Activation of Adenyl Cyclase by Glucagon in Cat and Human Heart

By Gerald S. Levey, M.D., and Stephen E. Epstein, M.D.

ABSTRACT

The purpose of this investigation was to determine the direct effects of glucagon on adenyl cyclase activity in cat and human heart particles and to elucidate the role of the beta receptor in any glucagon-mediated activation of adenyl cyclase. At the peak of its dose-response curve, crystalline glucagon increased the conversion of ATP to cyclic 3', 5'-AMP in particulate fractions of both cat and human heart homogenates by approximately 70%. The activation of adenyl cyclase was dose-related over a concentration ranging from $1 \times 10^{-7} \text{M}$ to $1 \times 10^{-5} \text{M}$. Half maximal activity was observed at $8 \times 10^{-7} \text{M}$. DL-propranolol, $1 \times 10^{-5} \text{M}$, did not block the activation of adenyl cyclase produced by glucagon, $1 \times 10^{-6} \text{M}$ or $1 \times 10^{-5} \text{M}$. However, the same concentration of propranolol blocked adenyl cyclase activation induced by norepinephrine, $1 \times 10^{-5} \text{M}$ and $1 \times 10^{-4} \text{M}$. Combined maximal doses of glucagon and norepinephrine did not produce additive effects on the activation of adenyl cyclase. The failure of DL-propranolol to block the glucagon-mediated activation of adenyl cyclase suggests that there are at least two receptor sites in myocardial tissue responsible for the activation of adenyl cyclase, one responsive to glucagon and one to norepinephrine. Moreover, since combined maximal doses of glucagon and norepinephrine failed to produce an additive response, it appears that in the heart there is probably only a single adenyl cyclase enzyme responsive to these hormones.

ADDITIONAL KEY WORDS

- cyclic 3',5'-AMP
- beta receptor
- propranolol
- phosphodiesterase
- inotropic agent
- norepinephrine

The activity of many hormones can be related to their ability to increase the levels of cyclic 3',5'-AMP (cyclic AMP) in their target organs (1), a change that is believed to occur as a result of activation of adenyl cyclase. The similarity of the actions of epinephrine and glucagon in stimulating the production of cyclic AMP in liver and adipose tissue (2, 3) led to a series of investigations concerned with the effects of glucagon on the heart. These studies demonstrated that glucagon had marked positive inotropic and chronotropic actions on the isolated heart muscle of the dog and cat and on the intact heart of the dog and man (4-6).

Although there is strong evidence that the cardiac effects of catecholamines are mediated by activation of adenyl cyclase (1), the results of studies of the effects of glucagon on cardiac cyclic AMP levels are conflicting; thus, while it has been reported that glucagon does not elevate cyclic AMP levels in rat papillary muscle (7), Murad and Vaughan recently demonstrated that glucagon increased cyclic AMP levels in rat heart muscle (8). Moreover, although the beta-receptor blocking agent, propranolol, blocks the positive inotropic action of the catecholamines, it does not inhibit the positive inotropic response to glucagon (4, 5). This finding suggests that if the cardiac effects of glucagon are mediated by adenyl cyclase, either two different receptor systems or two different enzymes must be present, one responsive to glucagon and one to the catecholamines.

The purpose of the present investigation was to determine the direct effects of glucagon on adenyl cyclase in the particulate fractions...
of cat and human heart homogenates and to clarify the role of the beta receptor in any glucagon-mediated activation of adenyl cyclase.

**Methods and Materials**

Left ventricular muscle was obtained from normal cats; a single cat was used for each experiment. After anesthesia with pentobarbital, 25 to 35 mg/kg, the heart was quickly excised. The left ventricle was dissected free of endocardium and epicardium and approximately 220 mg of left ventricular muscle was homogenized in 4.5 ml of cold 0.25M sucrose with a motor-driven homogenizer at 1°C. The homogenate was centrifuged at 12,000 g for 10 minutes at 4°C and the supernatant fluid decanted; the particles were washed with cold 0.25M sucrose and resuspended and recentrifuged at 12,000 g for 10 minutes. The washed particles were resuspended and rehomogenized in the cold 0.25M sucrose. Protein was determined by the method of Lowry et al. (9); the method solubilized all of the insoluble protein. Adenyl cyclase was assayed by a recently developed method (10, 11). The particulate fraction, containing 0.08 to 0.09 mg protein in a total volume of 0.06 ml, was incubated at 37°C for 3 minutes with adenosine 5'-triphosphate disodium salt (ATP), 1.6 mM (P-L Biochemicals); ATP32P, 2-3 X 10^9 counts/min (550 mc/mmole, International Chemicals and Nuclear Corporation); theophylline, 8 mM; MgCl2, 2mM; tris-Cl, 21 mM (pH 7.7); human serum albumin, 0.8 mg/ml (Pentex); and glucagon (Eli Lilly Laboratories) or norepinephrine (L-norepinephrine bitartrate from Mann Laboratories) at concentrations stated in the text. The incubations were started by adding the particulate fraction, which had been kept at 1°C to the other components which were at 23°C. Glucagon or norepinephrine were added to the particles just before beginning the incubations. DL-propranolol (Ayerst Laboratories), when present, was added immediately before the addition of hormone. After 3 minutes the incubations were stopped by adding 0.1 ml of a solution containing 4 μmoles of ATP, 1.25 μmoles of cyclic 3',5'-AMP, and 0.15 μc of 3H-cyclic 3',5'-AMP (1 c/mmole, Schwarz Bioresearch). The mixture was boiled for 3 minutes. The 3H-cyclic 3',5'-AMP served to determine the recovery of cyclic 3',5'-AMP during the procedure; recoveries were 30% to 35%. After boiling, 0.4 ml of water was added, the precipitate removed by centrifugation, and the supernatant fluid applied to a 0.5 X 2.0 cm Dowex-50 column (Dowex 50W-X8, 100-200 mesh, Calbiochem). The column was washed with water, and the eluate, between 3.0 and 6.0 ml, was collected and precipitated with 0.17M ZnSO4 and 0.15M Ba(OH)2. The mixture was centrifuged at 2,000 g for 10 minutes and the cyclic 3',5'-AMP32P and 3H-cyclic 3',5'-AMP, which were in the supernatant fluid, were then counted in a liquid scintillation spectrometer (9).

Human heart muscle was obtained from two patients undergoing open-heart surgery, one for repair of a ventricular septal defect and one for mitral valve replacement. Particles from the homogenates were prepared as described for the cat. For the determination of phosphodiesterase activity, 3H-cyclic 3',5'-AMP (200 picomoles) was added to each reaction mixture which contained approximately 0.09 mg of protein. Incubation conditions were identical to those noted above except that ATP32P was omitted. Glucagon was present at 1 X 10^-5M. After boiling, the precipitate was removed by centrifugation; the supernatant fluid was mixed with 3.4 ml of water followed by 0.2 ml of 0.17M ZnSO4 and 0.2 ml of 0.15M Ba(OH)2. After centrifugation, 3 ml of the supernatant fluid was added to 17 ml of Bray's solution and the radioactivity measured in a liquid-scintillation spectrometer.

**Results**

Glucagon, 10^-4M, increased the conversion of ATP32P to cyclic 3',5'-AMP32P in the particulate fraction of homogenates of both cat and human myocardium by an average of approximately 70% (p < 0.001 and p < 0.025,

<p>| TABLE 1 |
| Effect of Glucagon on Adenyl Cyclase in Cat Heart |</p>
<table>
<thead>
<tr>
<th>No. of samples</th>
<th>No. of cats</th>
<th>Cyclic 3',5'-AMP32P accumulated* (picomoles/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Glucagon (10^-5M)</td>
<td>22</td>
<td>12.6 ± 0.6†</td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Norepinephrine (10^-5M)</td>
<td>14</td>
<td>10.5 ± 1.1†</td>
</tr>
</tbody>
</table>

*Mean ± se; †P < 0.001.
TABLE 2

Effect of Glucagon on Adenyl Cyclase of Human Cardiac Muscle

<table>
<thead>
<tr>
<th></th>
<th>Cyclic 3',5'-AMP^32P accumulated (picomoles/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Control</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>Glucagon (10^-6 M)</td>
<td>11.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>Glucagon (10^-6 M)</td>
<td>13.5 ± 0.9</td>
</tr>
<tr>
<td>Norepinephrine (10^-6 M)</td>
<td>12.2 ± 1.3</td>
</tr>
</tbody>
</table>

In A, left ventricular papillary muscle was removed during the insertion of a prosthetic mitral valve. In B, right ventricular muscle was obtained during correction of a ventricular septal defect. The values represent the mean ± SE of three samples in A and the mean ± SE of five samples in B. The combined data are significant at the 0.025 level.

The effect of glucagon on adenyl cyclase in cat heart. Each point represents the mean of eight samples obtained from three cats; vertical lines = ±SE.

The effect of glucagon on adenyl cyclase in cat heart. Each point represents the mean of eight samples obtained from three cats; vertical lines = ±SE.

The effect of incubation time on the activation of adenyl cyclase in cat heart by glucagon. Each point represents the mean of two samples. Glucagon was present at 1 X 10^-6 M. The reaction mixture contained 0.08 mg of protein.

respectively (Tables 1 and 2). An equimolar concentration of norepinephrine produced a somewhat greater accumulation of cyclic 3',

5'-AMP^32P in the cat preparations (114%) and an equal accumulation in the human. The increase in cyclic 3',5'-AMP^32P produced by glucagon was dose-related over a concentration ranging from 1 X 10^-7 M to 1 X 10^-3 M (Fig. 1). Higher concentrations gave no greater response. The concentration of glucagon that produced 50% of the maximal response was 8 X 10^-7 M. The increase in cyclic 3',5'-AMP^32P was seen as early as 30 seconds after the initiation of the reaction (Fig. 2).

Although activation of adenyl cyclase occurred with crystalline glucagon, the commercial preparation of glucagon, containing phenol and large amounts of lactose, produced no effect on adenyl cyclase activation.

Fluoride, 8 X 10^-3 M, produced a 500% increase in cyclic 3',5'-AMP^32P accumulation in both the cat and human preparations.

Effect of Glucagon on Phosphodiesterase Activity

Since adenyl cyclase activity is assayed as the accumulation of cyclic 3',5'-AMP^32P, a decrease in phosphodiesterase activity would decrease the rate of cyclic AMP destruction and thereby increase the net accumulation of cyclic AMP. However, under the conditions of this experiment glucagon did not signifi-
sently alter phosphodiesterase activity (Table 3), indicating that the effect of this hormone was on adenyl cyclase activation.

Effects of DL-Propranolol on Activation of Adenyl Cyclase by Glucagon and Norepinephrine

The beta-receptor blocking agent, DL-propranolol, at $1 \times 10^{-5}$M, did not block the activation of adenyl cyclase produced by glucagon, $1 \times 10^{-4}$M or $1 \times 10^{-5}$M (Fig. 3). However, the same concentration of propranolol blocked the activation of adenyl cyclase induced by norepinephrine, $1 \times 10^{-4}$M and $1 \times 10^{-5}$M (Fig. 3).

Effect of Combined Maximal Doses of Glucagon and Norepinephrine on Adenyl Cyclase

An additive response obtained by combining maximal doses of glucagon and norepinephrine in a single incubation would suggest the presence of separate adenyl cyclases for each hormone in the heart. However, no such additive response was observed (Fig. 4).

Effect of Other Polypeptide Hormones on Cat Heart Adenyl Cyclase

Two other polypeptide hormones, thyroid-stimulating hormone and adrenocorticotropic hormone (ACTH), failed to stimulate adenyl cyclase in a preparation in which glucagon was active (Table 4).

Discussion

The results of this investigation clearly indicate that glucagon activates adenyl cyclase in particulate fractions of cat and human heart homogenates. The increase in cyclic 3',5'-AMP is detected as early as 30 seconds after the initiation of the reaction, and the response is dose-related. Since the thyroid-stimulating hormone and ACTH did not stimulate myocardial adenyl cyclase, it appears that the enzyme does not respond in a nonspecific way to all polypeptide hormones.

### TABLE 3

<table>
<thead>
<tr>
<th></th>
<th>Cyclic 3',5'-AMP hydrolyzed (picomoles/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.2 ± 2.0</td>
</tr>
<tr>
<td>Glucagon (10^{-5}M)</td>
<td>8.0 ± 1.6</td>
</tr>
</tbody>
</table>

Mean ± se of eight samples. The differences between the control and glucagon studies are not statistically significant.
GLUCAGON ACTIVATION OF ADENYL CYCLASE

FIGURE 4
The effects of separate and combined maximal doses of glucagon and norepinephrine on adenyl cyclase in cat heart. Glucagon and norepinephrine were each present at $1 \times 10^{-5}M$. The means ± se of three samples are given.

TABLE 4
Effect of Other Polypeptide Hormones on Adenyl Cyclase in Cat Heart Particles

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Cyclic 3',5'-AMP accumulated (picomoles/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.4</td>
</tr>
<tr>
<td>Glucagon ($10^{-5}M$)</td>
<td>8.7</td>
</tr>
<tr>
<td>Thyroid-stimulating hormone ($10^{-5}M$)</td>
<td>3.9</td>
</tr>
<tr>
<td>ACTH ($10^{-5}M$)</td>
<td>3.5</td>
</tr>
</tbody>
</table>

The values represent the mean of two samples. Each reaction mixture contained 0.09 mg of protein.

Activation of adenyl cyclase could be achieved only with pure crystalline glucagon; the commercial preparation available for clinical use was inactive in our broken cell preparation. The lack of activity was probably due to the chemical composition of diluent or to the large amounts of lactose used as a preservative for the crystalline glucagon. However, both types of glucagon produced comparable inotropic responses in the isolated right ventricular papillary muscle of the cat (Skelton, C. L., unpublished observations).

A wide variety of polypeptide hormones have been shown to increase cyclic 3',5'-AMP levels in their target tissues (1), and Sutherland has postulated a "two-messenger theory" of polypeptide hormone action in which the hormone is the first messenger, adenyl cyclase the receptor site or closely related to it, and cyclic AMP the second messenger. The second messenger serves to activate one or more biochemical pathways which ultimately results in the expected end organ response. The prototype of such a reaction is the increase in cyclic AMP levels and augmentation of myocardial contractility produced by epi- nephrine (1). It is generally accepted that these biochemical and mechanical changes are mediated by beta receptors; it has been further suggested that the adenyl cyclase system is in fact the beta receptor (12).

Since, like epinephrine, glucagon activates adenyl cyclase and augments myocardial contractility, it might appear that glucagon also acts by stimulating the beta receptor. However, data have been recently reported suggesting that two separate adenyl cyclase systems exist in liver, one stimulated by glucagon, the other by epinephrine (13). On the other hand, although ACTH, glucagon, and epinephrine were shown to stimulate separate
receptor sites in fat cells (14), the effects of the hormones did not summate at maximal concentrations, indicating that there was only one adenyl cyclase system in this tissue. In our study, the failure of combined maximal doses of glucagon and norepinephrine to produce an additive response suggests that the heart also contains only one adenyl cyclase enzyme responsive to these hormones. Furthermore, the observation that propranolol blocks the activation of adenyl cyclase produced by norepinephrine but does not impair the response to glucagon is compatible with the hypothesis that adenyl cyclase itself is not the beta receptor; rather, the heart appears to contain more than one receptor site responsible for activation of adenyl cyclase. Thus, if activation of adenyl cyclase plays a role in the augmentation of myocardial contractility, our results would appear to provide the biochemical explanation for the observations of Glick et al. (4) and Lucchesi (5) who found that although glucagon had a marked positive inotropic effect on cardiac muscle, this response was not blocked by propranolol.

Acknowledgments
We are grateful to Dr. Robert Reis for obtaining the surgical specimens of human heart and to Mrs. Hope Cook for her expert technical assistance.

References
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Circ Res. 1969;24:151-156
doi: 10.1161/01.RES.24.2.151

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