarrow \] ADENYL CYCLASE \[ \text{Mg}^{++} \] ADENOSINE-3', 5'-MONOPHOSPHATE (C-AMP) + 3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ADENOSINE-5'-MONOPHOSPHATE (5-AMP) \[ \text{(Mg}^{++}) \] PYROPHOSPHATE
The Role of C-AMP

Glycogenolysis

The role of C-AMP in skeletal muscle glycogenolysis is probably the best known mechanism to date. In skeletal muscle production of C-AMP initiates activation of a protein kinase, phosphorylase b kinase kinase, which is the link between C-AMP and glycogenolysis. When activated this kinase is capable of phosphorylating another kinase, phosphorylase b kinase, in the presence of ATP and Mg++, to the active form. Phosphorylase b kinase in turn, in the active form, converts inactive phosphorylase b to active phosphorylase a in the presence of ATP, Mg++ and calcium ion (Ca++). Active phosphorylase a catalyzes the conversion of glycogen to glucose-1-phosphate, which has an important role in cellular metabolism (Krebs, et al. 1966). The necessity of Ca++ for activation of phosphorylase b to a has initiated several speculations, however further investigation is necessary to make any definitive correlations (Robison, et al. 1971).

Liver glycogenolysis is also linked to the action of intracellular C-AMP. Again the link is the activation of a protein kinase, which appears to be analogous to phosphorylase b kinase. At present phosphorylase b kinase kinase has not been isolated from liver, however investigators feel the presence of such a kinase is a likely possibility (Langan, 1968).
Lipolysis

C-AMP is intimately associated with the breakdown of triglycerides to free fatty acids and glycerol. The great number of agents which affect lipolysis creates a great deal of confusion and makes generalization of mechanisms difficult. However, most hormones affecting lipolysis or fat deposition have been shown to involve C-AMP directly or indirectly, and C-AMP has been shown to influence the triglyceride-lipase system (Robison, et al. 1971). The role of C-AMP in lipolysis is physiologically important in that it has been estimated that oxidation of free fatty acids by peripheral tissues could account for 80 percent of basal oxygen consumption in man (Carlson, 1968).

Steroidogenesis and protein synthesis

The role of C-AMP in steroidogenesis has been hypothesized in papers by Lefkowitz, et al. (1971) and Garren, et al. (1971). Lefkowitz reports that the catalytic production of C-AMP in the adrenal cortex is specific for adrenocorticotropic hormone (ACTH). Garren et al. (1971) by the use of column chromatography and sucrose gradient centrifugation, demonstrated that C-AMP is bound to a C-AMP dependent receptor unit of a protein kinase. The binding of C-AMP to this receptor unit, functionally frees the catalytic unit of the kinase, thus activating it. This allows phosphorylation of a ribosomal protein, which in turn allows translation of messenger RNA and thus protein synthesis. Previous work had shown that protein
synthesis was an essential step in steroidogenesis (Garrén, et al. 1965). An involvement of C-AMP with protein synthesis has also been shown in bacteria. Size limitations of bacteria restrict the number of enzymes which they can carry at any one time, but they do carry the genetic potential for synthesizing many more when needed. The term inducible enzyme refers to this type of enzyme which is synthesized in response to an inducer. The inducer is usually a substrate for one of the enzymes. One of the essential roles of C-AMP is to regulate which enzyme is synthesized in response to a mixture of substrates. Further information on proposed mechanisms is presented by Pastan and Perlman (1970).

Other effects

In addition to those described above, C-AMP has been implicated in nearly every physiological function investigated, although most of the mechanisms of interaction are unknown. The involvement of C-AMP in muscle contraction appears to be understood, however cellular mechanisms are unknown. Speculation on Ca++ mobilization in muscle cells are many and varied. A recent report shows that C-AMP may inhibit a Ca++ activated ATPase of the sarcolemma of the heart which is involved in the active transport of Ca++ across the sarcolemma (Dietze and Hepp, 1972). This ability to inhibit this ATPase could result in changes in the flux or mobilization of Ca++ (Hurowitz and Suria, 1971). However, increased C-AMP levels in cardiac tissues result in increased contraction whereas in smooth muscles
this increase results in relaxation. This is only one example of actions which makes generalization of mechanisms difficult. Rasmussen (1970) has postulated interactions of C-AMP with Ca++ and intracellular organelles in various functions, including secretion, although little experimental evidence has been generated to support his hypothesis.

Many recent experiments regarding new roles of C-AMP have been summarized by Greengard and Costa (1970), and by Robison, Nahas and Triner (1971).

Formation and Degradation of C-AMP

The formation of C-AMP is dependent on the enzyme adenyl cyclase while degradation of C-AMP is dependent on a phosphodiesterase specific for the 3',5'-cyclic nucleotide.

Adenyl cyclase

Adenyl cyclase is the membrane bound enzyme which catalyzes the intracellular formation of C-AMP from ATP in the presence of Mg++, (Figure 1). Evidence suggests that the Mg++ is present as a Mg-ATP complex. Several studies have shown competition between Mg++ and other divalent cations (Rosen and Rosen, 1969; Drummond, et al. 1971). Drummond and Duncan (1970) have shown Ca++ to be competitive with Mg++, thus inhibiting myocardial adenyl cyclase in the guinea pig.

The distribution of adenyl cyclase is widespread. It has been identified in all mammalian tissues studied and in many lower forms.
Neither adenyl cyclase nor C-AMP has been found in higher plants, (Robison, et al. 1971). The enzyme is particulate in nature and is associated with the plasma membrane and microsomal membranes (Rabinowitz, et al. 1965). Separation of adenyl cyclase from other cellular components has to date been unsuccessful with the exception of a bacterial form of the enzyme (Hirata and Hayaishe, 1967). It is unclear whether the enzyme found in this lower form is closely related to the enzyme found in higher forms. Many questions will be difficult to investigate until a purification is accomplished. Difficulty in assessing results stems from contamination by other enzymes and agents in crude and partially purified preparations. Levey (1971a), has solubilized preparations of heart adenyl cyclase, but lost hormone responsiveness of the enzyme. By the addition of phosphatidylserine the response to glucagon was restored, and the addition of phosphatidylinositol restored norepinephrine (NE) responsiveness (Levey, 1971b). Work of this nature indicates that components of the plasma membrane are necessary for hormone responsiveness.

Early work postulated adenyl cyclase to be the beta-adrenergic receptor on the basis of many of its responses, and also to be associated with the alpha-receptor (Robison, et al. 1967). Several lines of evidence indicate that the adenyl cyclase enzyme is of a single type, which, however, may be associated with several distinct receptors. Butcher, et al. (1968) showed with work on fat cells.
that even though many hormones were able to activate adenyl cyclase, combinations of hormones were never additive. The ability to block one hormone without effecting the other was evidence of multiple receptors. A specific example in cardiac tissue is the actions of catecholamines and glucagon (Glick, et al. 1968). Their actions are similar in the activation of adenyl cyclase but not additive. Catecholamines can be efficiently blocked by propranolol, a beta-adrenergic blocking agent, but the glucagon is unaffected. The same correlation can exist for catecholamines and prostaglandins (Klein and Levey, 1971). Thus, it appears the adenyl cyclase system is composed of at least two parts, a membrane receptor or receptors and the catalytic unit. Stimulation by fluoride appears to be by a mechanism unlike the conventional receptor system (Robison, et al. 1971). The method of adenyl cyclase activation by fluoride is poorly understood. However, it is commonly used in experiments to elicit up to a 20-fold increase in activity over unstimulated activity (basal activity).

Cyclic-3',5'-nucleotide phosphodiesterase (PDE)

Phosphodiesterase is the enzyme responsible for the catalytic breakdown of C-AMP to adenosine-5'-monophosphate (5'-AMP) by hydrolysis of the 3' bond (Figure 1). The presence of Mg++ is required, however the presence of other divalent cations is necessary for total activity. PDE exists in soluble as well as in a form associated with the plasma membranes of the cell. The particulate PDE was not
elutable under several conditions, including hypertonic and hypotonic washings of a membrane preparation. However, the particulate and soluble forms appear to be enzymatically similar in preliminary experiments (Sutherland and Rall, 1958). PDE has been identified in many tissue types (Butcher and Sutherland, 1962) but only a few have been studied in detail.

The most common inhibitors of PDE are the methylxanthines, of which theophylline is the most widely used. Data suggests that in addition to PDE inhibition, theophylline also may inhibit adenyl cyclase under certain conditions (Lucchesi, 1968). Robison, et al. (1971) attests to this problem and summarizes in stating that the effects of methylxanthines are very complex, probably resulting in many actions other than PDE inhibition. Papaverine, which is a substituted isoquinoline, has been used as an inhibitor of PDE in some recent work since it is at least 10 times more potent than theophylline (Pöch and Kukovetz, 1971).

Assays for C-AMP and Adenyl Cyclase

To assess the presence of the adenyl cyclase enzyme and its response to various agents and hormones a variety of assays have been presented in the literature.

Assays for C-AMP

Original methods for determining tissue levels of C-AMP proposed by Rall and Sutherland, (1958) and Posner, et al. (1964) have been revised and largely dismissed over several years due to additional
knowledge of cellular mechanisms and kinetics and to limitations which were inherent in these early methods. Wastila, *et al.* (1971) and Kuo and Greengard (1970) devised methods of C-AMP determination from the discovery of the requirement of C-AMP by phosphorylase b kinase kinase. Gilman (1970) has presented another method based on C-AMP binding to a skeletal muscle kinase which is much simpler than other methods of similar theory. The reported sensitivity and specificity of this assay are additional reasons for its common usage (Breckenridge, 1971). Other methods include radioimmunoassay by Steiner, *et al.* (1969), separation via gas chromatography by Krishna (1968) and high pressure anion exchange chromatography by Brooker (1970). These latter methods have limitation primarily in the sophistication of equipment and technique necessary for the procedure.

**Assays for adenyl cyclase activity**

Like assays for C-AMP the methods for assaying adenyl cyclase activity are abundant in the literature representing many techniques and modifications. Earlier methods were cumbersome and time consuming since once the C-AMP was generated via the adenyl cyclase reaction, one had to perform a second assay on the incubate to quantify the C-AMP generated, and the use of two separate assays introduced additional errors into the methodology. Alternatives became available with the advent of radiolabeled ATP, however the generated C-AMP still had to be separated from other labeled components of the reaction.
The most widely used method for determination of adenyl cyclase activity was introduced by Krishna, et al. (1968), and had as its basis the reaction of radiolabeled ATP in the presence of an active adenyl cyclase preparation to form labeled C-AMP. C-AMP was separated from ATP and other products of the reaction, prior to quantification by liquid scintillation counting, by the use of barium sulfate (BaSO₄) precipitation following ion exchange chromatography. A method employing a single step for the separation of C-AMP, an aluminum oxide column, was introduced by Ramachandran, (1971). The addition of phospho(enol)pyruvate and pyruvate kinase or similar ATP-generating system have been employed to minimize any effects of ATPase activity, thus assuring sufficient ATP as substrate (Drummond and Duncan, 1970).

Phosphodiesterase contamination

In view of the current inability to obtain a purified preparation of adenyl cyclase, preparations used in assays are certain to contain contaminants. Residual particulate PDE, if present, must be inhibited in any assay for adenyl cyclase activity or measurement of C-AMP may be unrealistic due to increased hydrolysis of C-AMP in the presence of PDE. As mentioned previously, theophylline is the most commonly used inhibitor of PDE activity. During the investigation leading to this paper, a published communication from Weinryb and Michel, (1971) indicated that in guinea pigs, theophylline inhibits adenyl cyclase from lung but not from heart. Thus, in controlling
the effects of residual PDE, under some conditions and in some tissues one may be creating unrealistic adenyl cyclase activity.

Statement of Problem

The unknown entities of the adenyl cyclase system are numerous and reported data is often conflicting and confusing. This may or may not be due to analytical procedures, but judging by the vast variation in techniques presented in the literature, it appears few investigators are satisfied with current procedures. This often necessitates repetition of basic experimentation before further studies can be conducted with any degree of confidence.

The recent discovery of Weinryb and Michel (1971) that theophylline inhibits adenyl cyclase activity differentially in guinea pigs is significant in that routine use of theophylline in adenyl cyclase assays is evident in published data.

The hypothesis of this investigation was that theophylline and papaverine may affect rat myocardial adenyl cyclase activity differentially under various conditions of activation. It was the purpose of this research to investigate some currently used methods of analysis of adenyl cyclase activity and utilize the procedure which appeared most satisfactory in evaluating the effects of theophylline and papaverine on adenyl cyclase activity at conditions of no activation (basal), maximal activation produced by fluoride stimulation and activity stimulated by isoproterenol, a beta-adrenergic agonist of the heart.
METHODS AND MATERIALS

Materials

The following materials are listed because of their importance in the various assays and procedures to be stated. The name of each material is followed by its common abbreviation. The supplier from whom it was purchased is also listed because of variation in quality of commercially available materials.

Bovine serum albumin (BSA); L-norepinephrine-HCl (NE); adenosine-5'-triphosphate disodium salt (ATP); crystalline adenosine-3',5'-cyclic monophosphate (C-AMP); adenosine-5'-monophosphate (5'-AMP); phosphodiesterase, 3',5'-cyclic nucleotide from beef heart (PDE); phospho(enol)pyruvate disodium salt, hydrate (PEP); pyruvate kinase, crystalline suspension in ammonium sulfate, and diglycine hydrochloride were obtained from Sigma Chemical Co., St. Louis, Mo.

ATP, tetra(triethylammonium)salt-alpha-32P; adenosine-14C(u)-5'-triphosphate, tetrasodium; adenosine-3H(g)-3',5'-cyclic phosphate, ammonium salt; Aquasol® and Liquiflor® were obtained from New England Nuclear Corp., Boston, Mass. Tham® Tris (hydroxymethyl) aminomethane, Fisher Scientific Co., New Jersey; aluminum oxide, Woelm neutral, activity grade 1, distributed by Waters Associates Inc., Framingham, Mass.; sucrose (reagent grade), Merck, New Jersey; Folin-Ciocalteu phenol reagent, Harleco, Philadelphia, Pa.; ethylenedinitrilotetraacetic acid disodium salt (EDTA), Eastman Kodak Co., Rochester, N.Y.;
ammonium sulfate (analytical grade), Mallinckrodt Chemical Works, St. Louis, Mo.; glass distilled toluene, Burdick and Jackson Laboratories, Muskegon, Mich. and Dowex 50W-X8 cation exchange resin, hydrogen form, 100 to 200 mesh, 1.7 meq/ml from BioRad Laboratories, Richmond, Cal.

Theophylline-cholate; papaverine-HCl; and isoproterenol were supplied by The Upjohn Co., Kalamazoo, Mich.

All other chemicals used were of the best available commercial grade.

Methods

Preparation of tissue

Ten (10) male Upjohn bred rats [UPJ-TUC(SD)spf] of approximately 300 grams were mechanically sacrificed and ventricles rapidly excised and exanguinated in ice cold saline. The pooled tissue was homogenized at high speed in a Waring Blender for 20 seconds in 10 volumes of 2 mM glycyl-glycine buffer, pH 7.4 containing 1 mM magnesium sulfate. A procedure for hypotonic particle preparation was followed as given by Murad, et al. (1962) with slight modifications and deletion of the suspension in potassium chloride and potassium phosphate. In this procedure tissue was homogenized in solutions hypotonic to the cells thus causing a lysis of the cells and subsequent washings resulted in a preparation of primarily cellular membrane fragments. The final suspension of 72 ml was apportioned to test tubes in 1 ml aliquots and quick frozen in dry-ice acetone and stored over liquid nitrogen at -70 degrees C.
Protein concentrations were determined by a colorimetric assay introduced by Lowry, et al. (1951). Optical density readings were taken on a Gilford Micro-Sample Spectrophotometer, Model 300, at 550 nm.

**Phosphodiesterase activity of the particle preparation**

To assess the amount of residual PDE activity present in the particle preparation the assay by Pöch (1971) was utilized. The basic premise of the PDE assay is the incubation of labeled C-AMP with tissue being tested for PDE activity and observe if the amount of labeled C-AMP has decreased at the end of the incubation due to degradation in the presence of PDE. Positive controls of commercially available purified PDE allow quantification of PDE present in the test sample.

**An assay for C-AMP**

The performance of the assay described by Gilman (1970) was relatively simple. The purifications of the necessary components of the assay were attained with considerably more labor and time. The protein kinase inhibitor was isolated as described by Appelman, et al. (1966) and protein kinase was isolated according to Miyomoto, et al. (1969) with modifications by Gilman (1970). Bovine submaxillary muscle, obtained from a local abattoir, was the source of both preparations. The assay has as its basis a C-AMP dependent protein kinase and a heat stable protein inhibitor as isolated above. C-AMP present in a sample is quantified by its competition with a labeled
quantity of C-AMP for binding sites on the C-AMP binding protein kinase. The more unlabeled C-AMP present the less labeled C-AMP bound. The kinase can be isolated on Millipore filters and with the use of a standard curve of various levels of unlabeled C-AMP, the unknown sample can be quantified. The inhibitor protein was found to increase the affinity of C-AMP for the kinase and thus is included in the assay for that purpose. The 0.2 ml incubation mixture contains; 1 mM magnesium sulfate (MgSO₄); 50 mM sodium acetate buffer, pH 4.0; ³H-C-AMP; inhibitor protein and sufficient protein kinase to bind less than 30 percent of the ³H-C-AMP. A standard curve of commercially available C-AMP accompanied the test samples. The reaction was initiated by the addition of the protein kinase to the other ingredients. Equilibrium was attained at 0 degrees C., after at least 60 minutes and the reaction was terminated by dilution to 1 ml with cold 20 mM potassium phosphate buffer, pH 6.0. The resulting solution was applied to a 25 mm cellulose ester (Millipore) filter, previously rinsed with 20 mM phosphate buffer, pH 6.0 and washed with 10 ml of the same buffer. The filter was allowed to drain over suction and put directly into a scintillation vial containing 1 ml methyl cellosolve, to dissolve the filter. Counting was done in a solution containing cellosolve, Liquiflor® and toluene in a Packard Tri-Carb Liquid Scintillation Counter, Model 3375.
Assays of adenyl cyclase activity

As stated previously the assay of Krishna, et al. (1968), is an adenyl cyclase assay introducing a BaSO₄ precipitation coupled with ion exchange chromatography for the isolation of generated C-AMP. The adenyl cyclase assay differs from a C-AMP assay in that the reaction involving the adenyl cyclase enzyme in the presence of labeled ATP forms a labeled C-AMP. Thus by separating the C-AMP from the ATP, and other breakdown products of the reaction, the adenyl cyclase activity can be expressed as pmoles of C-AMP formed/weight protein/time. Thus, even though the ultimate measurement in an adenyl cyclase assay is that of C-AMP it is the product of the adenyl cyclase activity. Blanks were prepared by addition of protein, denatured by boiling, in amounts equal to active protein. In Krishna's assay the incubation volume of 0.6 ml contained: 40 mM Tris-CL buffer, pH 7.3; 3.3 mM MgSO₄; 10 mM theophylline; 1 to 2 mM ATP; ¹⁴C or ³²P ATP (specific activity 5-50 mc/m mole); 1 to 10 mg enzyme and 10 mM NaF when present. Incubations were at 30 degrees C. for various times. Reactions were terminated by the addition of 0.1 ml of a solution of carrier C-AMP and boiled for two minutes. The tubes were centrifuged at 2,000 rpm for 10 minutes and the supernatant chromatographed on Dowex 50 resin, prepared by pipetting a 50/50 suspension of the resin into a 0.5 cm diameter glass column to a height of 2 cm and washing with water. The flow through and first 2 ml of eluate were discarded. The next 4 ml of eluate were collected and treated with 0.2 ml of 0.25 M ZnSO₄ and 0.2 ml of...
0.25 M Ba(OH)$_2$, mixed then centrifuged at 2,000 rpm for 5 minutes. Three ml of the supernatant was counted in 10 ml of Aquasol$^\circledR$. A sequential summary of this procedure is stated in Figure 2.

It should be noted here that a modification of this method was introduced by Levey and Epstein, (1969), using an incubation volume of 0.06 ml, with changes in the reaction mixture including: a higher specific activity of substrate; 21 mM Tris-CL at pH 7.7 and the addition of human serum albumin, 0.8 mg/ml. The reaction was terminated with the addition of 0.1 ml of a solution containing 4 micromoles (μmoles) ATP, 1.25 μmoles C-AMP and $^3$H-C-AMP and boiled for 3 minutes. The concentrations of ZnSO$_4$ and Ba(OH)$_2$ were reduced to 0.17 M and 0.15 M, respectively.

The assay introduced by Ramachandran (1971) differs only slightly from that of Krishna, et al. (1968) in the reaction mixture. (See Figure 2). The significant change is in the separation technique for C-AMP. Ramachandran terminates the reaction with 0.25 ml of a solution containing 10 mM Tris-CL, pH 7.4, 3 mM C-AMP and 0.05 microcuries $^3$H-C-AMP and boiled for 3 minutes. From each tube, 0.25 ml of supernatant was applied to a 0.5 x 6 cm column of dry neutral aluminum oxide, stoppered by a plug of glass wool. The column was washed with 3 ml of 10 mM Tris-CL pH 7.4 and the effluent was collected in scintillation vials and counted in 15 ml of Bray (1960) solution.
FLOW SEQUENCE OF ADENYL CYCLASE
ASSAY BY KRISHNA, et al. (1968)
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<th>STEP 1</th>
<th>STEP 2</th>
<th>STEP 3</th>
<th>STEP 4</th>
<th>STEP 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Mixture</td>
<td>Incubation</td>
<td>Termination of Reaction</td>
<td>Separation Procedures</td>
<td>Radioactive Counting Procedure</td>
</tr>
<tr>
<td>Containing:</td>
<td>Incubation was carried out at 30°C in a water bath for various times.</td>
<td>Reactions were terminated by the addition of a C-AMP solution and boiled for three minutes.</td>
<td>The C-AMP was separated from other components of the reaction mixture by:</td>
<td>The chromato-graphed and precipitated solution containing labeled C-AMP was counted in Aquasol® in a liquid scintillation counter.</td>
</tr>
<tr>
<td>- enzyme prepara-tion</td>
<td>- ion exchange chromatography on Dowex 50 columns and</td>
<td>- BaSO₄ precipitation.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- radiolabeled ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- tris-CL buffer</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- MgSO₄</td>
<td></td>
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<tr>
<td>- theophylline</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- activation when present</td>
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</table>
A modified adenyl cyclase assay

The assay used in this paper to determine the effects of PDE inhibitors on adenyl cyclase activity was basically that of Levey and Epstein (1969) with modifications of Drummond and Duncan (1970) and the separation procedures of Ramachandran (1971) with other modifications which will be discussed in RESULTS AND DISCUSSION.

This assay is of the same theory in determination of adenyl cyclase activity as the above, that is, the formation of labeled C-AMP from labeled ATP and subsequent separation of the C-AMP. (See Figure 2). A summary of the assay follows: an incubation mixture of 0.06 ml contains - 40 mM Tris-CL, pH 7.7; 5 mM MgCl₂; 0.5 mM C-AMP; 0.2 mM ATP (³²P-ATP at 75-125 cpm/pmole); 20 mM PEP; 80 micrograms pyruvate kinase; 0.1 percent BSA and 150-200 micrograms protein. Sodium fluoride and isoproterenol at concentrations of 10 mM and 1 x 10⁻⁵ M, respectively, when present. The incubation is carried out for 20 minutes at 37 degrees C, and terminated with the addition of 0.25 ml of a solution containing 10 mM Tris-CL, pH 7.4; 3 mM C-AMP and 3-4,000 cpm ³H-C-AMP and boiled for 3 minutes. The entire contents of the tube was applied to a 0.5 x 6 cm column of dry aluminum oxide and washed with 3 ml of 10 mM Tris-CL, pH 7.4. The effluent was collected in scintillation vials and counted in 15 ml of Brays solution.

Statistics

Mean differences were compared statistically utilizing Student's t test. Differences significant from controls were expressed as p <0.001.
RESULTS AND DISCUSSION

The importance of C-AMP in cellular regulation and metabolism was discussed in the INTRODUCTION. The adenyl cyclase enzyme is the only known enzyme catalyzing the formation of C-AMP, thus knowledge of the properties of adenyl cyclase is essential in that this enzyme regulates the amount of C-AMP formed and thus the effect on any physiological parameter. Studies can be conducted on two main levels; regulation of the formation of C-AMP, or intracellular effects of C-AMP once it is formed. It is the former which is investigated in conducting assays for adenyl cyclase activity. Knowing the concentration of radioactive labeled substrate, ATP, and counting radioactive labeled product, C-AMP, the activity of the catalyzing enzyme, adenyl cyclase, is determined. Assays for C-AMP tell only how much C-AMP is in a particular sample, unless other information is available on conditions under which it was generated.

Investigation of Various Assay Conditions

The first assay investigated was that of Krishna, et al. (1968), which was an adenyl cyclase assay based on the formation of labeled C-AMP and separation techniques of ion exchange chromatography and BaSO₄ precipitation. Initial experiments indicated that if the Dowex columns used in separation of C-AMP received any protein from the reaction mixture the recovery of C-AMP through the column was variable. Denatured protein was difficult to completely isolate from the supernatant by simple measures such as centrifugation. Initial
experiments were done on ventricular tissue slices. The use of tissue homogenates, instead of crude tissue slices, decreased the variability, but not to a completely satisfactory level.

A procedure for the homogenization of cardiac tissue in 0.25 M sucrose and a modified solution used to terminate the C-AMP forming reaction were reported by Levey and Epstein, (1969). These modifications were combined with features of Krishna's assay (Krishna, et al. 1968), and adenyl cyclase activity was detected. However, values were low compared to published data and basal activity only reflected a 2-3 fold stimulation by fluoride, which is less than the reported 5-10 fold increases of basal activity (Levey and Epstein, 1969; Drummond and Duncan, 1970).

Blank samples, those with boiled tissue, showed variability even at low specific activity, 5-20 cpm/p mole. Blanks were in the range of 200-300. Common quantities of C-AMP produced in the assay under unstimulated conditions were about 10 pmoles. Thus with specific activity at 5 cpm/p mole the total cpm of labeled product would be 50 cpm. This over blank is not sufficient for quantification of product. Much greater blank to sample ratios must prevail to obtain acceptable levels of precision and sensitivity. Reducing the volume of the C-AMP forming reaction reduced variability and size of the blank, but the blank to sample ratios were still not satisfactory.

The addition of an ATP-generating system (Drummond and Duncan, 1970) alone, or variation of ATP concentration alone had little effect
on adenyl cyclase activity. However using ATP levels lower than most published, 0.25-0.5 mM, along with the generating system, the activity was substantially higher than with 1-2 mM ATP.

In that 
Mg$^{++}$ is required for the conversion of ATP to C-AMP by adenyl cyclase, experiments to determine optimum Mg$^{++}$ concentration were conducted at various levels of ATP to determine whether absolute levels of Mg$^{++}$ or ATP/Mg$^{++}$ ratio was the essential factor. In tests run on ATP concentrations from 0.1 to 0.5 mM, Mg$^{++}$ in concentrations of 0.25 to 2.0 mM increased activity, then activity plateaued, indicating 2 mM was the optimal Mg$^{++}$ concentration under the conditions of this assay.

Other ingredients of the reaction mixture were tested in order to attain the proper concentrations to evoke the maximum adenyl cyclase activity. Adenyl cyclase activity was determined at various concentrations of Tris-CL buffer, PEP and pyruvate kinase. This experimentation provided evidence that 20 mM PEP was optimal, pyruvate kinase at 80 micrograms/ml was in excess and 40 mM Tris-CL buffer provided the maximal adenyl cyclase activity.

Because of the low activity it was speculated that perhaps the sucrose homogenized cardiac preparation was not optimally active under the conditions of this assay with the modifications as mentioned above. A hypotonic particle preparation was prepared as described earlier and activity was still unsatisfactory.

Others in the field of adenyl cyclase research have reported successful use of the C-AMP assay introduced by Gilman (1970). This
is an assay for C-AMP, thus would replace only the separation
procedure of C-AMP from other components of the assay of AC activity.
Preliminary data indicated good precision and sensitivity. Binding
of the C-AMP dependent kinase, referred to in Methods section was
linear on a log:log scale. Although Gilman makes note of timing
filtrations after incubation, it was shown in the process of this
investigation to be unjustified. Duplicate samples filtered 2 hours
apart showed negligible change. A series of experiments were
conducted to obtain optimum conditions of the assay. Five micrograms
of kinase and 15 micrograms of inhibitor with 1 pmoles of $^3$H-C-AMP as
the competitive binder, was the most desirable combination. A
standard curve of C-AMP from 1-10 pmoles in a solution comparable to
that of the adenyl cyclase reaction mixture was linear on a log:log
scale. Reactions were conducted generating unlabeled C-AMP from
unlabeled ATP under the same conditions mentioned above with the
omission of the radioactive labeled ATP. The data obtained using
the combination of the two assays showed activity approximately 6
times less than that reported by Krishna, et al. (1968), however, his
data was collected on brain adenyl cyclase preparations and Sutherland,
et al. (1962) stated that brain adenyl cyclase may be 5-6 times
greater than that of heart.

It was soon evident by running the same standard curve with each
assay that something was causing variability in binding characteristics
of the C-AMP dependent kinase. Binding varied from day to day, some-
times by 50 percent. Investigation was undertaken into ingredients
of the assay and thawing and refreezing techniques. The presence of inhibitor protein showed some inhibition of kinase binding but only at concentrations 3 times that used in this experimentation. Mg\(^{++}\) at various concentrations had no effect up to 2 mM and only a slight effect up to 6 mM. Effects of 5'-AMP and ADP also were negligible. It was found that the technique of thawing the kinase from -70 degrees C. was critical in the binding activity of the kinase and also the time kept at 0 degrees effected the binding characteristics. Allowing the protein to stand in ice reduced its binding affinity for C-AMP.

In view of the lability of the C-AMP binding kinase and the necessity of combining two separate assays, one for generation of C-AMP and another for measurement of the C-AMP formed and the time involved in isolation of the C-AMP binding kinase from muscle, an alternate method was sought.

Two methods have been published with reportedly good separation of C-AMP from other components of the reaction mixture of an adenyl cyclase assay. White and Zenser (1971), and Ramachandran (1971) reported similar techniques utilizing aluminum oxide as the basic component of the separation technique. In pilot experiments the method of Ramachandran was selected because of the low blanks obtained, in spite of the slightly lower recovery it showed over the other method. Less than 0.02 percent of the ATP applied to the column came through with 3 mls of wash and approximately 90 percent of the C-AMP came through. The reaction was modified to contain 0.5 mM C-AMP and a higher specific activity of substrate was used. Preliminary data
indicated excellent blank to sample ratios of about 1:25, but activity
was not as high as shown in other work (Krishna, et al. 1968; Levey
and Epstein, 1969).

A substrate linearity study showed peak efficiency to be at 0.2
mM ATP under conditions of this assay. Mg^{++} curves showed potentia-
tion which plateaued at 5-6 mM.

Attempts to stimulate cardiac adenyl cyclase with prostaglandin
E_1 (PGE_1) were unsuccessful on all occasions. A review of the recent
literature revealed a communication by Vergroesen, et al. (1969)
which presented data to suggest the PGE_1 series to be inactive in rat
heart adenyl cyclase, even though this series is active in guinea pig
heart adenyl cyclase (Klein and Levey, 1971). The stimulatory effect
of 10 mM NaF and 1 \times 10^{-5} \text{ M isoproterenol were 1000 percent and 50
percent respectively, over basal activity.}

The adenyl cyclase assay used in the following experimentation
includes modifications stated above and summarized in Methods. The
assay was linear with time from 5 to 20 minutes and with enzyme from
100-200 micrograms at both basal and fluoride stimulated conditions.
Sensitivity is to at least 0.5 pmoles and specificity is inherent due
to a specifically labeled substrate. The precision is demonstrated
in the results of the data to follow.

The Effects of Theophylline and Papaverine on
Adenyl Cyclase Activity

Using the above described modified adenyl cyclase assay, including
0.5 mM C-AMP to dilute any activity of residual PDE activity AC
activity was determined. The rational for adding unlabeled C-AMP was that any degradation of accumulated product due to PDE activity would be diluted by breakdown of the unlabeled C-AMP which is in great excess over the labeled C-AMP. Adenyl cyclase activity was determined with and without 8 mM theophylline at basal activity; activity elicited by 10 mM fluoride and that by $1 \times 10^{-5}$ M isoproterenol (Table 1). The rational for using 8 mM theophylline was that this was the minimum concentration used in most assays (Krishna, et al. 1968; Levey and Epstein, 1969; Drummond and Duncan, 1970; Klein and Levey, 1971).

Isoproterenol was used instead of NE because preliminary data indicated a slightly higher stimulation of cardiac adenyl cyclase than NE and both are beta-adrenergic agonists of the heart. The effect of theophylline on basal activity was to increase it about 25 percent. The effect on fluoride stimulated activity was a decrease of nearly 20 percent. The effect on isoproterenol stimulated activity was an increase of approximately 15 percent. Percent error in all determinations ranged from 3.9 to 8.0, demonstrating the precision of the assay. Percent error is defined as the standard deviation divided by the mean times 100.

Table 2 reflects the effects of 1 mM papaverine under identical conditions as the above study. Basal activity was decreased 20 percent; fluoride stimulated activity was increased about 25 percent; and stimulation by isoproterenol resulted in a 15 percent decrease. Thus the action of theophylline and papaverine on a preparation of hypotonic.
TABLE 1
The Effects of Theophylline on Basal, Fluoride and Isoproterenol Stimulated Activity of Rat Heart Adenyl Cyclase

<table>
<thead>
<tr>
<th></th>
<th>BASAL ACTIVITY</th>
<th>Fluoride Stimulated Activity</th>
<th>Isoproterenol Stimulated Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- theophylline</td>
<td>+ theophylline</td>
<td>- theophylline + theophylline</td>
</tr>
<tr>
<td>Mean (n=6)</td>
<td>40.56</td>
<td>51.67**</td>
<td>356.22**</td>
</tr>
<tr>
<td>Stnd. Dev.</td>
<td>1.72</td>
<td>2.33</td>
<td>26.50</td>
</tr>
<tr>
<td>% Change</td>
<td>Increased</td>
<td>27.4</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Values expressed as pmoles/mg protein/20 minutes.

**Significantly different from control, p < 0.001.
TABLE 2

The Effects of Papaverine on Basal, Fluoride and Isoproterenol Stimulated Activity of Rat Heart Adenyl Cyclase

<table>
<thead>
<tr>
<th></th>
<th>BASAL ACTIVITY</th>
<th>Fluoride Stimulated Activity</th>
<th>Isoproterenol Stimulated Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- papaverine</td>
<td>+ papaverine</td>
<td>- papaverine</td>
</tr>
<tr>
<td>Mean (n=6)</td>
<td>50.78</td>
<td>40.44**</td>
<td>518.89</td>
</tr>
<tr>
<td>Stnd. Dev.</td>
<td>2.05</td>
<td>2.61</td>
<td>27.28</td>
</tr>
<tr>
<td>% Change</td>
<td>Decreased</td>
<td>20.4</td>
<td>Increased</td>
</tr>
</tbody>
</table>

Values expressed as pmoles/mg protein/20 minutes.

**Significantly different from control, p <0.001.
rat heart tissue under the conditions of this assay were exactly opposite. In basal activity theophylline increased adenyl cyclase activity over 25 percent whereas under the same conditions papaverine decreased activity about 20 percent. With fluoride stimulation theophylline decreased activity almost 20 percent while papaverine increased activity nearly 25 percent. With isoproterenol stimulation, theophylline increased activity nearly 15 percent and papaverine decreased activity nearly 15 percent.

It was interesting to note that with both PDE inhibitors the fluoride stimulated activity was effected opposite of the basal or isoproterenol stimulated conditions. Fluoride stimulated activity has been reported as being by some mechanism other than the normal receptor mechanism. Thus, one might speculate that the effect of the two PDE inhibitors on this unknown fluoride mechanism was creating a differential effect causing an increase in one case and a decrease in the other. The opposing effect of papaverine and theophylline at both basal and isoproterenol activated conditions can be speculated as a differential effect at the receptor level. The receptor is at least in part composed of lipoproteins of the plasma membrane and thus would possess definite solubility characteristics (Levey, 1971a and 1971b). This lipoprotein component would dictate solubility properties at the receptor site. In that theophylline is many times more water soluble than papaverine, it could be speculated that the differential effects seen could be a result of different interactions at the receptor site. A further speculation might involve a steric
effect on the membrane receptor. Theophylline, a xanthine, is a two
ring structure, whereas papaverine, an isoquinoline, is a three ring
structure. It seems reasonable to speculate that a configurational
change in the receptor due to interaction with either of the two
compounds may cause differential results when the altered receptor is
stimulated by a given activator.

At this point in time, because of the lack of knowledge of
receptor mechanism and structure, further speculation would be
meaningless. However, additional work in the area of differential
effects of activators in interaction with other compounds may shed
light on membrane structure and mechanism.

The Effects of C-AMP on Adenyl Cyclase Activity

In experiments testing the effects of theophylline and papa­
verine, 0.5 mM C-AMP was added to the labeled C-AMP generating
reaction mixture to dilute the effects of any residual PDE activity.
The effect of C-AMP was investigated to determine whether this
addition had any effect on adenyl cyclase activity. It was found
that in the absence of the unlabeled C-AMP very little activity was
present. Basal activity was less than 10 percent of that seen with
0.5 mM C-AMP added. Fluoride stimulated activity was approximately
10-15 percent of that seen with C-AMP added. This indicates that
either PDE is degrading the labeled C-AMP formed or the C-AMP is
being bound to some component of the assay and lost in the separation
procedures. Both situations would be elevated in the presence of 0.5
mM C-AMP, in that it dilutes the labeled C-AMP by at least 300:1.
The following attempts at detecting PDE activity were negative or very slight, not nearly enough to account for the loss seen. The standard PDE assay by Pöch (1971) on the particle preparation, as described in Methods section, detected no PDE activity. A PDE assay on conditions duplicating adenyl cyclase conditions also detected no PDE activity. The latter was accomplished by the addition of \(^3\text{H-}\text{C-AMP}\) to the ingredients of the adenyl cyclase assay which normally generates a labeled product. Thus a depletion in cpm in sample with active tissue as compared to control with no tissue would indicate PDE activity. However, as mentioned above, no difference was seen between sample and control groups. Positive controls of 20 micrograms of commercially obtained PDE degraded over 90 percent of the C-AMP present.

To determine whether an element of the adenyl cyclase assay was binding C-AMP, an experiment was designed duplicating adenyl cyclase conditions but again adding \(^3\text{H-}\text{C-AMP}\). After the completion of the normal adenyl cyclase reaction, some samples with tissue and some without were filtered through Millipore filters and others chromatographed on aluminum oxide columns. Results showed no binding except to the particle preparation, and that was only approximately 0.5 percent of the total. Boiling of the particle preparation reduced the binding to the particle preparation below this 0.5 percent level.

General Discussion

The lack of adenyl cyclase activity which was apparent when attempting to repeat the assays of Krishna et al. (1968) and Levey
and Epstein, (1969) was probably due to imbalance of certain ingredients of the adenyl cyclase assay which were to a large part elevated by the changes indicated earlier in this section, to include modification in concentration of Mg$$^{++}$$, ATP and the addition of an ATP-generating system. It would appear however, that some data published reflects blank to sample ratios which makes precise data difficult, as previously described. Judging by personal experience of obtaining high and variable blanks is was not surprising that some of the results of Levey and Epstein, (1969) reflect a percent error of over 30 percent. Even though significance was shown, the quantification of adenyl cyclase activity was dubious.

The preparation and use of Dowex columns was time consuming and variable. The variability was due primarily to overlapping of elution peaks of ATP and C-AMP, thus making resin volumes, packing consistancy and elution volumes critical.

The Gilman method superficially appeared to be excellent, however, personal experimentation indicated caution must be exercised to run proper standard curves with each experiment, due to the lability of the kinase preparation. The necessity of the standard curve reduced the number of test samples that can be assayed in a given time.

The most complete separation of C-AMP was demonstrated with columns of aluminum oxide. Low blanks of less than 100 were obtained with samples containing 1 to 2 x 10^6 cpm of labeled ATP. The procedure was highly reproducible. Recovery of C-AMP is consistant and
high, between 70-80 percent. Aluminum oxide is very hydrophilic therefore caution must be exercised in maintaining dry resin. Differences can be detected between a newly opened cannister of resin and one which has been exposed to air.

The procedure was less laborious and time consuming than Dowex columns and equally as simple as the Gilman method.

The effects of theophylline and papaverine on adenyl cyclase activity introduce interesting questions for anyone utilizing either of them in an adenyl cyclase assay. Under the conditions of this experiment it was shown that if one were to compare results using similar systems, but one using theophylline and the other papaverine, the results could vary by 50 percent. As a hypothetical example, suppose two sets of results appeared in the literature on adenyl cyclase activity of the same tissue, prepared the same way, from the same species. One group used theophylline and the other papaverine. If they compared the effects of isoproterenol on adenyl cyclase activity their results could vary by 30%. Tables 1 and 2 show the opposite effects of theophylline and papaverine not only for isoproterenol but for fluoride stimulated as well as basal activity. This is significant especially considering how little attention is given to the PDE inhibitor used in the assay of adenyl cyclase.

The degradation of C-AMP seen was puzzling in light of the lack of detectable PDE activity or binding. The most likely speculation would be the presence of a PDE which was not being detected by the assay employed. It seems feasible that the activity seen in the
purified standard PDE may not be relatable to the crude preparation attempting to assay. Robison et al. (1971) regarding the enzymatic properties of the soluble versus the particulate PDE was undefinitive enough to allow speculation regarding differences.

Regardless, the addition of unlabeled C-AMP in PDE inhibitor studies was sufficient to negate any effect of PDE activity on labeled C-AMP, thus have no effect on the results, because as mentioned earlier, the 300:1 dilution of labeled C-AMP by unlabeled C-AMP would dilute the degradation of the labeled product.

The inhibitory action on adenyl cyclase activity stated above could have conceivably been linked to an effect of theophylline or papaverine on the pyruvate kinase added to the reaction mixture. By inhibiting the activity of pyruvate kinase, less substrate, ATP, would be available. However, an effect on pyruvate kinase could not account for potentiation of adenyl cyclase activity in that ATP was in excess under conditions lacking the PDE inhibitors.
SUMMARY

The assay used for determination of activity of rat myocardial adenyl cyclase in this paper is believed by the author to be one which surpassed many in present literature on the basis of its sensitivity, reproducability and simplicity. The results obtained on the effects of not only theophylline but also papaverine on adenyl cyclase activity are very interesting in light of recent reports on theophylline activity. During the course of this investigation a communication by Weinryb and Michel (1971) reported theophylline to be inhibitory on lung adenyl cyclase but not heart of guinea pigs.

The present paper shows definite effects in the cardiac tissue of the rat for both theophylline and papaverine, thus the lack of activity in guinea pig cardiac adenyl cyclase indicated species differentiation. Also, this study shows that in a single tissue type, different conditions of activation change the effect of the PDE inhibitors. Theophylline increased basal activity and activity stimulated by isoproterenol, but decreased adenyl cyclase activity stimulated by fluoride. Papaverine decreased basal and isoproterenol stimulated activity while increasing fluoride stimulated adenyl cyclase activity. Thus, it is imperative to determine the effect that a particular PDE inhibitor may have on adenyl cyclase activity when stimulated by various chemical agents.
LITERATURE CITED


