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Research Article

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Evidence for Delayed Development of the Glucagon Receptor of Adenylate Cyclase in the Fetal and Neonatal Rat Heart

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ABSTRACT The effects, in vivo, of epinephrine, glucagon, and dibutyryl cyclic adenosine 3′,5′-monophosphate (cyclic AMP) on the glycogen content of rat heart and liver and, in vitro, upon adenylate cyclase activity in homogenates of rat heart and liver were determined during the latter third of gestation and the neonatal period. Hepatic glycogen was depleted by epinephrine, glucagon, and dibutyryl cyclic adenosine 3′,5′-monophosphate, but myocardial glycogen was depleted only by epinephrine and dibutyryl cyclic AMP in the neonates. Hepatic adenylate cyclase activity was augmented by both epinephrine (10^{-5} M) and glucagon (10^{-5} M), and myocardial cyclase was increased only by epinephrine in tissue obtained from 16, 18, and 20 day fetal rats. Myocardial adenylate cyclase responsiveness to glucagon was present in tissue obtained from rats 4 wk of age and older. It is concluded that in contrast to hepatic adenylate cyclase, myocardial adenylate cyclase in the rat is not responsive to glucagon during gestation and that responsiveness to glucagon and the associated ability of glucagon to deplete myocardial glycogen do not develop until well after birth.

INTRODUCTION

Epinephrine and glucagon exhibit similar pharmacologic effects in the adult rat heart, positive chronotropic and inotropic effects and glycogenolysis (1–3). These effects appear to be secondary to the stimulation of adenylate cyclase with the consequent increase in intracellular concentration of cyclic adenosine 3′,5′-monophosphate (cyclic AMP). Evidence supporting the role of cyclic AMP in the action of these hormones has recently been reviewed (4).

There is evidence that whereas the same adenylate cyclase system in myocardial tissue is activated by epinephrine and glucagon, the receptors for the two hormones are different (5, 6). Thus, beta adrenergic blockade inhibits activation of myocardial adenylate cyclase by epinephrine but not by glucagon. In the course of studies concerned with the regulation of glycogen metabolism in the developing fetal rat heart, it was noted that epinephrine and dibutyryl cyclic AMP but not glucagon produced glycogenolysis. This observation suggested that the glucagon receptor was not operative in the fetal rat heart. In the present communication evidence is presented that adenylate cyclase activity and activation by epinephrine occur early in the course of fetal development but that activation by glucagon does not appear until several weeks after birth. For comparison, studies of hormonal activation of adenylate cyclase and hormone-induced depletion of glycogen in liver are also described.

METHODS

Animals. Pregnant rats of known gestational age were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass. and maintained on Purina Rat Chow. Gestational age of the fetuses was confirmed by their weight (7).

In vivo glycogenolysis. Neonatal rats, 48 h or less in age, were injected intraperitoneally with 0.1 ml of 0.9% sodium chloride alone or with 6 μg of glucagon, 10 μg of epinephrine or 2.5 mg of dibutyryl cyclic AMP. At specified time intervals the animals were decapitated, and blood was collected in pour plates containing a small amount of powered heparin. The hearts and livers were then excised, frozen on dry ice, weighed, and analyzed for glycogen as described below. The heparinized blood was centrifuged, and

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TABLE I

Effects at 2 h of Dibutyryl Cyclic AMP, Epinephrine, and Glucagon on Neonatal Plasma Glucose, Myocardial Glycogen Content and Hepatic Glycogen Content

Injection	N	Plasma Glucose	N	Myocardial Glycogen	N	Hepatic Glycogen
		mM		*μmol/g wet weight*	N	*μmol/g wet weight*
Physiologic saline (0.1 ml/rat)	6	4.5±0.7	5	188±12	6	88±18
Dibutyryl cyclic AMP (2.5 mg/rat)	6	2.5±1.4 (NS)	6	99±15 (<0.01)	6	1±1 (<0.001)
Epinephrine (10 μg/rat)	6	19.4±2.0 (<0.05)	6	134±19 (<0.05)	6	2±1 (<0.001)
Glucagon (6 μg/rat)	5	7.0±0.7 (<0.05)	6	205±22 (NS)	5	6±2 (<0.001)

All rats were born on the same date and studied in a single experiment less than 24 h after birth. The animals were sacrificed 2 h after injection. The *P* values (given in parenthesis) are compared to the saline-injected animals. The mean ±SEM are presented. *N* is the number of observations.

a 50 lambda sample of plasma was analyzed for glucose as described below.

Analyses. The hearts and livers were digested in a solution of hot 30% KOH and 0.5% Na_2SO_4, and glycogen was isolated by the method of Good, Kramer, and Somogyi (8). The glycogen was dissolved in 5 N H_2SO_4, and the solution was neutralized to a phenophthalein end point with 2 N NaOH. Glucose was measured by a glucose oxidase method (Glucostate, Worthington Biochemical Corp., Freehold, N. J.). The concentration of glycogen is expressed as micromoles of glucose equivalent per gram wet weight. Plasma proteins were precipitated (Somogyi), and glucose was measured as above. Protein was measured by the biuret method (9).

Adenylate cyclase assay. Maternal rats were lightly anesthesized with ether; the fetuses were delivered by cesarean section, and the hearts were rapidly excised and kept at 4°C. Neonatal and adult animals were decapitated. Because of the small size of the hearts, several were pooled before homogenization. The number of hearts per homogenate ranged from over 100 at 16 days gestation to 20 at birth. Adenylate cyclase activity was assayed in homogenates of tissue with ATP[^{14}C] as substrate as described previously (10). Briefly, samples of tissue homogenates were centrifuged at 600 × *g* at 4°C. The pellets were resuspended and incubated in Tris buffer (0.05 M, pH 7.4) containing $MgCl_2$ (1.4 μmol); ATP-8[^{14}C] (0.4 μmol, 0.5 μCi); theophylline (5.0 μmol trisodium-2-phosphoenol pyruvate (3.0 μmol); pyruvate kinase (1.5 U); [^{3}H]cyclic AMP (1 nmol, 0.1 μCi); and the designated hormone or NaF. Crystalline glucagon was stored at −20°C in 0.01 M HCl at 2.5×10^{-3} M. It was diluted with Tris buffer just before use. The final volume was 0.5 ml. After incubation in a metabolic shaker at 37° for 15 min, the reaction was terminated by boiling, and the cyclic AMP was isolated by the method of Krishna, Weiss, and Brodie (11). 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 ATP[^{14}C]. In each experiment three blanks using boiled homogenate were carried through the entire incubation and isolation procedure. ^{14}C counts from these blanks ranged from 10 to 40 cpm above background. The mean value for the blanks in all experiments was 30% of the control value and ranged from 13 to 60%. The mean

TABLE II

Effects of Dibutyryl Cyclic AMP, Epinephrine, and Glucagon on Neonatal Plasma Glucose as a Function of Time

Time	N	Saline	N	Dibutyryl cyclic AMP	N	Epinephrine	N	Glucagon
min				*2.5 mg/rat*		*10 μg/rat*		*6 μg/rat*
0	6	4.6±0.5	12	4.6±0.4	6	4.5±0.7	3	5.4±0.6
30	5	4.8±0.9 (NS)	12	6.8±0.9 (<0.05)	6	18.6±1.1 (<0.001)	5	12.6±1.9 (<0.02)
60	6	2.9±0.5 (<0.05)	12	5.7±0.6 (NS)	6	22.8±3.2 (<0.001)	6	10.8±0.6 (<0.01)
90	4	3.9±0.3 (NS)	11	6.3±1.4 (NS)	6	16.8±0.8 (<0.001)	6	11.3±2.0 (<0.05)
120	5	4.0±0.3 (NS)	9	4.8±1.0 (NS)	6	19.4±2.0 (<0.001)	5	8.9±1.4 (NS)

The time-course for each substance was determined on animals born on the same day in a single experiment. The neonates were studied within 48 h of birth. Results are expressed as mM. *N* is the number of observations. The mean ±SEM are presented. The numbers in parenthesis are *P* values as compared to zero time.

TABLE III

Effects of Dibutyryl Cyclic AMP, Epinephrine, and Glucagon on Neonatal Myocardial Glycogen Content as a Function of Time

Time	N	Physiologic saline	N	Dibutyryl cyclic AMP	N	Epinephrine	N	Glucagon
min		*0.1 ml/rat*		*2.5 mg/rat*		*10 μg/rat*		*6 μg/rat*
				μmol glycogen/g wet wt				
0	6	87±11	3	83±6	4	82±6	6	65±6
30	6	91±5	4	55±11	6	52±7	5	99±13
		(NS)		(NS)		(<0.02)		(<0.05)
60	6	92±7	4	57±7	5	59±7	6	92±10
		(NS)		(<0.05)		(<0.05)		(<0.05)
90	6	100±7	4	62±9	5	69±6	6	65±6
		(NS)		(NS)		(NS)		(NS)
120	6	113±12	4	53±7	5	79±5	6	83±9
		(NS)		(<0.02)		(NS)		(NS)

The time-course for each substance was determined on animals born on the same day in a single experiment. The differing zero time values reflect the variability in base line glycogen after birth. The neonates were studied within 48 h of birth. Results are expressed as μmoles of glucose equivalent. *N* is the number of observations. The mean ±SEM are presented. The numbers in parenthesis are *P* values as compared to zero time.

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 pmoles cyclic AMP formed/milligram protein per 15 min.

Statistical analyses were performed with Student's *t* test.

RESULTS

Effects of epinephrine, glucagon, and dibutyryl cyclic AMP on plasma glucose. At 2 h both epinephrine and glucagon significantly increased plasma glucose above the saline-injected control, whereas dibutyryl cyclic AMP did not (Table I). The effects of the injection of these substances on plasma glucose as a function of time are summarized in Table II. The plasma glucose had fallen significantly by 60 min in the saline-injected controls. It subsequently returned toward basal values. Dibutyryl cyclic AMP, glucagon, and epinephrine each caused a significant increase in plasma glucose by 30 min. The increase caused by dibutyryl cyclic AMP was quantitatively the smallest and was no longer significant by 60 min and thereafter (Table II). Epinephrine caused quantitatively the greatest increase in plasma glucose, and glucagon was intermediate in its effect.

Effects of epinephrine, glucagon, and dibutyryl cyclic AMP on glycogenolysis. Glycogen content of both heart and liver vary widely during the neonatal period (12, 13). In order to minimize the effects of these changes in basal glycogen content, two types of experiments were performed. In one series of experiments all

TABLE IV

Effects of Dibutyryl Cyclic AMP, Epinephrine, and Glucagon on Neonatal Hepatic Glycogen Content as a Function of Time

Time	N	Physiologic saline	N	Dibutyryl cyclic AMP	N	Epinephrine	N	Glucagon
min		*0.1 ml/rat*		*2.5 mg/rat*		*10 μg/rat*		*6 μg/rat*
				μmol glycogen/g wet wt				
0	6	105±35	4	162±9	6	70±5	4	154±17
30	6	109±43	4	85±20	6	14±2	5	155±23
		(NS)		(<0.02)		(<0.001)		(NS)
60	6	106±45	4	45±3	6	17±3	6	91±25
		(NS)		(<0.001)		(<0.001)		(NS)
90	6	108±28	4	32±2	6	13±5	5	32±14
		(NS)		(<0.001)		(<0.001)		(<0.001)
120	6	109±29	4	64±11	6	7±2	6	10±2
		(NS)		(<0.001)		(<0.001)		(<0.001)

The time-course for each substance was determined on animals born on the same day in a single experiment. The differing zero time values reflect the variability in base line glycogen after birth. The neonates were studied within 48 h of birth. Results are expressed as μmoles of glucose equivalent. *N* is the number of observations. The mean ±SEM are presented. The numbers in parenthesis are *P* values as compared to zero time.

TABLE V

Effects of Epinephrine, Glucagon, and NaF on Fetal Myocardial Adenylate Cyclase

	Fetal age	Adenylate cyclase	N	P value*
	days	*pmol/mg protein per 15 min*		
Experiment 1	16			
Control		429±17	2	
Epinephrine, 10^{-5} mol/liter		72.3±6	2	<0.001
NaF, 10^{-2} mol/liter		1,982	1	
Experiment 2				
Control		240±7	3	
Epinephrine, 10^{-5} mol/liter		322±12	5	<0.001
NaF, 10^{-2} mol/liter		1,693	1	
Experiment 3				
Control		678±27	4	
Epinephrine, 10^{-5} mol/liter		930±22	7	<0.001
NaF, 10^{-2} mol/liter		4,003	1	
Experiment 1	18			
Control		204±8	2	
Epinephrine, 10^{-5} mol/liter		491±3	2	<0.001
NaF, 10^{-2} mol/liter		1,460±3	2	<0.001
Experiment 2				
Control		157±5	3	
Glucagon, 10^{-5} mol/liter		147±7	5	NS
NaF, 10^{-2} mol/liter		865	1	
Experiment 3				
Control		166±10	4	
Epinephrine, 10^{-5} mol/liter		337±10	5	<0.001
Glucagon, 10^{-5} mol/liter		180±7	5	NS
NaF, 10^{-2} mol/liter		1,604	1	
Experiment 1	21			
Control		160±5	3	
Epinephrine, 10^{-5} mol/liter		282±18	3	<0.01
NaF, 10^{-2} mol/liter		1,300	1	
Experiment 2				
Control		93±1	3	
Glucagon, 10^{-5} mol/liter		91±3	4	NS
NaF, 10^{-2} mol/liter		830	1	

N is number of observations per homogenate. The number of hearts per homogenate ranged from 120 at 16 days to 25 at 21 days.

Mean ±SEM are presented.

* As compared to control.

injections were performed in a single experiment on animals born on the same day. In this series of experiments, the animals were killed 2 h after the injection. A representative experiment is summarized in Table I. At 2 h, dibutyryl cyclic AMP, epinephrine, and glucagon each lowered hepatic glycogen significantly as compared to the saline-injected controls. Whereas dibutyryl cyclic AMP and epinephrine also caused significant mean decreases in myocardial glycogen content, glucagon did not (Table I).

In the second series of experiments, the time-course of myocardial and hepatic glycogen after injection was studied. In this series of experiments, the animals in each treatment group were studied on the same day. Representative experiments are summarized in Tables III and IV. Intraperitoneal administration of 2.5 mg

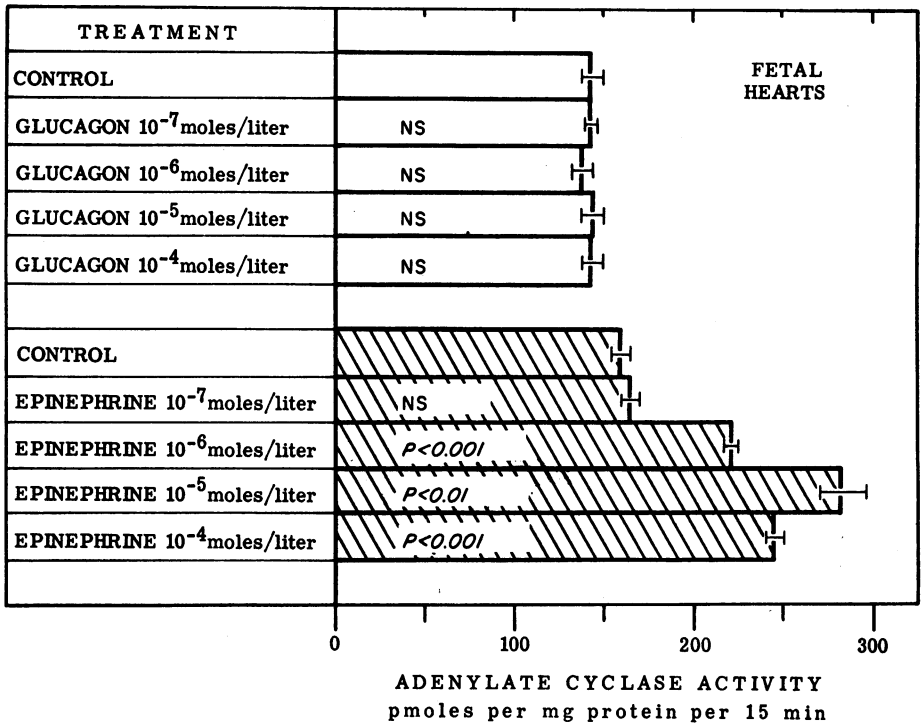

FIGURE 1 Effects of epinephrine and glucagon on adenylate cyclase activity from fetal rat hearts. Each hormone was studied at different concentrations on homogenates from the same animals. The mean (boxes) and SEM (horizontal bars) of six observations are presented.

of dibutyryl cyclic AMP or 10 μg of epinephrine was followed by significant mean decreases in cardiac glycogen by 60 min (Table III). In contrast, glucagon injection resulted in significant mean increases in myocardial glycogen content. This suggested unresponsiveness of myocardial adenylate cyclase to glucagon which in conjunction with mobilization of hepatic glycogen might result in increased myocardial glycogen. Each of the three substances caused hepatic glycogenolysis (Table IV).

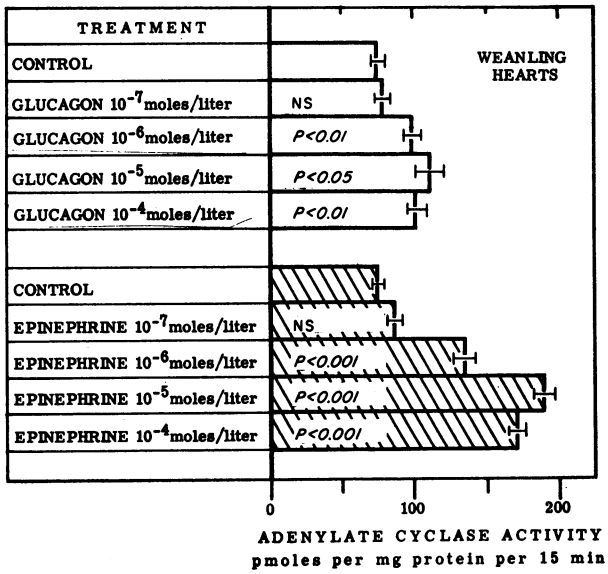

FIGURE 2 Effects of epinephrine and glucagon on adenylate cyclase activity from weanling rat hearts. Each hormone was studied at different concentrations on homogenates from the same animals. The mean (boxes) and SEM (horizontal bars) of six observations are presented.

Effects of epinephrine and glucagon on adenylate cyclase activity. Representative experiments summarizing the effects of glucagon and epinephrine on adenylate cyclase activity in fetal rat hearts on the 16th, 18th, and 21st days of a 22½ day gestation are depicted in Table V and Fig. 1. No stimulating effect of glucagon was detected. Epinephrine 10^{-5} mol/liter, stimulated adenylate cyclase at all ages; significant mean increases were seen at concentrations of 10^{-6} mol/liter and above.

However, both epinephrine and glucagon stimulated adenylate cyclase in heart tissue homogenates from weanling rats (Fig. 2) at concentrations of 10^{-6} mol/liter and above. In Table VI are summarized representative experiments depicting the effects of epinephrine and glucagon on myocardial adenylate cyclase from neonatal, weanling, and adult rats. Epinephrine, 10^{-5} mol/liter, stimulated adenylate cyclase at all ages studied. Myocardial adenylate cyclase responsiveness to gluca-

TABLE VI

Effects of Epinephrine, Glucagon, and NaF on Neonatal, Weanling, and Adult Myocardial Adenylate Cyclase

	Age	Adenylate cyclase	N	P value*
		pmol/mg protein per 15 min		
Experiment 1	Newborn			
Control	(0–7 days)	138±2	3	
Epinephrine, 10^{-5} mol/liter		228±13	3	<0.001
Glucagon, 10^{-5} mol/liter		131±8	3	NS
NaF, 10^{-2} mol/liter		980	3	
Experiment 2				
Control		104±8	3	
Glucagon, 10^{-5} mol/liter		94±4	5	NS
NaF, 10^{-2} mol/liter		1,261	1	
Experiment 3				
Control		152±6	5	
Glucagon, 10^{-5} mol/liter		166±14	6	NS
Experiment 1	Weanling			
Control	(28 days)	75±4	3	
Epinephrine, 10^{-5} mol/liter		191±1	3	<0.001
Glucagon, 10^{-5} mol/liter		112±10	3	<0.05
NaF, 10^{-2} mol/liter		812	1	
Experiment 2				
Control		68±3	3	
Glucagon, 10^{-5} mol/liter		77±2	5	<0.05
Experiment 1	Adult			
Control		93±4	3	
Epinephrine, 10^{-5} mol/liter		229±10	3	<0.001
NaF, 10^{-2} mol/liter		780	1	
Experiment 2				
Control		73±2	3	
Glucagon, 10^{-5} mol/liter		100±6	5	<0.01
NaF, 10^{-2} mol/liter		972	1	
Experiment 3				
Control		178±8	4	
Glucagon, 10^{-5} mol/liter		236±7	4	<0.001

N is number of observations per homogenate. The number of hearts per homogenate ranged from 15 in the neonate to 2 in the adults.

Mean ±SEM are presented.

* As compared to control.

gon, 10^{-5} mol/liter, was not present in neonatal tissue but had appeared by the 4th wk after birth. Adenylate cyclase activation by epinephrine was consistently greater than that produced by glucagon. In all studies in which the effects of NaF, 10^{-2} mol/liter, were examined, significant augmentation occurred (Tables V and VI).

Hepatic adenylate cyclase activity in tissues from 16, 18, and 20 day old fetal rats was significantly increased by epinephrine, 10^{-4} mol/liter, glucagon, 10^{-4} mol/liter, and NaF, 10^{-2} mol/liter (Table VII).

DISCUSSION

Glucagon, epinephrine, and dibutyryl cyclic AMP given to the neonatal rat in vivo resulted in increased glycogenolysis in the liver, whereas epinephrine and dibutyryl cyclic AMP but not glucagon were effective in the heart. The opposing effects of epinephrine and glucagon

TABLE VII

Effects of Epinephrine, Glucagon, and NaF on Fetal Hepatic Adenylate Cyclase

	Fetal age	Adenylate cyclase	N	P value*
	days	*pmol/mg protein per 15 min*		
Control	16	112±7	3	
Epinephrine, 10^{-4} mol/liter		253±5	5	<0.001
Glucagon, 10^{-4} mol/liter		349±8	5	<0.001
NaF, 10^{-2} mol/liter		2,387	1	
Control	18	173±4	4	
Epinephrine, 10^{-5} mol/liter		508±8	5	<0.001
Glucagon, 10^{-5} mol/liter		413±13	5	<0.001
Control	20	401±9	4	
Epinephrine, 10^{-4} mol/liter		1,253±18	4	<0.001
Glucagon, 10^{-4} mol/liter		810±9	4	<0.001
NaF, 10^{-2} mol/liter		1,738±34	4	<0.001

N is number of observations per homogenate. The number of livers per homogenate ranged from 100 at 16 days to 30 at 20 days.

Mean ±SEM are presented.

* As compared to control.

on myocardial glycogen content could have resulted from pharmacologic effects of the hormones district from their effect on myocardial adenylate cyclase. If, for example, epinephrine inhibited while glucagon stimulated pancreatic insulin release in these animals as they do in vitro (14), then the resultant insulin-induced myocardial glycogen synthesis (13) could have obscured the glycolytic effect of glucagon. This interpretation seems unlikely, however, since insulin release would also have been expected to obscure the effect of glucagon on hepatic glycogen. In addition, the dibutyryl cyclic AMP would also have been expected to cause insulin release (14) and should have had effects on myocardial glycogen similar to glucagon. This was not the case. A more likely explanation for these results is that the glycagon receptor on the adenylate cyclase enzyme system either had not developed or was inoperative in the neonatal rat heart, whereas the epinephrine receptor was already functional.

In vitro both epinephrine and glucagon stimulated adenylate cyclase activity in hearts from weanling and adult rats. Whereas the concentration at which glucagon stimulated adenylate cyclase in our studies is similar to that observed by Murad and Vaughn (3), the magnitude of increase in adenylate cyclase activity produced by glucagon is less. There are several differences between our studies and those of Murad and Vaughan which may explain these differences. Whereas we used a resuspended $600 \times g$ pellet, they used a partially purified membrane preparation. It is possible that a specific inhibitor of glucagon activation of adenylate cyclase is present in the rat heart similar to that previously described in the rat liver (15). In this case purification of the membrane would remove the inhibitor and, thus, increase the activation of adenylate cyclase by glucagon in the adult rat heart. We were reluctant to carry out purifications since liability of fetal myocardial glucagon receptor has been demonstrated in fetal tissues (16). In these studies performed in the lamb, Friedman, Sobel, and Cooper (16) showed that partial purification of fetal myocardial membranes abolished activation of adenylate cyclase by glucagon. We therefore chose to manipulate the membranes minimally. Under the experimentally conditions described, epinephrine significantly increased myocardial adenylate cyclase activity in tissues from both fetal and neonatal animals, and epinephrine and glucagon each stimulated adenylate cyclase activity in hearts from weanling and adult rats.

In contrast, glucagon did not activate the adenylate cyclase in hearts from either fetal or neonatal rats.

The finding that in fetal rat heart both glycogenolysis and adenylate cyclase activity can be increased by epinephrine suggest that the epinephrine receptor is operative in this fetal tissue. The failure of glucagon to augment myocardial glycogenolysis in vivo and adenylate cyclase activity in vitro in the fetus and newborn suggests that the glucagon receptor is not operative in these tissues. These studies do not reveal the precise defects in the glucagon receptor in the fetal myocardium. Failure of the development of the receptor until after birth or the presence of an inhibitor are equally possible.

The results from fetal rat liver suggest that both glycogenolysis and adenylate cyclase activity can be

increased by both epinephrine and by glucagon indicating that both receptors are operative in this fetal tissue. Augmentation of fetal hepatic adenylate cyclase by epinephrine and glucagon has been observed previously by other investigators (17, 18).

Previously it has been suggested that epinephrine and glucagon activate a single myocardial adenylate cyclase enzyme by interacting with separate receptors (4–6). The present results indicate that the glucagon receptor for adenylate cyclase in the rat does not become operative until after the appearance of the enzyme and beta adrenergic receptor. Also, it has been observed experimentally in cats that the ability of glucagon but not epinephrine to stimulate myocardial adenylate cyclase activity is lost during chronic heart failure (4). Thus, in addition to its developmental distinctiveness, the glucagon receptor may be altered or inactivated apparently without any changes in the enzyme or beta adrenergic receptor.

A final question raised by these studies is whether differential development of glucagon activation of adenylate cyclase serves a physiologically useful purpose. A definite physiologic role of glucagon on the myocardium is not presently substantiated. Most studies including the present one have utilized pharmacologic doses of glucagon to obtain myocardial effects. These results, however, have not established a physiologic role for glucagon on the myocardium. Mayer, Namm, and Rice (2) have observed effects on contractile amplitude in the isolated perfused rat heart at glucagon concentrations as low as 10^{-10}M which is in the physiologic range for the neonatal rats (19). It also has been observed that there are unequivocal increases in plasma glucagon in the rat at birth (19). These data suggest that glucagon could serve to mobilize hepatic glycogen stores in the neonatal period and, thus, to stabilize plasma glucose at birth.

Further studies to determine the physiologic role of glucagon in the neonatal period appear warranted.

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