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^{125}I-Glucagon Binding and Adenylate Cyclase Activation in the Fetal Rat Heart[1] (38119)

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The polypeptide hormone, glucagon, initiates a number of physiological responses including glycogenolysis, lipolysis, insulin release, and increases in heart rate and myocardial contractility. The actions, like those of many other hormones on their respective target tissues, are thought to be mediated by the activation of the membrane-bound enzyme, adenylate cyclase and the resultant increase in the intracellular concentration of adenosine 3', 5'-monophosphate (cyclic AMP). Following the classic description of the glucagon and epinephrine mediated increases in hepatic cyclic AMP by Sutherland and Rall(1) and the delineation of cyclic AMP as the mediator of the action of these hormones on glycogenolysis, Sutherland and his coworkers proposed four criteria which should be fulfilled before concluding that the effects of a hormone on its target organ were mediated by cyclic AMP (2). These criteria have been fulfilled for the actions of glucagon on the heart. One, glucagon activates adenylate cyclase in broken cell preparations of myocardium (3, 4). Two, glucagon increases cyclic AMP levels in intact, isolated perfused hearts (5). Three, the inotropic effects of glucagon are potentiated by the phosphodiesterase inhibitor, theophylline (6). Four, the dibutyryl derivative of cyclic AMP has a positive inotropic effect on heart muscle similar to that of glucagon (7, 8).

It is of interest that all of these studies have utilized adult animals. Furthermore, relatively little information is available pertaining to hormonal effects on fetal heart muscle (9–14). Recently, Wildenthal reported a detailed study of the chronotropic responsiveness of fetal mouse hearts to a variety of hormones including the catecholamines and glucagon (15). He found that chronotropic responses to catecholamines were maximal at 18 days of fetal life. Responsiveness to glucagon did not appear until after day 17 of fetal life. In another investigation he found that fetal rat hearts did not respond to glucagon until term (day 22) in marked contrast to what was observed with catecholamines (16). Clark, Beatty, and Allen showed that rat myocardial adenylate cyclase was not activated by glucagon until approximately 28 days after birth (17). Martin, Levey, and Levey, have recently showed that fetal rat myocardial adenylate cyclase specifically binds ^{3}H-norepinephrine and is fully activated by norepinephrine by the thirteenth day of fetal life (18).

The purpose of the present investigation was to examine specific glucagon binding to myocardial receptors in fetal rat heart and activation of adenylate cyclase in these same hearts.

Materials and Methods. Crystalline glucagon was a gift from Eli Lilly and Co., Indianapolis, Indiana. Lubrol-PX was a gift from ICI America, Inc., Stamford, Connecticut. Bovine serum albumin, 4X-crystalline, was purchased from Nutritional Biochemicals, Cleveland, Ohio. Alpha-labeled ^{32}P-ATP was obtained from International Chemical and Nuclear Corp., Irvine, California; carrier-free-$Na^{125}I$ (in 0.1 *N* NaOH) from Union Carbide Company, Tuxedo, New York.

Pregnant rats of known gestational age (± 12 hr) were obtained from Holtzman Com-

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pany, Madison, Wisconsin. The presence of sperm is detected by vaginal smears within 12 hr after mating. The pregnant rats were anesthetized with approximately 25–35 mg/kg pentobarbital intrathoracically and the fetuses rapidly removed. The heart from each fetus was removed with the aid of a dissection microscope (Bausch and Lomb). Only the hearts which were beating at the time of removal were used for these studies. In addition, random hearts were selected for histological study to confirm the identity of the tissue removed.

Approximately 20–30 hearts from the 13-day-old fetuses, and 8–12 hearts from the 18-day-old fetuses were added to 1.0 ml of 0.25*M* sucrose and homogenized in a Dounce homogenizer (glass on glass) with 5–7 strokes of the pestle. For the experiments with adult rats approximately 200 mg of left ventricular muscle was added to 4.5 ml of sucrose and homogenized as previously described (5). The homogenates were utilized for the assay of adenylate cyclase in which the conversion of alpha-labeled ^{32}P-ATP to ^{32}P-cyclic AMP was measured by the method of Krishna *et al.* (19) under the precise conditions described previously from our laboratory (5). The fractions for assay containing 0.045–0.075 mg protein in a total volume of 0.06 ml were incubated at 37° for 10 min with ATP 1.6 m*M*, α-^{32}p-ATP, 2.5–3.5 $\times$ 10^6 cpm, theophylline, 8 m*M*; Mg Cl_2, 2 m*M*; Tris–HCl, 21 m*M*, pH 7.7; and bovine serum albumin, 0.8 mg/ml. Crystalline glucagon, dissolved for use in the 10 m*M* Tris–HCl, pH 8.7, and warmed at 37° was added to the enzyme at 1° at final concentrations stated in the text. The incubations were initiated by adding the enzyme-glucagon mixture at 1°, to the other components which were at 23°. After 10 min the incubations were stopped and the ^{32}P-cyclic 3', 5'-AMP accumulated was determined (5).

Heart muscle adenylate cyclase was solubilized as previously described (20). Approximately 20 hearts from the 13-day-old fetuses and 10 hearts from the 18-day-old fetuses were solubilized in a homogenizing solution containing in final concentration sucrose, 0.25*M*; Tris–HCl, 10 m*M*; pH 7.7; Lubrol-PX, 20 m*M*, and EDTA-magnesium chloride, 1 m*M*. For the adult hearts approximately 300 mg of left ventricular muscle was added to 4.5 ml of an identical Lubrol-PX solution. The homogenate was centrifuged at 12,000 *g* for 10 min and the supernatant containing the solubilized adenylate cyclase utilized for the ^{125}I-glucagon binding experiments.

Glucagon was iodinated by a modification of the procedure of Hunter and Greenwood (21). The following solutions were added successively into a 10 $\times$ 75 mm flint glass tube: 20 μl $NaPO_4$, 0.6 *M*, pH 7.4; 10 μl (35 μg) crystalline glucagon, 3.5 mg/ml, 0.01*N* HCl, ^{125}Iodine, 2.5 to 3.0 mCi; 10 μl chloramine T, 3.5 mg/ml, 0.05 *M* $NaPO_4$, pH 7.0, and 25 μl Na Metabisulfite, 2.4 mg/ml, 0.05 *M* $NaPO_4$, pH 7.0. The specific activity of the iodinated glucagon was 330 μc/nmole, which is less than one atom per mole of glucagon (22). Five μl of the iodination mixture is then added to a solution of 100 μl of Veronal 0.1 *M*, pH 8.6 and 15 μl plasma containing bromphenol blue. This mixture was applied to Whitman 3M paper, and electrophoresed for 90 min at 25°, dried and strip scanned on a Nuclear Chicago Actigraph to determine the relative efficience of glucagon iodination. The iodination generally was 90–95% complete, the remainder being free iodine. ^{125}I-glucagon was purified on a column of cellulose powder as described by Rodbell *et al.* (22). The ^{125}I-glucagon was applied to a 2.5 cm cellulose column prepared in a Pasteur pipette of 0.6 cm diameter and prewashed with 1% albumin in 10 m*M* sodium phosphate, pH 7.5. The iodinated glucagon was applied and the column washed with 3.0 ml of a solution of 1% albumin in 10 m*M* sodium phosphate adjusted to pH 7.5, and then eluted with 0.6 ml of the same solution adjusted to pH 10.0 with concentrated ammonium hydroxide. The ^{125}I-glucagon eluted in this manner was biologically active as determined by its ability to activate the particulate myocardial adenylate cyclase.

^{125}I-glucagon was assayed by a modification of the method of Rodbell *et al.* (22) as previously described from our laboratory (23). The specific fractions of solubilized enzyme were incubated at 37° in a final volume of 100 μl containing 1.0% albumin in 10 m*M* Tris–HCl, pH 7.7, and ^{125}I-glucagon (0.370 μc/pmole). After 60 min the incubation mixture was added to dry 2.5 cm cellulose columns in a disposable Pasteur pipette with an inside diameter of 0.6 cm and washed with

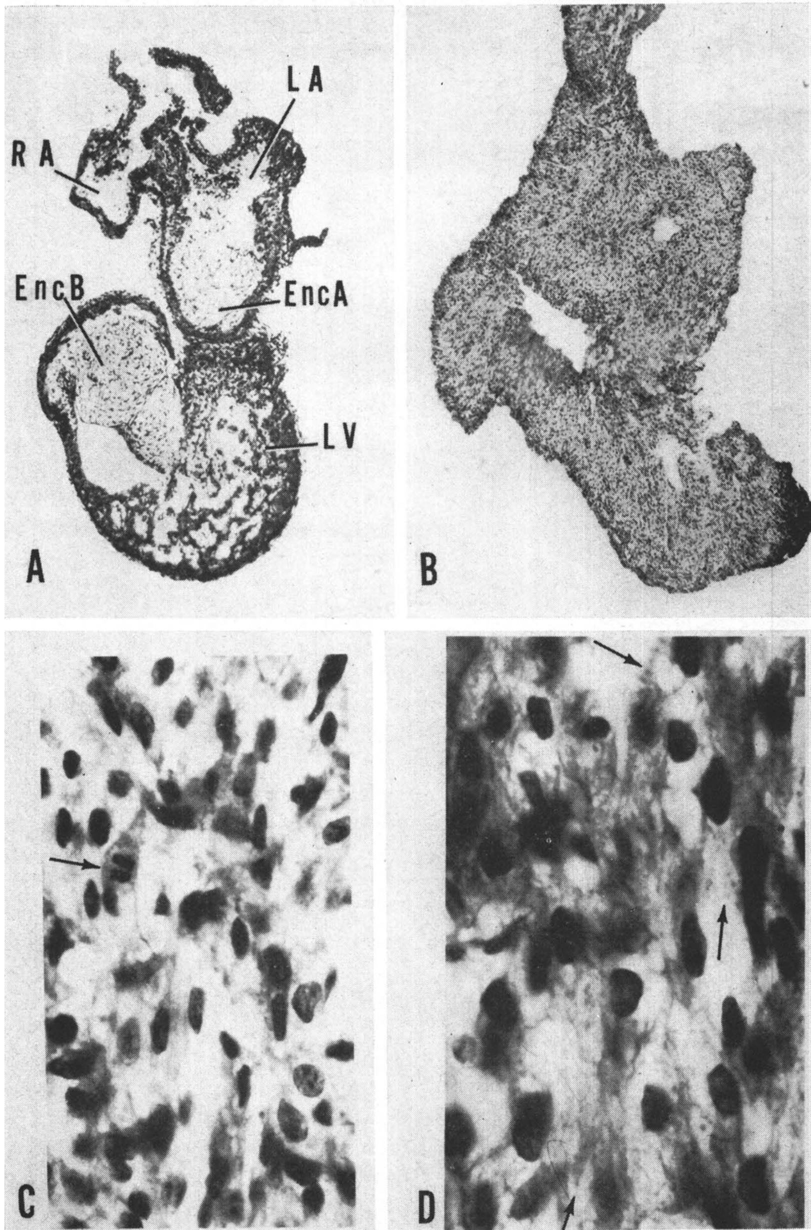

FIG. 1. *Photomicrographs of hematoxylin and eosin stained sections of fetal rat hearts randomly selected from specimens utilized for biochemical studies.* (A) Section of 13 day fetal heart. RA, right atrium; LA, left atrium; EncA, endocardial cushing tissue of atrioventricular junction, tangential cut; LV, left ventricle; EncB, endocardial cushion tissue of bulbous cordis. (B) Section of 18 day fetal heart, ventricle only. (C) Higher magnification from the same 18-day specimen showing muscle cell in mitosis (arrow). (D) Enlargement of a field from the same series showing cross-banded myofibrils (arrows). A and B × 62; C, × 630; and D, × 1150.

1.4 ml of 1% albumin in 10 m*M* Tris–HCl, pH 7.7. Bound ^{125}I-glucagon did not adsorb to the column whereas free (unbound) ^{125}I-glucagon did. The eluate was then counted in a Nuclear Chicago Auto-gamma. This method removes more than 90% of the free (unbound) ^{125}I-glucagon as determined by the number of counts found in the control sam-

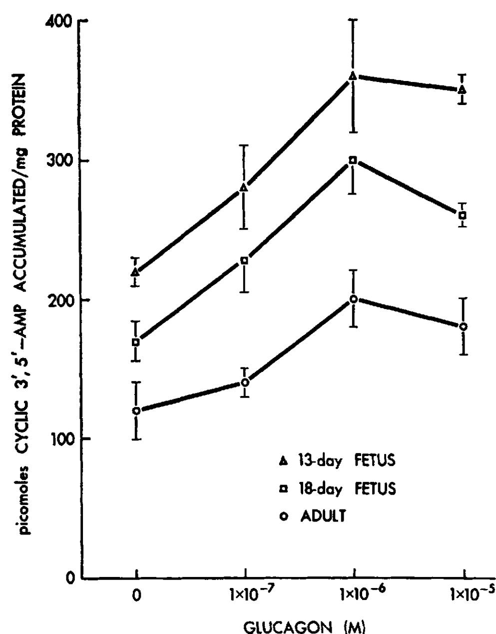

FIG. 2. *Glucagon activation of adenylate cyclase in fetal and adult rat heart.* Each value represents the mean ± S.E. of 6–8 samples from 9 litters (3 experiments) for the fetal hearts and the mean ± S.E. of 4 samples from 2 adult hearts.

ples of identical composition incubated simultaneously using 10 m*M* Tris–HCl, pH 7.7, and Lubrol-PX in place of the enzyme fraction. All experimental values were corrected by subtracting the blank values obtained from incubations in the absence of the solubilized binding fraction.

Results. Although the fetal hearts at days 13 and 18 are well-defined, beat after removal from the fetus and are readily dissected with the aid of a microscope, it seemed appropriate to examine randomly selected specimens of 13- and 18-day-old fetal hearts histologically. Figure 1A shows the characteristic interrelationships of the different chambers of an excised 13-day heart as seen at low magnification. Note particularly the pattern of the ventricular muscle, with bands of muscle (trabeculae carneae) interdigitating with endothelial-lined channels which are continuous with the central portion of the ventricular lumen. Considerable differentiation of the muscle occurs by the eighteenth day as seen in Figs. 1B, 1C, and 1D. This tissue sample was sectioned in its entirety (118 sections of 10 μm thickness), and it was composed entirely of ventricles. The myocardium is less trabeculated than at 13 days (compare Figs. 1A and 1B). Myofibrils are more numerous than in the muscle cells of the 13-day specimens, but they are still irregularly arranged and course singly or in small bundles. Under the oil immersion objective, the fibrils are seen to be cross-striated by A (dark) and I (light) bands (Fig. 1D arrows). The endocardium consists of endothelium resting closely on the myocardium and the epicardium is composed of simple squamous epithelium (mesothelium) resting on the outer surface of the myocardium. Connective tissue is very sparse.

Figure 2 shows that glucagon activated adenylate cyclase in homogenates of 13- and 18-day fetal hearts and in adult hearts. The increases in cyclic AMP accumulation were observed over the concentration ranges $1 \times 10^{-7}M$–$1 \times 10^{-5}M$. Half-maximal activation occurred at similar concentrations of glucagon in all preparations, 2 to $5 \times 10^{-7}M$. Adenylate cyclase activity per mg of protein was highest in the 13-day fetal preparation and lowest in the adult.

We have recently described the binding of biologically-active ^{125}I-glucagon to soluble preparations of adult cat heart preparations (23). Figure 3 shows that the binding of ^{125}I-glucagon occurred in all preparations over concentration ranges similar to those observed for the activation of myocardial adenylate cyclase, $5 \times 10^{-8}M$–$5 \times 10^{-6}M$. The specificity of the ^{125}I-glucagon binding was demonstrated by the observation that approximately 90% of the glucagon binding was displaceable by unlabeled glucagon, the remainder presumably representing nonspecific binding. Displacement occurred over the concentration range $10^{-7}M$–$10^{-5}M$.

Discussion. The data obtained from this investigation clearly demonstrate specific glucagon binding to a solubilized preparation of 13- and 18-day fetal rat hearts and a glucagon-responsive adenylate cyclase in homogenates from these same hearts. There is relatively little physiological information pertaining to the hormone responsiveness of the fetal heart and in general this derives from studies in chickens, rats and mice (9–14). The rat heart begins to beat with a slow, fixed heart rate at about the ninth or tenth day of fetal

life. The rate progressively increases with age. In fetal mice, acetylcholine produces a marked bradycardia as early as the twelfth day, a time of minimal responsiveness to catecholamines (15). The catecholamines produce significant chronotropic effects by the sixteenth to eighteenth day, when innervation of the sinus node occurs. A catecholamine-sensitive adenylate cyclase is present at this time (17) and as early as the thirteenth day in fetal rats (18). Glucagon responsiveness (chronotropic) of the fetal mouse heart is not observed until after the seventeenth day of fetal life, with the maximum response occurring somewhere between 19 and 22 (term) days (16). Theophylline, which inhibits phosphodiesterase, the enzyme catalyzing the breakdown of cyclic AMP to 5'-AMP has a significant chronotropic effect on the thirteenth to fourteenth day. Inotropic responsiveness to glucagon is not found before term in fetal rat heart (16). Friedman *et al.*, found that glucagon exerted a negative inotropic effect on the term fetal lamb and a small positive inotropic effect in newborns (24). In addition, they found that glucagon activated adenylate cyclase in fetal lamb heart homogenates.

The major question to be resolved concerns the role cyclic AMP serves in mediating the inotropic and chronotropic responses to hormones. Current literature strongly supports such a role; Sutherland's four criteria having been fulfilled for the catecholamines and glucagon and partially fulfilled for histamine, prostaglandins, and thyroid hormones (25). However, several studies have raised notes of caution (26, 27) and suggested cyclic AMP may not entirely account for the inotropic and chronotropic effects of these hormones. It seems clear that a catecholamine-responsive adenylate cyclase and specific catecholamine-binding sites are present in the myocardium of 13–14-day-old rat fetuses, a time when inotropic and chronotropic responses are minimal. These findings are consistent with the interpretation that one of the many steps beyond cyclic AMP, which are required to initiate inotropic and chronotropic responses may not yet be fully developed (28).

The data obtained with glucagon are more difficult to explain since both catecholamines and glucagon activate adenylate cyclase, bind specifically to cardiac receptors, are both thought to act via cyclic AMP but appear to have vastly different times of onset of their inotropic and chronotropic responses in the fetus. Assuming the pathway following cyclic AMP generation to initiation of the physiologic response is identical for both, how can the physiologic discrepancy be explained? Current evidence suggests that adenylate cyclase as it is situated in the cell membrane

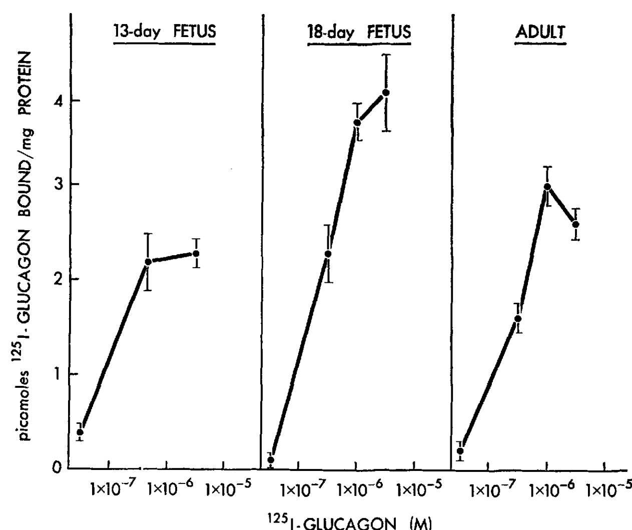

FIG. 3. *^{125}I-glucagon binding in fetal rat heart.* Each value represents the mean ± S.E. of 6 samples from 9 litters (3 experiments) and the mean ± S.E. of 3 samples from an adult heart.

consists of several subunits; a catalytic subunit on the interior of the cell surface, a coupler subunit, and a receptor or regulatory subunit which serves as the specific binding site for the hormone (29). The configuration of the enzyme in the membrane would determine which receptor site(s) are available for hormone-binding and activation of the enzyme. It seems possible that the fetal glucagon receptor *in vivo* may not be available for binding and activation of the adenylate cyclase. Disruption of the cell membrane might enable these sites to become accessible to glucagon. In addition, some antagonist to glucagon action may be also present *in vivo* which is removed or destroyed in the *in vitro* system. On the other hand, the data could be interpreted to indicate that cyclic AMP mediates only a part or none of the inotropic and chronotropic effects of glucagon in the fetus despite the fulfillment of Sutherland's four criteria in adult animals.

It should be noted that the glucagon-activation of adenylate cyclase demonstrated in this report, and the activation of adenylate cyclase in homogenates of fetal lamb hearts (24) are in marked contrast to the results of Clark *et al.* (17), who found glucagon-unresponsiveness of fetal rat myocardial adenylate cyclase until about 28 days after birth. The reason for this discrepancy is unknown but the assays for the enzyme are complex, multifactorial, and may involve such things as lability of receptors, mode of preparation, and type of animal.

Summary. Hearts from 13- and 18-day rat fetuses were shown to specifically bind ^{125}I-glucagon and to have a glucagon-sensitive adenylate cyclase. ^{125}I-glucagon binding was observed over the concentration range $5 \times 10^{-8}M$–$5 \times 10^{-6}M$. Glucagon activation of adenylate cyclase occurred over a concentration range $10^{-7}M$–$10^{-5}M$. Similar results for both ^{125}I-glucagon binding and adenylate cyclase activation were obtained in adult rat hearts. In addition, the data in the present study demonstrate that glucagon activation of adenylate cyclase in the fetal rat occurs on day 18, when physiological and biochemical studies have shown insignificant inotropic and chronotropic responses to catecholamines, activation of cardiac adenylate cyclase by catecholamines, but no significant inotropic or chronotropic responses to glucagon. Several explanations for this discrepancy are considered including the possibility that cyclic AMP may not mediate the inotropic and chronotropic responses to glucagon in the fetal rat heart.

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