JCI The Journal of Clinical Investigation

Responsiveness to glucagon in fetal hearts. Species variability and apparent disparities between changes in beating, adenylate cyclase activation, and cyclic AMP concentration.

K Wildenthal, … , J R Wakeland, C M Clark Jr

J Clin Invest. 1976;57(3):551-558. https://doi.org/10.1172/JCI108310.

Research Article

Previous studies of the ability of the immature heart to respond to glucagon have yielded conflicting results. To test the possibility that the apparent discrepancies might be explained in part by species variability, isolated hearts of fetal mice and rats (13-22 days' gestational age) were studied under identical conditions in vitro. Changes in atrial rate and ventricular contractility were measured in spontaneously beating hearts exposed to glucagon, and activation of adenylate cyclase was assayed in cardiac homogenates. In mice of 16 days' gestational age or less, there was no change in heart rate in response to glucagon; at 17-18 days, minimal responsiveness was present; and after 19 days, 10muM glucagon caused an increase in spontaneous atrial rate of 30 +/- 4% (SEM) (P less than 0.001). Measurement of the extent and speed of volume displacement of the isotonically contracting hearts with a specially constructed capacitance transducer revealed that ventricular inotropic responsiveness also appeared after 17-19 days. Cardiac stores of glycogen were reduced in older hearts exposed to glucagon, but not in those aged less than 16 days. In contrast, glucagon failed to activate adenylate cyclase in homogenates of hearts of fetal mice at any age. Furthermore, glucagon failed to elicit an increase in the concentration of cyclic AMP in spontaneously beating hearts that developed tachycardia. Responses in hearts of fetal […]

Responsiveness to Glucagon in Fetal Hearts

SPECIES VARIABILITY AND APPARENT DISPARITIES BETWEEN CHANGES IN BEATING, ADENYLATE CYCLASE ACTIVATION, AND CYCLIC AMP CONCENTRATION

KERN WILDENTHAL, DONALD O. ALLEN, JAN KARLSSON, JACQULINE R. WAKELAND, and CHARLES M. CLARK, JR.

From the Pauline and Adolph Weinberger Laboratory for Cardiopulmonary Research, Departments of Physiology and Internal Medicine, University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, Texas 75235; and Departments of Internal Medicine and Pharmacology, University of Indiana Medical School, Indianapolis, Indiana 46204

ABSTRACT Previous studies of the ability of the immature heart to respond to glucagon have yielded conflicting results. To test the possibility that the apparent discrepancies might be explained in part by species variability, isolated hearts of fetal mice and rats (13-22 days' gestational age) were studied under identical conditions in vitro. Changes in atrial rate and ventricular contractility were measured in spontaneously beating hearts exposed to glucagon, and activation of adenylate cyclase was assayed in cardiac homogenates. In mice of 16 days' gestational age or less, there was no change in heart rate in response to glucagon; at 17-18 days, minimal responsiveness was present; and after 19 days, 10 μM glucagon caused an increase in spontaneous atrial rate of 30±4% (SEM) ($P < 0.001$). Measurement of the extent and speed of volume displacement of the isotonically contracting hearts with a specially constructed capacitance transducer revealed that ventricular inotropic responsiveness also appeared after 17-19 days. Cardiac stores of glycogen were reduced in older hearts exposed to glucagon, but not in those aged less than 16 days. In contrast, glucagon failed to activate adenylate cyclase in homogenates of hearts of fetal mice at any age. Furthermore, glucagon failed to elicit an increase in the concentration of cyclic AMP in spontaneously beating hearts that developed tachycardia.

Responses in hearts of fetal rats were distinctly different from those in mouse hearts: at no age was there any change in heart rate, strength of contraction, glycogen content, or adenylate cyclase activation.

Thus, there are major species differences in cardiac pharmacological maturation. Although the mouse heart develops the ability to increase its rate and strength of contraction and to undergo glycogenolysis in response to glucagon well before birth, the rat heart does not. In addition, there is an apparent disparity in late fetal mouse hearts between the ability of glucagon to induce functional responses and its ability to stimulate adenylate cyclase and increase cyclic AMP levels. It is impossible, of course, to rule out absolutely the possibility that localized increases in a critical cyclic AMP pool were present but too small to measure in the entire tissue. Nevertheless, the most obvious interpretation of our results is that they are compatible with the hypothesis that glucagon may exert some of its hemodynamic effects independently from the adenylate cyclase-cyclic AMP system in the late-fetal mouse heart.

Dr. Wildenthal and Dr. Allen were supported by U. S. Public Health Service Research Career Development Awards HL 70125 and AM 50316, and Dr. Clark by a Clinical Investigatorship for the Veterans Administration. Dr. Karlsson's present address is Department of Physiology, GIH, Stockholm, Sweden.

Received for publication 10 January 1975 and in revised form 31 July 1975.

INTRODUCTION

As the immature heart develops, it gradually acquires the ability to respond to cardioactive agents. The point during development at which pharmacological responsiveness appears may vary widely for different agents. For example, cardiac responsiveness to the physiological neurotransmitters, acetylcholine and norepinephrine, ac-

tually precedes innervation of the heart (1, 2), whereas such agents as tyramine have no effect until later (2). Analysis of the patterns of maturation of contractile and metabolic responsiveness to various drugs has been suggested as a useful means for gaining insight into possible mechanisms of action of the drugs (2).

Responsiveness to glucagon has been reported to appear especially late in cardiac development. Indeed, some experiments have indicated that the heart remains completely insensitive to the drug until long after birth (3-5). On the other hand, glucagon has been said to cause an increase in the spontaneous beating rate of hearts of fetal mice well before term (2). The experiments that yielded these apparently conflicting results were performed in different species as well as under different protocols; accordingly, to resolve the discrepancies, the present protocol was designed to evaluate in detail the comparative maturation of chronotropic, inotropic, and metabolic responses to glucagon in hearts of fetal mice and of fetal rats of various gestational ages. In addition, experiments were made to test the ability of glucagon to activate myocardial adenylate cyclase at various stages of fetal development in the two species in order to correlate the presence of enzyme activation with the ability of the hearts to alter their beating. Finally, the effect of glucagon on cyclic AMP concentration in fetal mouse hearts was studied as well.

METHODS

Experiments were conducted in three separate phases. Measurements of cardiac contraction and glycogen content were made in Dallas by techniques described by Wildenthal et al. (2, 6) and Karlsson (7). Adenylate cyclase activity in fresh cardiac homogenates was measured in Indianapolis by techniques described below. Measurements of cyclic AMP were made after treating spontaneously beating hearts with glucagon or norepinephrine and freezing them in liquid nitrogen in Dallas, and assaying the tissue for cyclic AMP in Indianapolis.

Studies of cardiac beating and glycogen content. Intact hearts were removed from mouse and rat fetuses of known gestational ages ranging between 13 and 22 days (term is 20–22 days in both species). The hearts were dissected free of surrounding tissue and transferred to specially constructed chambers where they lay on stainless steel grids with their lower surfaces exposed to liquid medium 199 (Grand Island Biological Co., Grand Island, N. Y.) and their top surfaces protruding into the atmosphere (6).

For studies of beating rate, the entire heart with both atria intact was used; for measurements of inotropism and of glycogen content, the atria were removed and the isolated ventricles alone were transferred to the chamber. In either instance, the myocardium beat spontaneously and rhythmically, with a stable rate and strength of contraction throughout the experiment (2, 6). After initial stabilization in an incubator for 2 h at 37°C, 95% O_2, and pH 7.4, the chamber with its heart was transferred to a water bath in room air. The medium was replaced with fresh control medium, and the rate and strength of contraction were recorded. Then the medium was changed to an identical solution supplemented with 10 μM glucagon (Eli Lilly and Company, Indianapolis, Ind., or Sigma Chemical Co., St. Louis, Mo.) and repeat recordings were made after a 45–60-s period of stabilization. This concentration of glucagon was determined in preliminary studies to provide a maximal response: lower concentrations gave submaximal responses, higher levels failed to augment further the rate or strength of contraction at any age, and concentrations of 0.1 mM or more occasionally even induced toxic effects (i.e., arrhythmias or depressed contractility). In some experiments a maximally effective concentration of norepinephrine (10 μM) was tested in a similar manner for comparison to the glucagon response, and both agents were also tested in the presence of 0.1 mM propranolol.

Recordings of contractions were obtained with a specially constructed microcapacitance-probe that has been described previously (6). The device detects movements in space of the isotonically beating hearts; changes in the speed and extent of displacement of the hearts can be recorded as a function of changes in inotropic state while beating rate is monitored simultaneously.

For analyses of cardiac glycogen content after glucagon treatment, hearts were allowed to stabilize for 2 h as described above. Then, the medium for the experimental heart was replaced with solution containing 10 μM glucagon, while the medium for a control heart (taken from a matched littermate of the experimental animal and prepared identically was simultaneously replaced with fresh control medium. The hearts were then reincubated at 37°C, 95% O_2, and pH 7.4 for 30 min, after which they were frozen in liquid nitrogen and subsequently assayed for glycogen as described by Karlsson (7).

Studies of adenylate cyclase activity in cardiac homogenates. Analyses of adenylate cyclase activity were performed as previously described (5, 8). Briefly, maternal rats were lightly anesthetized with ether; the fetuses were delivered by Caesarean section, and the hearts were rapidly excised and kept at 4°C. Because of the small size of the hearts, several were pooled before homogenization. The hearts were homogenized in 0.25 M sucrose that contained 10 mM Tris at pH 7.4. Samples of tissue homogenates were centrifuged at 600 *g* at 4°C. The pellets were resuspended in the sucrose Tris buffer and incubated in the assay media. The assay media contained 3.2 mM [α-^{32}P]ATP (10–20 cpm/pmol), 5 mM magnesium chloride, 1 mM EDTA, 25 mM Tris-HCl, pH 7.6, 0.8 mM theophylline, and an ATP-regenerating system consisting of 20 mM phosphoenopyruvate and 4 μU/ml pyruvate kinase. The hormones were diluted with a 12.5% bovine serum albumin solution before use. The reaction was initiated by adding 100–200 μg of tissue protein in a volume of 10 μl to a final assay volume of 50 μl. At the end of a 15-min incubation at 30°C, the reaction was stopped by the addition of 100 μl of recovery mix containing 40 mM ATP and 21.5 mM tritiated cyclic AMP (0.2 μCi/μM), before boiling for 3.5 min. 1 ml of 50 mM Tris-HCl, pH 7.6, was then added to each tube, before centrifugation at 1,500 *g* for 10 min. The cyclic AMP formed was isolated on an 0.8 × 4 cm alumina column (9). After addition of the sample, the column was washed with 0.8 ml of sucrose Tris buffer and then the following 2 ml were collected. Recovery of the tritiated cyclic AMP ranged from 40 to 65%. Tritium and ^{14}C were measured by liquid scintillation counting. Final values were calculated from efficiency of counting, recovery of cyclic AMP, and specific activity of the [^{32}P]ATP. In each experiment, three blanks with boiled homogenate were carried throughout the entire incubation and isolation procedure; ^{32}P counts from these

blanks ranged from 60–100 cpm above background. The mean value of the blanks in all experiments reported was less than 50% of the control values. The mean value for the blanks in each experiment was subtracted from the value for each of the control and experimental samples. The results are expressed as picomoles cyclic AMP formed per milligram protein per 15 minutes. Statistical analyses were performed with Student's *t* test.

Comparison studies were made with liver homogenates from 18-day fetal mice. Conditions were similar to those described above for heart and those described previously for fetal rat liver (5).

Studies of tissue levels of cyclic AMP. Intact hearts from 20–21 day fetal mice were prepared as if for studies of beating (see above). To provide sufficient tissue for the assays, six hearts were placed on each grid and subsequently pooled as one sample. After stabilization for 2–3 h in 95% oxygen, the hearts were exposed to fresh control medium, 10 μM glucagon, or 10 μM norepinephrine. Beating rates were monitored over the next 20–180 s, after which the hearts were rapidly frozen in liquid nitrogen. It required less than 0.5 s to transfer each individual beating heart from the grid to the liquid nitrogen, but since multiple hearts were transferred for each sample, the difference in the duration of exposure to the drug from the first to the last heart of each sample spanned approximately 10 s. To test for time differences in the responses, samples were collected after exposures of 20–40 s (hereafter labeled "30 s"), 50–70 s (1 min), 100–130 s (2 min), and 160–190 s (3 min). Subsequently the frozen hearts were shipped to Indianapolis, where they were assayed for cyclic AMP. Briefly, hearts were powdered under liquid nitrogen and homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, N. Y., model Pt-10) at full speed in 5% trichloroacetic acid. The samples were acidified with 0.1 ml

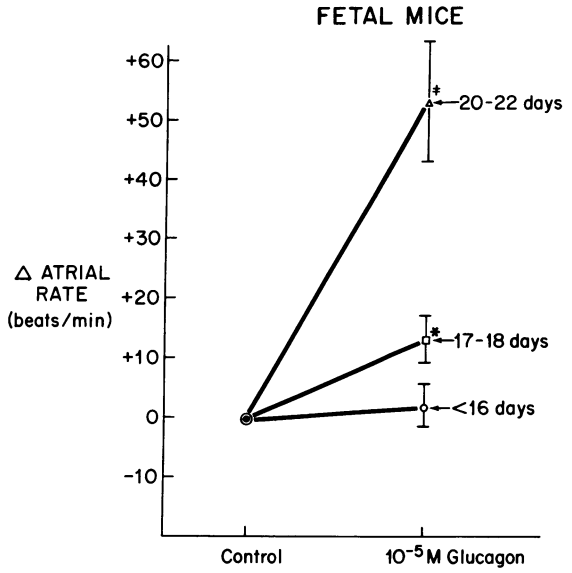

FIGURE 1 Atrial rate response to glucagon in fetal mouse hearts. Each point represents the mean of 16 hearts, and the vertical bars represent±1 SEM.
*$P < 0.05$ compared to control (Student's *t* test for paired observations).
‡$P < 0.01$.

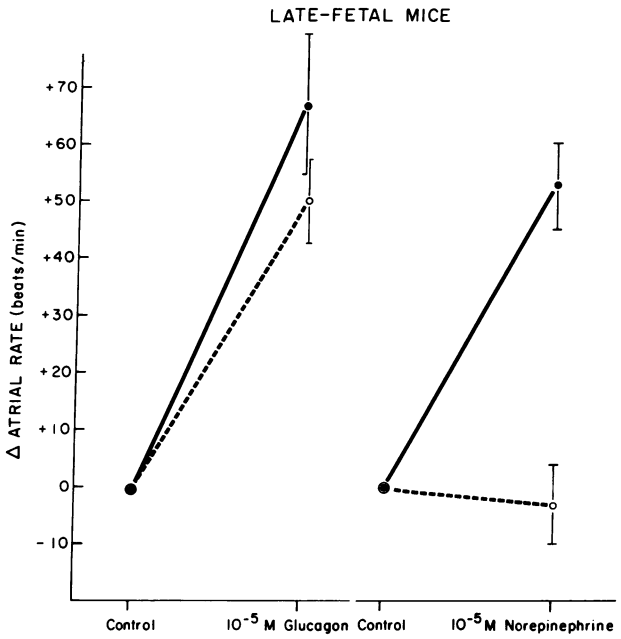

FIGURE 2 Atrial rate response to glucagon and norepinephrine in 21-day fetal mouse hearts. Hearts of matched littermates were tested with glucagon in the presence ($n = 6$) or absence ($n = 4$) of propranolol or with norepinephrine in the presence ($n = 4$) or absence ($n = 4$) of propranolol. Propranolol had no significant effect on the atrial response to glucagon ($P > 0.20$), but significantly inhibited the response to norepinephrine ($P < 0.01$). Symbols as in Fig. 1. ●——●, no propranolol; ○- - -○, 100 μM propanolol.

1 N HCl, [H^3]cyclic AMP (0.5 pmol; 0.1 μCi) was added, and the samples were centrifuged at 2,500 *g* for 10 min. The supernate was extracted five times with 2.5 vol of water-saturated diethyl ether. The samples were further purified by ion exchange chromatography (10) and assayed for cyclic AMP by the method of Gilman (11) with cyclic AMP-binding protein and "inhibitor protein" isolated from rabbit skeletal muscle. Levels of cyclic AMP were corrected for recovery (65–75%) and expressed as picomoles per milligram wet weight.

RESULTS

Mice. The spontaneous atrial rate in hearts of early-fetal mice (less than 16 days) was not altered by glucagon, but tachycardia in response to the drug began to appear by 17-18 days of gestation (Fig. 1). The magnitude of the response continued to increase as gestation proceeded, and glucagon caused a 30% increase in atrial rate in hearts of mice near term. The response to glucagon was not a result of release of endogenous catecholamines or beta-receptor stimulation, since high concentrations of propranolol failed to block the response even though norepinephrine-induced tachycardia was completely inhibited (Fig. 2).

Ventricular contractile characteristics also failed to change in response to glucagon in early-fetal mouse

TABLE I

Effect of Glucagon on Glycogen Content in Hearts of Fetal Mice and Fetal Rats

	Glycogen content				
	Control	10 μM Glucagon	Difference±1 SEM (%)	*n*	*P*
	nmol glucose/mg wet wt				
Fetal mice					
<16 days gestational age	105	108	+3±6.3 (+3%)	4	NS
>19 days gestational age	87	76	−11±3.2 (−13%)	4	<0.05
Fetal rats					
<16 days gestational age	114	110	−4±4.6 (−4%)	4	NS
>19 days gestational age	93	94	+1±3.6 (+1%)	4	NS

hearts, whereas hearts from fetuses of more than 19 days' gestational age always responded. At 17-18 days, responses were small and inconsistent. As is apparent in Fig. 3, changes in the older hearts were characterized by increases in the total extent of displacement and in the maximal rate of displacement of the ventricles; simultaneously, there was little or no change in the time to peak displacement or in the total duration of contraction. These changes in the presence of glucagon are indicative of an improvement in inotropic state and are similar to those normally encountered in myocardium tested with conventional techniques. Most of the isolated ventricles from late-fetal mice increased their rate of beating as well as their strength of contraction after exposure to glucagon. However, in a few, such as the one illustrated in Fig. 3, there was only a minimal change in heart rate; nevertheless, there still occurred an increase in contractility. Thus, the inotropic alteration could not be explained merely as a passive consequence of tachycardia; rather, it apparently represented a primary change in the contractile state of the heart induced by glucagon. Usually the increased contractility began gradually after a latent period of 20-30 s and peaked at 1-2 min after exposure to glucagon. This gradual development of increased strength of contraction (also observed for norepinephrine in this system) presumably reflected in part the fact that hearts of older fetal mice are 1 mm or more in diameter, and the drug cannot diffuse to all the cells instantaneously.

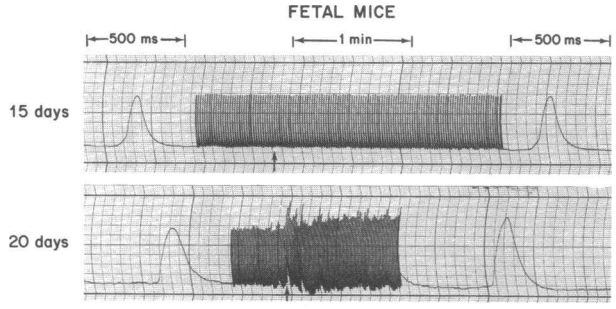

FIGURE 3 Inotropic response to glucagon in fetal mouse hearts. Isolated ventricles of 15-day (upper panel) and 20-day (lower panel) fetuses were exposed to 10 μM glucagon at the arrows, while contractions were recorded by a capacitance probe that measured motion of the heart in space (6).

The effects of glucagon on the glycogen content of early and later-fetal mouse hearts are shown in Table I. Before 17 days' gestational age there was no change in glycogen after a 30-min exposure to glucagon; after 19 days, on the other hand, cardiac glycogen levels fell by 13% under similar conditions.

In the younger mouse hearts glucagon failed to activate adenylate cyclase, just as it failed to alter beating at that age (Table II). Unlike the situation with chronotropic and inotropic responsiveness, however, further fetal maturation was not accompanied by the appearance of adenylate cyclase activation in response to the drug. Rather, even in the oldest fetal mouse hearts tested, glucagon had no effect on adenylate cyclase ac-

TABLE II

Effects of Glucagon, Epinephrine, and NaF on Fetal Myocardial Adenylate Cyclase Activity in the Mouse

	Fetal age	Adenylate cyclase	*n*	*P*
	days	*pmol/mg protein/15 min*		
Experiment 1	18			
Control		88±7 (SEM)	4	
Epinephrine, *10 μmol/liter*		226±8	4	<0.001
Glucagon, *10 μmol/liter*		83±6	4	NS
NaF, *10 mmol/liter*		755±10	2	<0.001
Experiment 2	22			
Control		296±24	4	
Epinephrine, *10 μmol/liter*		638±25	4	<0.001
Glucagon, *10 μmol/liter*		269±21	4	NS
NaF, *10 mmol/liter*		4,668±127	2	<0.001

TABLE III

Effects of Glucagon and NaF on Hepatic Adenylate Cyclase Activity in the 18-Day Fetal Mouse

	Adenylate cyclase	n	P
	pmol/mg protein/15 min		
Control	44±4 (SEM)	3	
NaF, 10 mmol/liter	567±25	2	<0.001
Glucagon, 0.2 nmol/liter	40±4	3	NS
Glucagon, 2 nmol/liter	64±4	3	<0.05
Glucagon, 20 nmol/liter	73±5	2	<0.02
Glucagon, 0.2 μmol/liter	95±2	3	<0.001
Glucagon, 2 μmol/liter	137±11	3	<0.01
Glucagon, 20 μmol/liter	170±10	3	<0.001

tivity (Table II). Results were similar at all glucagon concentrations tested (0.2 nM–20 μM).

The absence of responsiveness to glucagon was not due to an unspecific lack of adenylate cyclase responsiveness in homogenates of fetal mouse hearts, inasmuch as fluoride and epinephrine caused typical increases in enzyme activity (Table II). Furthermore, there was no evidence that fetal mouse tissues in general were unresponsive to glucagon: liver homogenates from 18-day fetuses displayed concentration-dependent increases of adenylate cyclase activity over a range of 2 nM–20 μM glucagon, with a fourfold rise at maximal concentrations (Table III).

The apparent disparity between the ability of glucagon to stimulate beating in late-fetal mouse hearts and its inability to stimulate cardiac adenylate cyclase in vitro might imply that the action of the enzyme is not important in mediating the hormone's chronotropic and inotropic effects. On the other hand, it might simply mean that the glucagon receptor is labile in this tissue and susceptible to damage or solubilization during the trauma of homogenization. To distinguish between these possibilities it seemed necessary to demonstrate the presence or absence of increased cyclic AMP formation in beating hearts that developed tachycardia and increased contractility in response to glucagon. Accordingly, hearts were frozen 30 s-3 min after exposure to glucagon and subsequently assayed for cyclic AMP. Results for all samples are shown in Table IV. Concentrations of cyclic AMP were not significantly increased after exposure to glucagon, despite the fact that beating rate and contractile strength were observed to increase in the same hearts. This was not a general unresponsiveness, since in other experiments norepinephrine caused consistent increases in tissue cyclic AMP under identical circumstances (Table IV).

As shown in Fig. 4, concentrations of cyclic AMP were unchanged after exposure to glucagon for 30 s (i.e., just as increased contractility and heart rate were becoming apparent), 1 min (when maximal ventricular responses were appearing), 2 min (by which time all hearts were responding maximally), or 3 min. In contrast, increases in cyclic AMP after norepinephrine averaged 33±6 pmol/mg after 30 s-1 min (six hearts), 23±8 pmol/mg after 2 min (five hearts), and 18±7 pmol/mg after 3 min (three hearts).

Rats. Hearts of rat fetuses aged 16 days responded to glucagon exactly as did mouse hearts of the same age; that is, they displayed no changes in beating or glycogen content. Responses of late-fetal hearts were distinctly different in the two species, however: unlike mouse hearts, hearts from fetal rats remained completely insensitive to the drug even late in gestation.

Atrial rate was not altered significantly by 10 μM glucagon at 16 days gestation (mean change, −3±2 beats/min), at 17-18 days (mean change, +1±3 beats/min), or at term (mean change, −1±3 beats/min). Similarly, neither the amplitude nor the duration of contraction, as measured from recordings of ventricular motion with the capacitance probe, was affected by glucagon at any fetal age. Glycogen content also remained unchanged (Table I).

Finally, in agreement with previous experiments from the laboratory of Clark et al. (5), adenylate cyclase activity in homogenates of fetal rat hearts was not significantly increased in the presence of glucagon, even though epinephrine and NaF exerted their usual stimulatory effect. Thus, cyclase activity in cardiac homogenates from 18-day fetal rats was 166±10 pmol/mg protein/15 min under unstimulated basal conditions, as compared to 180±7 after 10 μM glucagon ($n=4$ in each group; $P > 0.10$), while epinephrine (10 μM) increased activity to 337±10 and NaF (10 mM) increased activity to 1,064 ($P < 0.001$ for both). In 21-22 day fetal hearts, the comparable values were 244±6

TABLE IV

Effects of Glucagon and Norepinephrine on Tissue Concentrations of Cyclic AMP in Matched Groups of Spontaneously Beating Hearts of Late Fetal Mice

	n	Cyclic AMP	P
		pmol/mg wet wt	
Control	28	0.77±0.06 (SEM)	
Glucagon (10 μM)	28	0.75±0.05	
Difference from matched controls		−0.02±0.07 (−3%)	NS
Control	14	0.68±0.06	
Norepinephrine (10 μM)	14	0.94±0.06	
Difference from matched controls		+0.26±0.04 (+38%)	<0.001

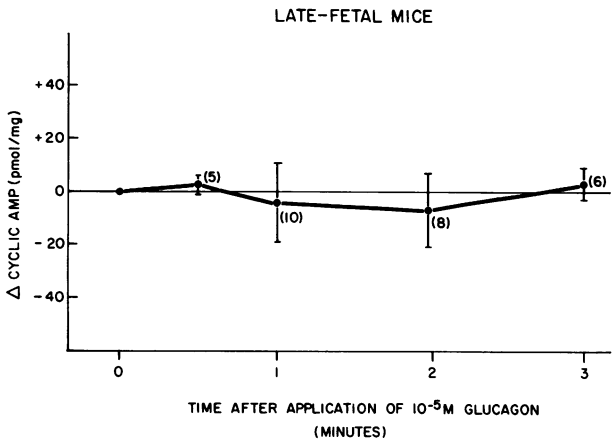

FIGURE 4 Effects of glucagon (10 μM) on cardiac cyclic AMP content at various intervals after exposure to the drug. Each point represents the mean change from matched controls, and the bars represent ±1 SEM. The number of samples tested at each interval is given in parentheses. There were no significant differences between control values and any post-glucagon values.

(control), 295±29 (glucagon, $P > 0.10$), 369±8 (epinephrine, $P < 0.001$), and 1,467 (NaF, $P < 0.001$).

DISCUSSION

The data obtained in this study demonstrate that the appearance of cardiac responsiveness to glucagon occurs at around 17-18 days' gestational age in the mouse fetus, well before term. In contrast, as suggested previously by Clark et al. (5), functional maturation of cardiac responsiveness to glucagon in the rat appears not to occur until after birth, although some laboratories have reported binding of glucagon and activation of adenylate cyclase in homogenates of fetal rat hearts (12). Previous studies from other laboratories have suggested that hearts of lambs, like those of rats, fail to respond functionally to glucagon in the fetal and newborn period (3, 4). Thus, apparent discrepancies in previous reports of glucagon's actions in fetal and newborn hearts may be simply a function of species differences. By extrapolation these findings suggest that evaluations of pharmacological maturation and differentiation of receptor sites should always be controlled for species variability, whatever the drug being tested. It is also essential to document accurately the exact age of the animal being studied. Major changes in responsiveness may occur in just a few days, so that unmodified descriptions of "fetal responsiveness" can be potentially misleading if the exact stage of gestation is not defined precisely.

A corollary of this point is that species and age differences might serve as a great aid in efforts to dissect out the mechanism of action of drugs. Thus, as Friedman and his colleagues (4) have implied, observations that the onset of inotropic and chronotropic responsiveness to glucagon coincide with the appearance of adenylate cyclase activation may lend support to (but certainly do not prove) the hypothesis that the drug's effects on beating are mediated through adenylate cyclase (13). On the other hand, it has been suggested (2) that if a species could be identified in which maturation of chronotropic and inotropic responsiveness truly precedes the ability of the enzyme to be activated by a drug, that would provide impressive evidence that the enzyme's actions do not cause the observed changes in beating.

The appearance of marked chronotropic and inotropic responsiveness to glucagon by 19-21 days' gestation in the mouse implies that membrane receptor sites and all other essential components of the cellular machinery involved with mediating the drug's actions on beating are fairly mature by that time. Simultaneously, the drug apparently cannot activate adenylate cyclase in homogenates of the cardiac tissue. This disparity might imply that the action of the enzyme is not vital to glucagon's effects on beating, or it might merely reflect an unusual lability of the enzyme in fetal hearts. The inability to demonstrate an increase in cyclic AMP levels of beating hearts lends support to the possibility that the former alternative may be correct. It also should be pointed out, however, that one cannot rule out the possibility that increases in a particular pool of cyclic AMP may have occurred that were too small for detection grossly but which could have been of critical importance for beating. Such a critically important pool could occur theoretically in a small subcellular compartment of all cells or, as suggested by the work of Venter et al. (14), possibly even in only a few cells from which an impulse for increased strength of contraction might somehow be propogated throughout the tissue without all cells actually being exposed to the hormone. Whatever the ultimate explanation may be for the observed disparity between glucagon's biochemical and contractile effects in fetal mouse hearts, the present results indicate that the interrelation between the drug's ability to augment beating and its ability in many species to activate cyclase and cause gross increases in cyclic AMP may be, at the very least, more complex than is often supposed.

Preliminary data indicate that the apparent inability of homogenates of mouse hearts to display activation of adenylate cyclase after exposure to glucagon extends even into adulthood (15), suggesting that the disparities observed may not be peculiar to the fetus but may occur at all ages in this species. It is of interest in this regard that Henry and his co-workers (16) have presented convincing evidence that the guinea pig is another species that displays a disparity between glucagon's effects on beating and its effects on adenylate

cyclase and cyclic AMP. Thus glucagon causes a maximal increase in contractility at 10 μM in the adult guinea pig heart, but has no significant effect on adenylate cyclase activity or total cyclic AMP content (16). In the adult rat heart, on the other hand, maximal inotropic changes also occur at 10 μM, but these changes, which are of a similar magnitude as in guinea pig, are accompanied by significant dose-dependent increases in cyclase activity and cyclic AMP content (5, 16, 17). Again, in the guinea pig heart as in fetal mouse heart, available data cannot yet rule out the possibility of immeasureably small but critically important increases in cyclic AMP in an isolated pool.

The present data demonstrate a disparity not only between activation of the adenylate cyclase system by glucagon and changes in beating but also between cyclase activation and glycogen depletion. We do not feel, however, that the present data are sufficient to suggest a conclusion that glucagon-induced glycogenolysis is not mediated by cyclic AMP accumulation: the amount of glycogen depletion observed was small and could easily have occurred secondarily, because of increased energy demands by the rapidly beating hearts, rather than as a primary metabolic action of glucagon. Furthermore, we have found that adult mouse hearts fail to respond to glucagon with an increase in phosphorylase activity or glycogenolysis in vivo, despite a positive chronotropic response (Clark et al., unpublished data), suggesting that the glycogenolysis observed in fetal hearts in vitro was not a direct consequence of glucagon's metabolic actions.

Finally, it should be mentioned that the concentration of glucagon required to affect the heart optimally in this study, as in other previous ones, was much higher than occurs physiologically. Thus, the present observations probably have more applicability to cardiac pharmacology and to evaluations of receptor differentiation than to normal physiology. However, the recent incursion of clinical cardiologists into this area has insured that evaluations of pharmacological glucagon responsiveness have more than theoretical relevance: as long as glucagon continues to be proposed and used clinically as an inotropic drug, the need to define the conditions under which it is effective and the mechanisms by which it acts on the heart will remain vital (18).

ACKNOWLEDGMENTS

The advice and help of Drs. G. Glick, G. S. Levey, G. H. Templeton, F. Vinicor, and J. T. Willerson are gratefully acknowledged. We wish to thank Mr. Donald Waller, Ms. Diane Kohalmi, Ms. Phyllis Morton, and Ms. Susan Jasinski for technical assistance and Ms. Sharon Smith, Sandra Weathers, and Olive Evans for assistance in preparation of the manuscript.

This work was supported by grants from the American Heart Association, NIH (HL 06296, HL 14706, AM 73-831), Indiana Heart Association, Moss Heart Fund, and the Veterans Administration.

REFERENCES

1. Hall, E. K. 1957. Acetylcholine and epinephrine effects on the embryonic rat heart. *J. Cell. Comp. Physiol.* **49**: 187–200.
2. Wildenthal, K. 1973. Maturation of responsiveness to cardioactive drugs. Differential effects of acetylcholine, norepinephrine, theophylline, tyramine, glucagon. and dibutyryl cyclic AMP on atrial rate in hearts of fetal mice. *J. Clin. Invest.* **52**: 2250–2258.
3. Downing, S. E., N. S. Talner, A. G. M. Campbell, K. H. Holloran, and H. B. Wax. 1969. Influence of sympathetic nerve stimulation on ventricular function in the newborn lamb. *Circ. Res.* **25**: 417–428.
4. Friedman, W., B. Sobel, and C. Cooper. 1969. The age dependent enhancement of cardiac contractility by glucagon: relationship to activation of the adenyl cyclase enzyme system. *Proc. Soc. Ped. Res.* **39**: 20. (Abstr.)
5. Clark, C. M., Jr., B. Beatty, and D. O. Allen. 1973. Evidence for delayed development of the glucagon receptor of adenylate cyclase in the fetal and neonatal rat heart. *J. Clin. Invest.* **52**: 1018–1025.
6. Wildenthal, K., D. R. Harrison, G. H. Templeton, and W. C. Reardon. 1973. Method for measuring the contractions of small hearts in organ culture. *Cardiovasc. Res.* **7**: 139–144.
7. Karlsson, J. 1971. Lactate and phosphagen concentrations in working muscle of man. *Acta Physiol. Scand. Suppl.* **358**: 1–72.
8. Pohl, S. L., L. Birnbaumer, and M. Rodbell. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. I. Properties. *J. Biol. Chem.* **246**: 1848–1856.
9. White, A. A., and T. V. Zenser. 1971. Separation of cyclic 3′,5′-nucleoside monophosphates from other nucleotides on aluminum oxide columns. Application to the assay of adenyl cyclase and guanyl cyclase. *Anal. Biochem.* **41**: 372–396.
10. Murad, F., V. Manganiello, and M. Vaughan. 1971. A simple, sensitive protein-binding assay for guanosine 3′:5′-monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 736–739.
11. Gilman, A. G. 1970. A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 305–312.
12. Levey, G. S., S. Martin, B. A. Levey, W. Copenhaver, and E. Ruiz. 1974. ^{125}I-glucagon binding and adenylate cyclase activation in the fetal rat heart. *Proc. Soc. Exp. Biol. Med.* **146**: 425–431.
13. Levey, G. S., and S. E. Epstein. 1969. Activation of adenyl cyclase by glucagon in cat and human heart. *Circ. Res.* **24**: 151–156.
14. Venter, J. C., J. Ross, Jr., and N. O. Kaplan. 1975. Lack of detectable change in cycle AMP during the cardiac inotropic response to isoproterenol immobilized on glass beads. *Proc. Natl. Acad. U. S. A. Sci.* **72**: 824–828.
15. Vinicor, F., D. Waller, D. Kohalmi, C. M. Clark, Jr., D. Allen, E. Yount, G. Levey, and K. Wildenthal. 1974. Studies of the mechanism of action of glucagon in the adult mouse heart. *Clin. Res.* **22**: 482A. (Abstr.)

16. Henry, P. D., J. G. Dobson, Jr., and B. E. Sobel. Dissociations between changes in myocardial cyclic adenosine monophosphate and contractility. *Circ. Res.* **36**: 392–400, 1975.
17. Mayer, S. E., D. H. Namm, and L. Rice. 1970. Effect of glucagon on cyclic 3′,5′-AMP, phosphaylase activity and contractility of heart muscle of the rat. *Circ. Res.* **26**: 225–233.
18. Glick, G. 1972. Glucagon: a perspective. *Circulation.* **45**: 513–515.