Effects of Epinephrine on Cardiac Cyclic 3′,5′-AMP, Phosphorylase Kinase, and Phosphorylase

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(Received August 14, 1967)

SUMMARY

The concentration of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) and the activities of phosphorylase kinase and phosphorylase were determined in biopsy samples from dog hearts and in whole rat hearts frozen *in situ*. Cyclic AMP was measured by the activation of muscle phosphorylase kinase employing a preincubation at 0° to improve the sensitivity of the assay. Dog heart phosphorylase kinase was measured on extracts of homogenates by comparing the activity of the enzyme at pH 6.0 to that at 8.2. Epinephrine markedly increased the activity at pH 6.0 at a dose that did not significantly elevate the percentage of phosphorylase *a*. The time course of the change in kinase activity was consistent with the hypothesis that activation of this enzyme was necessary for the formation of cardiac phosphorylase *a*.

No significant increase in the concentration of cyclic AMP was demonstrable in the dog heart at a dose of epinephrine that produced a marked inotropic effect. The possibility of an artifact in the biopsy technique was suggested by the finding that a rapid increase in cyclic AMP occurred in rat hearts frozen *in situ*, but not when samples were excised and frozen. Phosphorylase kinase activity increased as rapidly as did the cyclic AMP concentration and before any change in the phosphorylase activity occurred. Pronethalol blocked the epinephrine-induced rise in the cyclic AMP concentration.

These results are consistent with the hypothesis that the effect of catecholamines on cardiac phosphorylase is mediated through an action of cyclic AMP on phosphorylase kinase.

INTRODUCTION[2]

Cyclic 3′,5′-adenosine monophosphate, (cyclic AMP) has been suggested to be the mediator of the catecholamine-induced activation of cardiac glycogen phosphorylase and increased myocardial contractility (1, 2). This hypothesis of Sutherland and colleagues can be stated as: (a) The interaction of epinephrine with the myocardial cell results in activation of adenyl cyclase and consequently an increase in the production of cyclic AMP. (b) Cyclic AMP activates phosphorylase kinase which in turn activates phosphorylase. (c) Cyclic AMP may mediate the positive inotropic effect of epinephrine through an interaction with some component controlling the contractile process.

The interaction of catecholamines with heart adenyl cyclase is well documented. Murad *et al.* (3) showed that the catalytic activity of the heart enzyme was enhanced when exposed to a variety of β-adrenergic agonists *in vitro*. With regard to the second part of the hypothesis, an augmentation of phosphorylase kinase activity by cyclic

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[2] Publication No. 863 of the Division of Basic Health Sciences.

AMP was first demonstrated on purified skeletal muscle enzyme by Krebs *et al.* (4). This effect required ATP and Mg. More recently, such an effect was shown on the enzyme purified from beef heart by Drummond *et al.* (5). The speculation that cyclic AMP plays a role in the positive inotropic effect of catecholamines is based on indirect evidence which has been reviewed (2). There is, at the present time, no evidence of an effect of the nucleotide on the contractile process, on the factors that control this process, or on the contractility of the isolated, working, perfused rat heart (1).

The purpose of this investigation was to examine the effects of epinephrine on the cyclic AMP concentration of the heart *in situ* to determine whether changes in the phosphorylase activity and contractility could be correlated with changes in the concentration of this nucleotide.

METHODS AND MATERIALS

Physiological Preparations

Mongrel dogs weighing between 15 and 24 kg were used. Dogs were anesthetized with 20 mg/kg sodium pentobarbital and 220 mg/kg sodium barbital. Body temperature was maintained between 37° and 38°. Bilateral cervical vagotomy was performed on all dogs. The heart was exposed by a mid-sternal incision, and a strain gauge was sutured to the right ventricle to record contractile force. Artificial respiration was begun just before opening the chest. A positive pressure pump (Harvard) was adjusted to deliver 13 ml of air per kilogram of body weight, 18 times a minute. A polyethylene catheter was inserted in the femoral vein until its tip was about 2 cm below the right atrium. The catheter was prefilled with drug so that when the infusion pump was turned on, the drug was expelled immediately into the vein. Zero time in all experiments refers to the beginning of the constant-rate infusion.

Biopsy samples of the right ventricle of the dog were obtained by the method described by Mayer *et al.* (6). The myocardium was grasped with Cottle tissue forceps and cut with curved eye scissors. Samples of about 200 mg were rapidly immersed in liquid dichlorodifluoromethane (Freon 12) cooled to —150° in liquid nitrogen. A maximum of 3 biopsy samples was obtained from each dog.

Open-chested rats were prepared by the method of Williams and Mayer (7). Male Sprague-Dawley rats (150–225 g) were anesthetized with an intraperitoneal injection of chloralose (50 mg/kg) and sodium pentobarbital (35 mg/kg). Atropine, 1 mg/kg, was administered with the anesthetic solution. The animals were artificially respired with a mixture of 95% O_2 and 5% CO_2. Hearts were frozen *in situ* by clamping between two silver blocks cooled in liquid nitrogen. Drugs were administered through a polyethylene catheter inserted in the femoral vein.

Enzyme Assays

Cardiac phosphorylase activity was assayed by a method described previously (8). This assay also was used to determine phosphorylase activity as part of the cyclic AMP and phosphorylase kinase assays (see below). One unit of phosphorylase is defined as that amount of enzyme that produces 1 μmole of glucose 1-phosphate from glycogen per minute at 30°.

Dog heart phosphorylase kinase activity was measured by a modification of the method of Krebs *et al.* (4). The activity of the enzyme was measured at pH 6.0 (rather than 6.8) and 8.2 and the ratio of activities at these pH's is used as an index of the degree of activation of the enzyme. Dog heart samples (5–10 mg) were homogenized in 100 volumes of a solution containing 20 mM β-glycerophosphate, 20 mM NaF, 4 mM EDTA, 0.01% bovine plasma albumin, 15 mM mercaptoethanol, pH 6.8. Extracts were prepared by centrifugation of the homogenate at 3000 *g* for 10 min. The initial step in the assay, the conversion of phosphorylase *b* to *a*, was carried out at 30° in the following medium: 25 mM Tris-HCl, 25 mM β-glycerophosphate, 10 mM Mg acetate, 3 mM ATP, 7.5 units of phosphorylase *b*, and 25 μl of

heart extract in a total volume of 150 μl at either pH 6.0 or 8.2. Blank tubes which contained all the above components except the heart extract were run through the *b* to *a* conversion step at both pH's. The *b* to *a* conversion reaction was stopped by dilution with 1 ml of a solution containing 0.1 M maleic acid, 10 mM NaF, 40 mM mercaptoethanol, and 0.01% bovine plasma albumin, pH 6.5 at 0°. The amount of phosphorylase *a* activity was then determined. One unit of phosphorylase kinase was defined as that amount of enzyme that converts 1 unit of phosphorylase *b* to *a* in 1 minute at 30°.

Rat heart phosphorylase kinase activity was measured by a modification of the procedure described by Drummond and Duncan (9). Assays were carried out at pH 6.8 and 8.2. A blank containing heart extract, but not phosphorylase *b*, was run through the *b* to *a* conversion step and phosphorylase was added after dilution. This was necessary to correct for the formation from heart extract of an activator of phosphorylase *b* during the conversion from *b* to *a*. In addition 5′-AMP, formed from ATP during the conversion of *b* to *a*, was destroyed by adding 2 μl of adenylic deaminase (Sigma, "2 μM units/ml") to the diluted *b* to *a* reaction mixture and incubating for 5 min at 30°. Phosphorylase activity was measured as described previously (8).

The formation of 5′-AMP or other activators of phosphorylase *b* during incubation was not sufficient to interfere with the dog heart assay. Therefore, the less complicated dog heart procedure was used to obtain all data from this species.

The concentration of cyclic AMP in heart samples was measured by a modification of the method described by Hammermeister *et al.* (10). The tissue was powdered under liquid nitrogen in a stainless steel mortar. Approximately 150 mg of the powdered tissue was transferred to a glass tube and brought to —20° in a dry ice–alcohol bath. Hot 10 mM theophylline solution (2.5 volumes) was then added to the tubes and these were placed in a boiling water bath for 5 min. The samples were cooled and centrifuged, and the supernatant solution was put on a 0.7 × 2 cm Dowex 1 Cl column (100–200 mesh, 10% crosslinkage). After several washes with water, the nucleotides were eluted with 0.1 N HCl. The eluent was lyophilized, and the residue was taken up in 25 mM phosphate buffer, pH 8.15, yielding a final pH of 7.0. Twenty-five microliters of the extract was added to 100 μl of a solution containing: 10 mM Mg acetate, 3 mM ATP, 2 mM theophylline, 25 mM Tris-HCl, 25 mM β-glycerophosphate, pH 6.8, and the appropriate amount of rabbit skeletal muscle phosphorylase kinase. This reaction mixture was allowed to stand at 0° for 30 minutes, a procedure which increased the sensitivity of phosphorylase kinase to activation by cyclic AMP (see Fig. 1). Sensitization of the liver assay system to cyclic AMP by a cold incubation step has been demonstrated previously by Butcher *et al.* (11). The reaction mixture was then brought to 30° and 25 μl of a 240 units/ml solution of phosphorylase *b* was added to initiate the reaction converting phosphorylase *b* to *a*. The re-

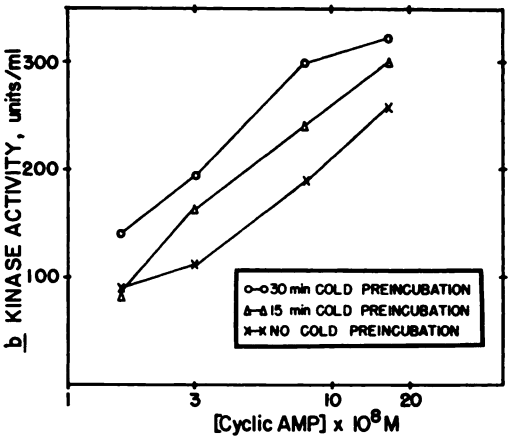

FIG. 1. *Standard curve for the cyclic AMP assay and the effect of the preliminary cold incubation*

The effect of the 0° incubation immediately preceding the kinase activation step is shown. The activation step in this example was run for 10 min. The activity of *b* kinase in the absence of cyclic AMP was not affected by cold preincubation, and in this example was 76 units/ml.

action was stopped after the appropriate time (this varied with the kinase preparation used, as described below) by adding 1 ml of the maleic acid buffer solution. The phosphorylase *a* activity was then assayed as described previously (8). Standard cyclic AMP solutions were carried through all the above procedures with each set of tissue samples, and a standard curve was obtained. An example of a standard curve and the effects of varying the time of the cold incubation step are shown in Fig. 1. The validity of the method was tested as suggested by Posner *et al.* (12).

There was considerable variability in the optimal timing of the kinase activation step in the cyclic AMP assay. One factor contributing to this variability was the phosphorylase kinase preparation. Activation by cyclic AMP was markedly reduced after storage of the enzyme at —20° for more than 6 weeks. The time course of the kinase activation was estimated on each new enzyme preparation and often with its routine use in the assay to assure optimal conditions. Figure 2 is an example of such a study. If the assumption is made that no activation of kinase by Mg-ATP took place in the first minute of the *b* to *a* reaction, the deviation of the "actual no C.A." line from the theoretical one is an index of the activation of the enzyme by Mg-ATP alone. This was considerable after about 20 min in the example illustrated. The optimal incubation period was that time interval at which a maximal difference was observed in the kinase activation by varying concentrations of cyclic AMP from that produced by Mg-ATP alone. This time varied from 5 to 30 min with the kinase preparations used in this study.

Cyclic AMP concentrations and all other biochemical data are expressed in terms of the wet weight of tissue.

Materials

Nucleotides were obtained from P-L Biochemicals, Milwaukee, Wisconsin; the enzymes used in the phosphorylase assay from Boehringer-Mannheim. Phosphorylase *b*, containing less than 1.5% of the *a* form, was prepared by a modification of the

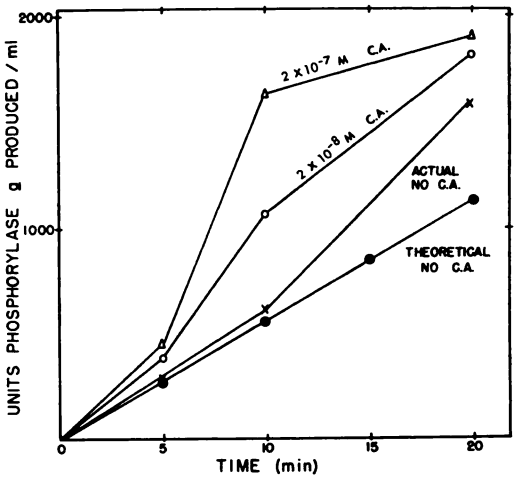

FIG. 2. *Time course of the activation of phosphorylase b kinase*

The "theoretical no cyclic AMP (C.A.)" line was extrapolated from experiments in which the conversion of phosphorylase *b* to *a* was limited to 1 min in the absence of cyclic AMP. The "actual no C.A." line is the *b* kinase activity measured over the 20-min period in the absence of cyclic AMP. The concentrations of cyclic AMP given are those in the reaction medium.

method of Fischer and Krebs (13) by DeLange *et al.* (14). Cyclic nucleotide phosphodiesterase was kindly supplied by R. W. Butcher, Vanderbilt University. Phosphorylase kinase was purified from rabbit skeletal muscle by the method of Krebs *et al.* (4) through the first ultracentrifugation step [78,000 *g* (average) for 90 min].

Drugs

Epinephrine bitartrate (Suprarenin) was diluted in 0.9% saline to a concentration which, when infused at 2 ml/min in the dog or 0.2 ml/min in the rat, provided the appropriate dose. Sodium metabisulfite (0.1%) was added to the drug solution to prevent deterioration of the catecholamine. Pronethalol (a gift of Dr. J. W. Black, Imperial Chemical Industries, Ltd.) was dissolved in 0.9% saline solution.

Statistical Methods

Statistical analysis was performed by means of the *t* test for either paired or

unpaired observations. The probability of 0.05 was taken as a level of significant difference.

RESULTS

Cardiac Phosphorylase b Kinase

Administration of epinephrine to rats has been shown to elevate the activity of skeletal muscle phosphorylase *b* kinase when this was expressed as the ratio of the activities at pH 6.8 to 8.2 (15). However, no clear effect of epinephrine was demonstrated in the perfused rabbit heart by Hammermeister *et al.* (10). Furthermore, these investigators found that the control value of pH 6.8:8.2 activities was much higher in heart than in skeletal muscle. They also showed that a large fraction of the heart enzyme sedimented from the whole homogenate after centrifugation at 10,000 *g* for 20 min. Drummond *et al.* (16) have recently shown that epinephrine did increase the pH 6.8:8.2 activity ratio of phosphorylase *b* kinase in extracts from perfused rat hearts, although some increase in pH 8.2 activity was also observed.

A preliminary study to characterize dog heart phosphorylase kinase was carried out because of the differing results obtained in other species by previous investigators. Activity of kinase was measured at pH 6.0 and 8.2. This resulted in lower control activity ratios than could be obtained at pH 6.8. Since one possible reason for the inability to demonstrate an effect of epinephrine on rabbit heart *b* kinase was the high control activity observed in that tissue (10), it seemed important to assay the enzyme under conditions providing a low control activity ratio.

Approximately three-quarters of the activity of the homogenate appeared in the 3000 *g* supernatant fractions of both control and epinephrine-treated hearts (Table 1). The pH 6.0:8.2 activity ratio was considerably lower in the extracts than the homogenates, and only in the extract could a significant effect of epinephrine be demonstrated.

TABLE 1

Properties of phosphorylase kinase of dog heart

Biopsy samples were taken from the right ventricle of dogs before and 30 sec after the beginning of an infusion of 10 μg/kg min^{-1} of epinephrine. Activity is expressed as units per gram wet weight of tissue ± the standard error of the mean. Standard errors of the differences (SED) between epinephrine (Epi.) and control values are given. Asterisks denote significant differences from control at $P < 0.05$. Each value represents the mean from six experiments.

	Homogenate			Extract[a]		
Parameter	Control	Epi.	SED	Control	Epi.	SED
Activity pH 6.0	39.4 ±3.7	49.3 ±4.4	3.8	18.0 ±3.0	28.5 ±3.1	2.4*
Activity pH 8.2	89.3 ±4.0	99.1 ±4.1	4.6	66.5 ±4.1	72.5 ±7.3	4.4
Activity ratio, pH 6.0:8.2	0.44 ±0.05	0.50 ±.05	0.02	0.27 ±.04	0.39 ±.05	0.04*

[a] Supernatant solution obtained from centrifugation of the homogenate at 3000 *g* for 10 min.

Effects of Epinephrine in the Open-Chested Dog

The effects of epinephrine on contractile force, percentage of phosphorylase *a*, phosphorylase kinase activity, and cyclic AMP concentrations are shown in Figs. 3 and 4. During an infusion of 1 μg/kg min^{-1} of epinephrine (Fig. 3), right ventricular contractile force was increased without a significant change in the percentage of phosphorylase *a*. There was, however, a significant increase in the activity of phosphorylase kinase beginning 30 sec after

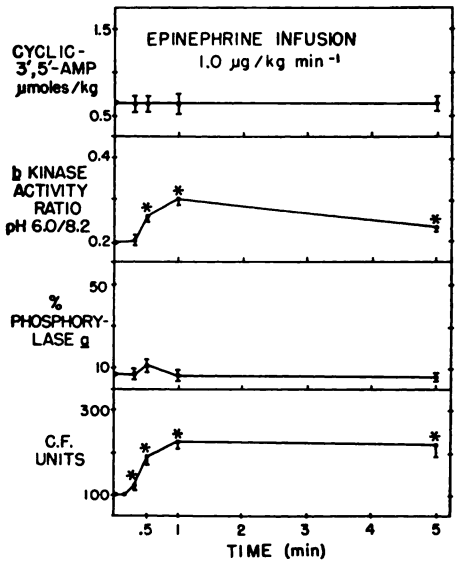

FIG. 3. *Effects of epinephrine, 1 μg/kg min⁻¹, on the dog heart in situ*

Each point represents the mean of at least 6 experiments. Asterisks denote significant differences, $P < 0.05$, from paired controls (biopsies taken before the epinephrine infusion). C.F. = contractile force. Bars represent 1 standard error of the differences from paired control data.

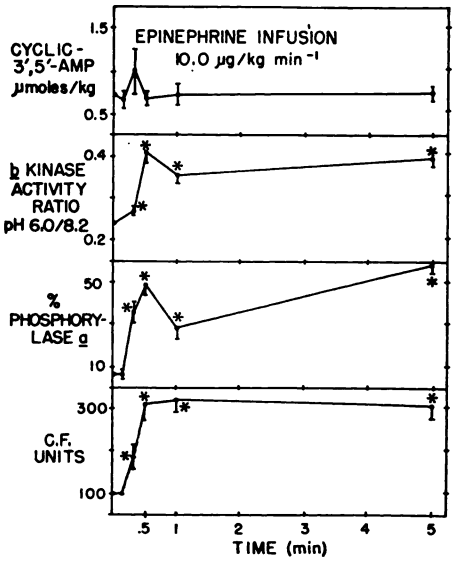

FIG. 4. *Effects of epinephrine, 10 μg/kg min⁻¹, on the dog heart in situ*

Each point represents the mean of at least 6 experiments. Asterisks denote significant differences, $P < 0.05$, from paired controls (biopsies taken before the epinephrine infusion). C.F. = contractile force. Bars represent 1 standard error of the differences from paired control data.

the onset of the infusion. No significant change in the cyclic AMP concentration occurred at any time during the infusion.

Epinephrine, 10 μg/kg min⁻¹ (Fig. 4) produced a larger increase in the contractile force. This dose also produced a marked increase in the percentage of phosphorylase *a*. Phosphorylase kinase activity increased to a higher peak value and was significantly elevated at 20 sec with this dose of epinephrine. The small increase in the cyclic AMP concentration in the first minute of the infusion was not significantly different from paired controls.

There was no significant change in either the total phosphorylase or phosphorylase kinase activities (pH 8.2) during the epinephrine infusions.

Effects of Epinephrine in the Open-Chested Rat

Robison *et al.* (1) have shown that epinephrine increased the cyclic AMP concentration in the perfused rat heart. Experiments in our laboratory have confirmed this observation (17). Three alternative hypotheses for our inability to see this effect in the open-chested dog were tested: (a) that the effect is species dependent; (b) that it is a consequence of the different conditions of the *in situ* vs. the *in vitro* preparations; (c) that it is due to the differences in the sampling techniques used in the two preparations.

Infusion of epinephrine, 2 μg/kg min⁻¹, caused a rapid and significant increase in the cyclic AMP concentration and phosphorylase kinase activity in rat hearts frozen *in situ* (Fig. 5). The effect on cyclic AMP and kinase was maximal at the earliest sampling time (15 sec). The change in the percentage of phosphorylase *a* not only followed a slower time course, with the first significant increase observed 30 sec after the beginning of the infusion, but phosphorylase *a* activity was still markedly elevated at 2 min when phosphorylase kinase activity had returned to

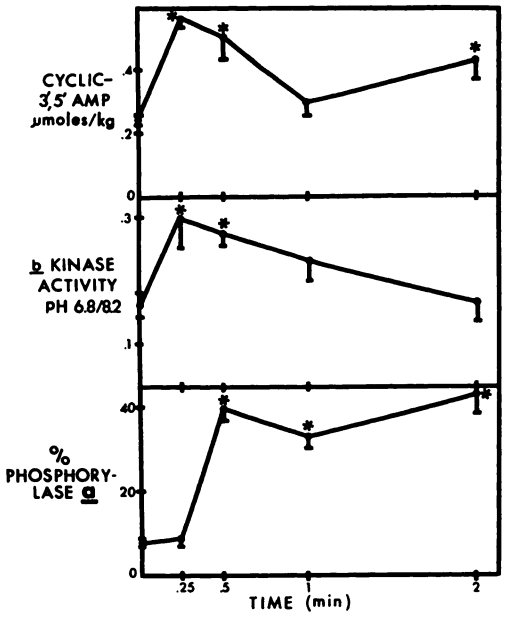

FIG. 5. *The effects of epinephrine, 2 μg/kg min⁻¹, on the in vivo rat heart*

Each point represent the mean of at least 3 experiments. Asterisks denote significant differences, $P < 0.05$, from control experiments. Bars represent 1 standard error.

control levels. A secondary, significant ($P < 0.02$) rise in cyclic AMP concentration was also noted at this time.

To simulate the dog heart biopsy method in rats, their ventricles were removed with scissors and frozen by immersion in dichlorodifluoromethane at —150° instead of using the chilled silver blocks and freezing *in situ*. Control hearts and those from rats that had been infused with 2 μg/kg min⁻¹ epinephrine for 15 sec were frozen by this procedure. The cyclic AMP concentration of control and epinephrine-treated hearts was 0.27 ± 0.01 and 0.27 ± 0.02 μmole/kg, respectively ($n = 3$).

Effects of Pronethalol on the Responses to Epinephrine in the Open-Chested Rat

Pronethalol, 20 mg/kg, was administered 30 min before an infusion of 2 μg/kg min⁻¹ of epinephrine. Control hearts were taken from rats which received only pronethalol. Hearts from epinephrine-treated rats were frozen 15 sec after the infusion had begun, since this appeared to be the time of peak response. Cyclic AMP levels in pronethalol controls were 0.27 ± 0.006 μmole/kg and in hearts from epinephrine-treated rats were 0.30 ± 0.004 μmole/kg ($n = 3$).

DISCUSSION

Krebs and his colleagues (4) have suggested that skeletal muscle phosphorylase kinase exists in two forms, an inactive form exhibiting very low activity at pH 6–7, and an active form with higher activity in this pH range. Experiments by these investigators on the effects of epinephrine and nerve stimulation on skeletal muscle *in situ* supported this hypothesis (15). These investigators later showed that the properties of the heart enzyme differed from those of the muscle enzyme, and conversion from an inactive to an active form of the enzyme was not clearly demonstrable (10). The effects of epinephrine on the phosphorylase kinase activity of the dog heart reported in this study support the hypothesis that activation of this enzyme is involved in the biochemical sequence leading to catecholamine-induced activation of cardiac phosphorylase *in vivo*. A significant increase in the pH 6.0 activity and the pH 6.0:8.2 activity ratio was found. This is consistent with the concept that there are two forms of the heart kinase and that epinephrine effects the conversion of an inactive form of the enzyme to an active one. The observation that epinephrine (1 μg/kg min⁻¹) increased the activity of cardiac phosphorylase kinase in the dog without producing a significant rise in the percentage of phosphorylase *a* suggests that a threshold activation of the kinase must be exceeded before net conversion of phosphorylase *b* to *a* occurs. Drummond *et al.* (16) have reported a similar dissociation between *b* kinase activation by epinephrine and phosphorylase activity in the perfused rat heart.

The study of the effects of epinephrine on the rat heart *in situ* supports the hypothesis that cyclic AMP is the mediator of the effects of catecholamines on cardiac phosphorylase through an activation of

phosphorylase kinase. Both the concentration of the cyclic nucleotide and the activity of phosphorylase kinase rose before a significant increase in the phosphorylase *a* activity was demonstrable. Cyclic AMP rapidly returned to the control value and then increased again. These rapid fluctuations in heart cyclic AMP during an *in vivo* infusion of epinephrine are similar to those reported by Williamson (18) in the perfused rat heart.

Phosphorylase kinase activity in rat hearts reached a peak 15 sec after the beginning of the infusion and then decayed to control values while the percentage of phosphorylase *a* remained high. This suggests that the conversion of phosphorylase *a* to *b*, catalyzed by phosphorylase phosphatase, occurs at a rate which is significantly less than the rate of the *b* to *a* conversion. This is consistent with the kinetics determined for the interconversion of the two forms of glycogen phosphorylase of skeletal muscle by Danforth *et al.* (19). These authors found that the rate of conversion of phosphorylase *b* to *a* was from two to ten times greater than the rate of the reverse reaction.

The rapidity of the rise of the concentration of cyclic AMP suggests that the cyclic nucleotide is involved in the inotropic effect of the catecholamine (1, 2). However, contractile force was not measured in the experiments with the open-chested rats. Until some valid relationship between cyclic AMP and the contractile process or factors governing the process is demonstrated, temporal relationships can only be of limited usefulness in determining cause and effect.

Experiments in which rat hearts were frozen in a manner similar to that used on dog hearts suggest that the sampling or freezing techniques in the dog studies were unsatisfactory for the subsequent measurement of tissue concentrations of cyclic AMP. Wollenberger *et al.* (20) showed that the cooling time of tissue frozen by a clamp prechilled in liquid nitrogen was about 10–30 times less than that of tissue frozen in isopentane at −150° exclusive of the actual time required to biopsy the tissue. This delay in freezing by the biopsy technique could allow newly formed cyclic AMP to be metabolized by the action of heart cyclic nucleotide phosphodiesterase. These experiments demonstrate the advisability of choosing the most rapid freezing technique in any study concerning the concentration of cyclic AMP.

Pronethalol produced essentially complete blockade of the effect of epinephrine on the cyclic AMP concentration in the rat heart. This is consistent with the hypothesis that the effect of catecholamines on heart adenyl cyclase is a result of the interaction of the drug with the β adrenergic receptor (1, 2).

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Margaret Maltbie for her excellent technical assistance.

This study was supported by Research Grant HE 04626 from the National Heart Institute, Career Development Award GM 6257 (S.E.M.), and the Interdepartmental Cardiovascular Training Program of Emory University School of Medicine sponsored by the National Heart Institute Training Grant 5T1 HE 5380.

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