Effect of Glucagon on Cyclic 3',5'-AMP, Phosphorylase Activity and Contractility of Heart Muscle of the Rat

By Steven E. Mayer, Ph.D., Donald H. Nomm, Ph.D., and Lucian Rice, B.S.

ABSTRACT

The purpose of this investigation was to contrast the effect of glucagon and that of epinephrine on the concentration of cyclic adenosine 3',5'-monophosphate (cyclic AMP), the activity of phosphorylase $a$ and the contractile amplitude of isolated perfused rat hearts. The two drugs were about equally effective except that the maximal augmentation of contractility by epinephrine ($5 \times 10^{-9}$ moles) was twice that produced by an equivalent dose of glucagon with a fourfold greater increase in cyclic AMP concentration. Combination of large doses of the two drugs caused increases in the cyclic nucleotide considerably greater than those required for maximal phosphorylase activation or associated with a maximal inotropic response. The effects of glucagon also developed more slowly than those of epinephrine. An increase in cyclic AMP was not detectable until after phosphorylase $a$ and contractile amplitude had increased.

The beta-receptor-blocking agents dichloroisoproterenol and pronethalol did not block the biochemical responses to glucagon in doses which abolished the epinephrine-induced increases in cyclic AMP and phosphorylase $a$. These results, along with those obtained by other investigators, indicate that glucagon can elicit the same biochemical responses in intact heart as have been obtained with epinephrine, but by action at a different receptor site.

ADDITIONAL KEY WORDS beta-receptor-blocking agents epinephrine catecholamines hormones metabolic control in heart rat heart metabolism pronethalol

Glucagon has biochemical and physiological effects on heart muscle similar to those produced by catecholamines. Farah and Tuttle were the first to demonstrate the positive inotropic and chronotropic effects of glucagon (1). These observations have recently been confirmed with several types of preparations of cardiac muscle (2-5) and in unanesthetized human volunteers (6). Glucagon also caused activation of glycogen phosphorylase and promoted glycogenolysis in the isolated perfused (5, 7, 8) and in-situ (9) rat heart. Neither the biochemical nor the physiological effects are likely to represent significant actions of glucagon as a hormone, since they occurred only at concentrations of the polypeptide that are greater than those normally found in plasma by radioimmunological assay (10). However, glucagon is receiving attention as a therapeutic agent because of its unique stimulatory action on the heart (6).

One of the purposes of the present study was to investigate the nature of the receptor or receptors at which glucagon produces its physiological and biochemical actions. The
observation that dichloroisoproterenol (DCI) blocked the effects of glucagon on cardiac rate and contractility led Farah and Tuttle (1) to conclude that these effects were mediated through the beta receptor. This interpretation has recently been challenged on the basis that the newer beta-receptor-blocking agents, propranolol and propranolol, did not interfere with the physiological effects of glucagon on the heart (2, 3, 5).

This report also describes the effect of glucagon on the concentration of cyclic 3',5'-adenosine phosphate (cyclic AMP) in the perfused rat heart. This nucleotide has been suggested as the mediator of the mechanical and metabolic effects of the catecholamines on the heart consequent to the stimulation of adenyl cyclase by these agents (11). Cyclic AMP also has been implicated in several metabolic effects of glucagon. Increases in the concentration of this nucleotide have been observed in association with the glycogenolytic effect of glucagon on liver (12) and in its lipolytic action on adipose tissue (13). Since glucagon and epinephrine have similar biochemical and physiological effects on the heart, cyclic AMP may be a common mediator for the effects of these drugs (14).

Several conflicting reports have appeared on the relationship between the nucleotide and the effects of glucagon on cardiac muscle. LaRaia et al. (15) were unable to detect any significant change in the cyclic AMP concentration of the perfused rat heart when glucagon was administered in a dose that produced considerable augmentation of contractility. However, both Levey and Epstein (16) and Murad and Vaughan (17) found that glucagon increased the rate of formation of cyclic AMP by particles prepared from myocardium. We have found that glucagon increased the concentration of cyclic AMP in the isolated perfused rat heart. A preliminary report of part of this work has been presented (5).

**Methods**

Perfused Hearts.—Rat hearts were excised under diethyl ether anesthesia and were perfused through the aortic cannula at a flow rate of 7 ml/min by a Holter pump. The medium contained NaCl, 119 mEq/liter; KCl, 5.6 mEq/liter; CaCl₂, 3.2 mEq/liter; MgCl₂, 2.0 mEq/liter; EDTA, 0.05 mM; dextrose, 10 mM; and NaHCO₃, 25 mM, equilibrated with 95% O₂-5% CO₂ to pH 7.4 at 37°. Contractility was measured with a force transducer connected to the apex of the heart by a string run through a pulley.

Drugs dissolved in the perfusion medium were injected into the aortic cannula in volumes of less than 0.2 ml. Blocking drugs were added to the reservoir of the perfusion medium. Hearts were frozen without prior removal from the perfusion apparatus with modified Wollenberger clamps (18).

Biochemical Measurements.—Phosphorylase activity was measured in the direction of glucose-1-phosphate production (19). Data on phosphorylase activity are reported as apparent fraction of phosphorylase in the a form (% a) as determined from the ratio of enzyme activity in the absence of added 5'-AMP to that in the presence of 1 mM 5'-AMP. Cyclic AMP was measured by modification (20) of the method of Hammermeister et al. (21). Ventricular muscle (200 mg) was powdered at −180°C and then homogenized at −10°C with 1 ml of 10% trichloroacetic acid. After centrifugation at 48,000 x g for 10 minutes, the acid was extracted from the supernatant solution with 4.5 volumes diethyl ether (saturated with water). Cyclic AMP was then partly separated by passage of the aqueous solution through a column of Dowex 1 Cl. The fraction eluted with 0.1N HCl was assayed after concentration by lyophilization by measuring the rate of formation of phosphorylase a in the presence of phosphorylase kinase under conditions in which the activity of the latter was proportional to the logₐ₀ concentration(582,780),(856,910) of cyclic AMP (20). The amount of ventricular cyclic AMP in 100 µl incubation solution was at least 10⁻¹⁴ moles where the limit of detection was 2 X 10⁻¹² moles with a standard error of 10%.

Phosphorylase kinase activity was measured as previously described (20).

Materials.—Nucleotides were obtained from P-L Biochemicals; the enzymes used in the phosphorylase assay were obtained from Boehringer-Mannheim. Muscle phosphorylase kinase and phosphorylase b were prepared as previously described (20).

Drugs.—Epinephrine bitartrate (Suprarenin) was diluted in perfusion medium containing 0.1% sodium metabisulfite. Doses are given as the concentration of the base. Crystalline glucagon¹ was stored at −20°C as a stock solution (0.5 mg/ml) in 1 mM Tris HCl, pH 8.5. Just before

¹Given by Eli Lilly Co.
GLUCAGON AND CARDIAC CYCLIC AMP

each experiment the stock solution of glucagon was diluted in perfusion medium.

Statistical Methods.—Statistical analysis was performed by Student's t-test. The probability of 0.05 was taken as the level of significant difference between two groups of data.

Results

Comparison of Glucagon and Epinephrine.

The time course of biochemical and contractile events was measured after a dose of 3 μg of glucagon (8 × 10^-10 moles) (Fig. 1). A more than twofold increase in the concentration of cyclic AMP was detected 20 seconds after injection of the polypeptide, at least 5 seconds later than the onset of the positive inotropic effect and augmentation of phosphorylase a activity. Epinephrine has been shown to increase the cyclic AMP concentration before (11) or simultaneously (18) with its inotropic effect, but after a larger dose (2 μg, 1.1 × 10^-8 moles).

The effects of varying the doses of epinephrine and of glucagon administered singly and in combination were determined 20 seconds after injection. The two agents were equally potent in increasing contractility of the perfused heart with doses of 3 × 10^-10 moles or less (Fig. 2). The ED50 was approximately 1 × 10^-10 moles. Comparable effects on phosphorylase activity were also noted (Table 1). No increases in cyclic AMP concentration

FIGURE 1

The effects of glucagon on cardiac cyclic AMP, phosphorylase and contractility. Three μg of glucagon were administered at 0 time. There were seven control, six 15-second, five 20-second and 40-second, and three 60-second experiments. Each point on the contractile amplitude curve differs from control (P < .01). The same is true of the phosphorylase data. The mean control was 5.8 ± 1.1%; the mean at 15 seconds was 11.4 ± 1.4. Cyclic AMP concentration (per kg wet weight of ventricle) differs from control at the 20-, 40- and 60-second points only (P < .01). A vertical line represents 1 SE.

Dose-response curves of the effects of glucagon and epinephrine on contractile amplitude of isolated perfused rat hearts. Diastolic tension of 2 g was applied and measurements begun after 20 minutes of equilibration. Percent changes were measured by comparing control tension just before administration of the drug to the peak tension, 20 seconds later. A complete dose response curve was determined in each of six hearts for each set of experiments. Epi = epinephrine; Glu = glucagon; Pro = pronethalol. The Epi only curve differs from the Epi & Glu curve only at the dose(s) of 5.5 × 10^-11 mole (11.2 ± 1.5% vs 16.6 ± 1.7%, P < .05).

FIGURE 2
TABLE 1
Effects of Epinephrine and Glucagon, Alone and in Combination, on Cyclic AMP in Perfused Rat Hearts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>No of expts</th>
<th>Increase contractile amplitude (%)</th>
<th>Phosphorylase (%)</th>
<th>Cyclic AMP (imoles/kg ventricle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td></td>
<td>21</td>
<td>5.9 ± 0.5</td>
<td>0.21 ± .01</td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>.03 μg (0.16 nmole)</td>
<td>6</td>
<td>19</td>
<td>18.6 ± 1.5*</td>
<td>0.23 ± .03</td>
</tr>
<tr>
<td></td>
<td>.1 μg (0.55 nmole)</td>
<td>6</td>
<td>25</td>
<td>64 ± 4.8*</td>
<td>0.53 ± .03*</td>
</tr>
<tr>
<td></td>
<td>1 μg (5.5 nmole)</td>
<td>11</td>
<td>30</td>
<td>65 ± 3.0*</td>
<td>1.1 ± .2*</td>
</tr>
<tr>
<td>Glucagon</td>
<td>1 μg (0.27 nmole)</td>
<td>6</td>
<td>22</td>
<td>24 ± 6.4*</td>
<td>0.19 ± .02</td>
</tr>
<tr>
<td></td>
<td>3 μg (0.81 nmole)</td>
<td>11</td>
<td>20</td>
<td>55 ± 4.0*</td>
<td>0.48 ± .02*</td>
</tr>
<tr>
<td>Epinephrine and Glucagon</td>
<td>0.1 + 3 μg (0.55 + 0.81 nmole)</td>
<td>6</td>
<td>20</td>
<td>65 ± 3.8*</td>
<td>0.76 ± 18*</td>
</tr>
<tr>
<td></td>
<td>1 + 3 μg (5.5 + 0.81 nmole)</td>
<td>11</td>
<td>20</td>
<td>69 ± 2.7*</td>
<td>1.8 ± .2*</td>
</tr>
</tbody>
</table>

Hearts were frozen 20 seconds after administration of the drugs, at the peak of the inotropic and cyclic AMP changes in response to glucagon. Results are expressed as means ± 1 se.

*Differs from control with P < .01.

**FIGURE 3**
The effect of pronethalol on cardiac phosphorylase activation induced by epinephrine and glucagon. Hearts were frozen at the time of peak phosphorylase activation: 30 seconds after the addition of epinephrine, 40 seconds after glucagon. Pronethalol alone did not affect the control %. Each bar represents the mean of at least four experiments; 1 se is indicated on each bar.
GLUCAGON AND CARDIAC CYCLIC AMP

TABLE 2

Effects of Beta-Receptor-Blocking Agents on Phosphorylase Activation and Cyclic AMP Formation Induced by Epinephrine and Glucagon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of expts</th>
<th>Phosphorylase a (%)</th>
<th>Cyclic AMP (pmoles/kg ventricle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>10</td>
<td>7.5 ± 0.8</td>
<td>0.32 ± .02</td>
</tr>
<tr>
<td>Epinephrine, 0.1 µg</td>
<td>6</td>
<td>64 ± 4.8*</td>
<td>0.53 ± .03*</td>
</tr>
<tr>
<td>Pronethalol, 0.5 µg/ml</td>
<td>3</td>
<td>8.0 ± 1.6</td>
<td>0.30 ± .02</td>
</tr>
<tr>
<td>Epinephrine, 0.1 µg, after Pronethalol</td>
<td>3</td>
<td>6.9 ± 0.9</td>
<td>0.33 ± .03</td>
</tr>
<tr>
<td>Glucagon, 3 µg</td>
<td>11</td>
<td>55 ± 4.0*</td>
<td>0.48 ± .02*</td>
</tr>
<tr>
<td>Glucagon, 3 µg, after Pronethalol</td>
<td>4</td>
<td>60 ± 5.0*</td>
<td>0.60 ± .06*</td>
</tr>
<tr>
<td>DC1, 3 µg/ml</td>
<td>3</td>
<td>14.9 ± 1.7*</td>
<td>0.29 ± .02</td>
</tr>
<tr>
<td>Epinephrine, 0.1 µg, after DC1</td>
<td>3</td>
<td>12.4 ± 1.0*</td>
<td>0.30 ± .02</td>
</tr>
<tr>
<td>Glucagon, 3 µg, after DC1</td>
<td>3</td>
<td>53 ± 2.8*</td>
<td>0.72 ± .17*</td>
</tr>
</tbody>
</table>

Blocking agents were added to the perfusion medium for 30 minutes. Hearts were frozen 20 seconds after administration of the agonists. Data are recorded as means ± 1 SE.

*Differs from no drug with P < .01.

were detectable after $2 \times 10^{-10}$ moles of the two agents, doses that did increase contractile amplitude and phosphorylase $a$. Glucagon and epinephrine were equally effective in altering all three variables at doses of 8.1 and $5.5 \times 10^{-10}$ moles, respectively. However, epinephrine produced a larger maximum response of contractile amplitude (Fig. 2) and a greater increase in formation of cyclic AMP (Table 1) than did glucagon.

Simultaneous administration of the two compounds produced a significant additive effect on contractile amplitude at only one dose of each substance tested, $8 \times 10^{-11}$ moles. No change in cyclic AMP concentration relative to control could be detected. Larger combined injections caused an increase in cyclic AMP concentration approximating the sum of the effects of the two agents administered separately. No additive effects on phosphorylase $a$ activity were noted. Maximal activation of the enzyme was produced by one agent or the other administered alone. Thus no rigorous correlation between cyclic AMP concentration and either increased contractile amplitude or phosphorylase activation was demonstrable. On the one hand, contractile

TABLE 3

Glucagon-Induced Phosphorylase Activation in the Isolated Rat Heart Perfused with Normal and Calcium-Free Medium

<table>
<thead>
<tr>
<th>Perfusion medium</th>
<th>Treatment</th>
<th>No. of expts</th>
<th>Phosphorylase a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>no drug</td>
<td>4</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>glucagon</td>
<td>3</td>
<td>43.5 ± 7.7</td>
</tr>
<tr>
<td>Calcium free</td>
<td>no drug</td>
<td>4</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>glucagon</td>
<td>5</td>
<td>7.8 ± 0.9</td>
</tr>
</tbody>
</table>

One group of hearts was perfused with normal medium for 15 minutes before administration of glucagon; the other group received an additional 5-minute perfusion with medium from which Ca$^{2+}$ had been omitted (18). The dose of glucagon was 2 µg. Hearts were frozen 1 minute after the injection of the polypeptide. Phosphorylase data are expressed as the mean ± SE.

Circulation Research, Vol. XXVI, February 1970

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$^2$The maximal activation of phosphorylase observed, 60-70% $a$, is consistent with the finding that crystalline muscle phosphorylase $a$ is about 0.7 as active in the absence of 5'-AMP as it is in the presence of the nucleotide (22).
amplitude and phosphorylase activities were altered in the absence of detectable changes in cyclic AMP. On the other hand, cyclic AMP increased to concentrations greater than those associated with either maximal augmentation of contractile amplitude or transformation of phosphorylase b to a.

Effects of Beta-Receptor-Blocking Agents.— In these experiments, pronethalol (0.5 or 2 μg/ml) was added to the medium and the hearts perfused for 30 minutes before other agents were administered. The positive inotropic response to epinephrine was partially blocked by 0.5 μg/ml pronethalol, while the response to glucagon was unaltered (Fig. 2). The marked increase in phosphorylase a activity produced by epinephrine was completely blocked by the lower dose of the blocking agent, whereas neither 0.5 nor 2 μg/ml of pronethalol had an effect on phosphorylase activation induced by glucagon (Fig. 3, Table 2). Finally, this blocking agent was ineffective in preventing the increased concentration of cyclic AMP induced by glucagon in perfused rat hearts (Table 2). Pronethalol has previously been shown to block this effect of epinephrine (11, 20).

Dichloroisoproterenol (DCI) (3 μg/ml) produced signs of sympathetic stimulation: tachycardia, an occasional small increase in contractile amplitude (10 to 15% of control), and activation of phosphorylase (Table 2). This concentration of DCI significantly reduced the positive inotropic effect of both epinephrine and glucagon but had no effect on the inotropic response to CaCl₂. However, while DCI abolished the epinephrine-induced increase in cyclic AMP concentration and activation of phosphorylase, this blocking agent was without effect on either response to glucagon (Table 2).

Phosphorylase Kinase Activation and Activity in Response to Glucagon.—The experiments reported so far indicate separate sites of action of epinephrine and glucagon in initiating activation of the sequence of enzymes that begins with adenyl cyclase, followed by the activation of phosphorylase kinase and the transformation of phosphorylase b to phosphorylase a. It seems likely that the last two steps are part of a common pathway. However, we were unable to obtain reproducible activation of phosphorylase kinase in the presence of glucagon. Epinephrine has been shown to produce such an effect in perfused isolated hearts (18, 21, 23) and hearts in situ (20). The reason for this difference is not known to us. On the other hand, evidence was obtained for the hypothesis that transformation of phosphorylase b to phosphorylase a occurred by the same mechanism whether epinephrine or glucagon was the initiating stimulus. When hearts were perfused with a medium from which Ca²⁺ had been omitted, glucagon no longer produced activation of phosphorylase (Table 3). This is similar to what we have reported for epinephrine (18), i.e., the catalytic activity of phosphorylase kinase depends on the availability of Ca²⁺ irrespective of the initiating stimulus.

Discussion

The effects of glucagon on the heart present an interesting contrast to those of epinephrine. It is evident from previous studies (2, 3) and the present one that the inotropic effects of these drugs are not mediated by the same putative receptor site. The anomalous effect of dichloroisoproterenol in blocking the inotropic effect of glucagon has been related by Lucchesi to the intrinsic sympathomimetic activity of the blocking agent (2). He showed that when this sympathomimetic effect was blocked by propranolol, DCI no longer affected the inotropic response to glucagon. That propranolol and pronethalol do not block glucagon is consistent with this interpretation of Lucchesi, since these beta-receptor-blocking agents have little or no sympathomimetic effect on the isolated perfused heart (24).

Our data indicate that glucagon-induced activation of cardiac phosphorylase also results from an action other than on the beta receptor. Mayer et al. (9) had previously reported that pronethalol blocked the phosphorylase activation produced by glucagon in the rat heart in situ. The discrepancy may be due to the difference in the doses of the blocking agent. In the experiments on the in-
situ hearts, the dose of pronethalol used (20 mg/kg) led to an estimated concentration 40 times larger than that obtained in the isolated rat hearts. Nonspecific blockade or an inhibition of Ca$^{2+}$ utilization by pronethalol (25) may have occurred in the former experiments.

While epinephrine and glucagon interact with different cardiac receptors, they probably act through a common pathway distal to the receptor to activate phosphorylase. Both agents increase cyclic AMP concentration and both are dependent on Ca$^{2+}$ for the conversion of phosphorylase $b$ to phosphorylase $a$ (18, 26).

A comparison of epinephrine and glucagon in terms of the relationship between doses and the biochemical and physiological responses that were measured shows that the two were approximately equally potent. The lowest doses of each agent used produced similar augmentation of contractility (Fig. 2) and phosphorylase activity (Table 1). Similar changes in cyclic AMP concentration were noted (although these required doses of epinephrine and glucagon producing larger inotropic and phosphorylase responses). These results agree with previously published studies. Glucagon and epinephrine had similar potency in stimulating the flux of metabolites through the glycolytic pathway of isolated perfused rat hearts (8). Like dose response curves were obtained when the two agents stimulated adenyl cyclase activity in particulate preparations from heart (16, 17). The range of effective doses ($5 \times 10^{-8}$ to $5 \times 10^{-6}$ M) was similar to that used in our study.

However, when the two agents are compared in terms of doses which caused maximal inotropic responses (5.5 nmoles epinephrine, 0.3 to 0.84 nmoles glucagon) epinephrine produced a greater effect on both contractility (Fig. 2) and cyclic AMP (Table 1, [18]). The biochemical response to large doses of glucagon was not measured, because of the depression of cardiac contractility observed. These results suggest that glucagon may augment cardiac contractility by the same mechanism as does epinephrine, but that its maximal effectiveness is limited by other, depressant, actions on the heart.

Another contrast between epinephrine and glucagon is that the former agent increased cyclic AMP concentration 5 seconds after administration, simultaneously with its effects on both contractility and phosphorylase activity (17). The effect of glucagon was delayed until 20 seconds, appearing at least 5 seconds after the first detectable increase in contractile amplitude. The hypothesis that the cardiac effects of glucagon and epinephrine are mediated through a single mechanism, the activation of adenyl cyclase (14), is also apparently not supported by the observations that both augmentation of contraction and of phosphorylase activation occurred with small doses of these agents where no increases in cyclic AMP concentration were detectable.

There is, however, an inherent danger in attempting to relate variables such as contractility and cyclic AMP measured by such different techniques. A change in contractile amplitude of 5% is readily detectable in our preparation. However, the cyclic AMP concentration would have to rise at least 16% to differ from control values at the 95% level of confidence. This is a minimum difference based on the assumption that variance does not increase with the elevation of the cyclic AMP concentration. Thus, our data cannot be interpreted as negating the hypothesis that glucagon enhances myocardial contractility through activation of adenyl cyclase.

Furthermore, while our data indicate that glucagon and epinephrine act at different receptor sites they do not permit speculation on whether the same or separate adenyl cyclase systems are involved. The possibility of the latter has been suggested for liver because the maximal increase in cyclase activity produced by the two hormones together was greater than the maximal effect of either alone (27) and hepatic cell membranes containing only the glucagon-sensitive enzyme have been isolated (28). However, in the present experiments it was not possible to induce maximal increases in cyclic AMP concentrations with either drug alone or in
combination. Toxic effects on contractility were observed with doses of glucagon or glucagon with epinephrine which were appreciably greater than those required for maximal effects on contractility and phosphorylase activation. Thus it was apparently not possible to saturate cardiac adenyl cyclase within the range of doses and dose combinations employed. This suggests that while the degree of activation of adenyl cyclase may be the limiting step in the sequence leading to activation of phosphorylase and perhaps augmented cardiac contraction, the capacity of adenyl cyclase to respond to a drug or hormone may not be limiting.

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