Effects of Glucagon on Adenosine 3′,5′-Monophosphate and Guanosine 3′,5′-Monophosphate in Human Plasma and Urine

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ABSTRACT Glucagon, infused intravenously into fasting, well-hydrated, normal men in doses of 25–200 ng/kg per min, induced up to 30-fold increases in both plasma and urinary cyclic AMP. Cyclic GMP levels were unaffected by glucagon. Simultaneous cyclic AMP and inulin clearance studies demonstrated that the glucagon-induced increase in urinary cyclic AMP was entirely due to glomerular filtration of the elevated plasma levels of the nucleotide.

The cyclic AMP response to glucagon was not mediated by parathyroid hormone or epinephrine, and trypsin-treated glucagon was completely inactive.

The perfused rat liver released cyclic AMP into the perfusate in response to glucagon, indicating that the liver is a possible source of the cyclic AMP entering the extracellular fluids in response to glucagon in vivo.

INTRODUCTION

Adenosine 3′,5′-monophosphate (cyclic AMP) plays its principal physiologic role in mammalian tissues as an intracellular mediator of the actions of a number of hormones (1). However, it is now apparent that some of the nucleotide escapes from its cells of origin into the extracellular fluids, including plasma (2–4), urine (2, 5), and cerebrospinal fluid (2, 3).

Antidiuretic hormone (6) and parathyroid hormone (7) were the first agents reported to alter urinary cyclic AMP, and each of the initial publications (6, 7) associating these two hormones with increased cyclic AMP excretion suggested that the hormone under study was the major determinant influencing urinary levels of the nucleotide. Since both parathyroid hormone and antidiuretic hormone have been shown to interact with renal adenyl cyclase systems (8), an implication of these studies was that most, if not all, of cyclic AMP in urine was formed by the kidney. However, we have found (3) that significant amounts of cyclic AMP occur in human plasma and that approximately two-thirds of the nucleotide excreted by normal human subjects under basal conditions is derived from plasma by glomerular filtration. These findings suggested that the major determinants of extracellular cyclic AMP might be agents which affect extrarenal tissues.

Glucagon has recently been shown to increase cyclic AMP excretion by rats (9). Several possible mechanisms could account for the increased urinary cyclic AMP produced by glucagon administration. First, glucagon has been shown to stimulate cyclic AMP formation by liver (10–13), cardiac muscle (14), adipose tissue (15), and perhaps by the pancreatic islets (16); and some of the cyclic AMP formed in these tissues might be released into the extracellular fluids, enter the circulation, and be excreted by glomerular filtration. Second, glucagon has been reported to exert a number of effects on renal function (17), and it is conceivable that glucagon might stimulate a renal adenyl cyclase system resulting in a direct release of cyclic AMP into the urine. Additionally, the elevation in glomerular filtration rate observed in response to glucagon administration (18) might contribute an increase in cyclic AMP excretion simply on the basis of an increase in filtered load of the nucleotide. Third, large doses of glucagon have been reported to produce hypocalcemia (19, 20) which could lead to increased circulating titers of parathyroid hormone, an agent known to increase cyclic AMP excretion (4, 7, 21).

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Finally, large doses of glucagon in man and laboratory animals have been shown to elevate circulating levels of the catecholamines (22–25), hormones which stimulate adenyl cyclase systems in a wide variety of tissues (1).

The present studies were undertaken in order (*a*) to define the effects of glucagon on plasma and urinary cyclic AMP, (*b*) to determine whether these effects were mediated by other hormones, and (*c*) to determine whether the liver, a known target tissue of glucagon, responds to this hormone with increased release of cyclic AMP into extracellular fluids.

Guanosine 3′,5′-monophosphate (cyclic GMP), which was first identified in urine (5, 9, 26, 27), is the only cyclic nucleotide other than cyclic AMP known to occur in nature. The biological role of cyclic GMP remains to be established. The plasma has been identified as the source of virtually all of the cyclic GMP excreted by normal human subjects under basal conditions (3), and thus, the extracellular levels of the nucleotide presumably reflect formation of the compound in extrarenal tissues. The effects of glucagon and other agents on plasma and urinary cyclic GMP were assessed in the present studies.

METHODS

Except for the following details, the methods employed in the present studies were as described in a companion publication (3).

Materials

Glucagon (control 2 EX68A) was generously provided by Doctors M. A. Root and J. A. Galloway of the Lilly Research Laboratories (Indianapolis, Ind.). Epinephrine (lot HJ 110) was purchased from Parke, Davis & Co. (Detroit, Mich.). Female thyroparathyroidectomized rats and normal rats of the same age were obtained from Hormone Assay Laboratories (Chicago, Ill.). Bovine serum albumin and trypsin (twice crystallized) were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). Soybean trypsin inhibitor was obtained from Worthington Biochemical Corporation (Freehold, N. J.).

Subjects and procedures

Volunteers selected were healthy, nonobese, nonhospitalized males between the ages of 21 and 34. All subjects had fasting blood glucose levels of less than 100 mg/100 ml. At least 240 g of dietary carbohydrate were ingested daily for 3 days before testing.

Glucagon was dissolved in its commercial diluent, diluted in isotonic saline, and administered by a constant flow infusion pump. Epinephrine was diluted in isotonic saline containing ascorbic acid (2.5 mg/ml) to protect against oxidation and was administered by gravity flow.

Rat liver perfusion studies

Livers from fed rats (100–120 g) were perfused in situ[1] at 37°C with 50 ml of recirculating medium by the technique of Mortimore (28) as modified by Exton and Park (29). The medium consisted of Krebs-Hanseleit bicarbonate buffer (pH 7.4) containing 3% bovine serum albumin and 20% washed bovine erythrocytes. Infusions (5 μl/min) of glucagon (0.005, 0.01, 0.02, 0.05, 0.5, and 5.0 μg/ml) or saline were begun after 30 min of preperfusion and continued for 30 min. Samples of liver were then removed, frozen within 5 sec in aluminum tongs (30) precooled in liquid N_2, and stored at −70°C until analysis. The medium was pumped out of the perfusion apparatus and processed for cyclic AMP content as described below for samples of human blood.

The livers were extracted for cyclic AMP analysis by a slight modification of the method of Lowry, Passonneau, Hasselberger, and Schulz (31); 0.005 μCi of cyclic AMP-^{3}H was added to the frozen powder for recovery determinations. The protein-free extracts from the liver samples were sequentially chromatographed over 60 × 0.60 cm and then 30 × 0.60 cm Dowex-50 columns, with a lyophilization step between the column steps.

Purification of samples for cyclic nucleotide assay

Urine. After addition of 0.01 μCi of cyclic GMP-^{3}H and/or cyclic AMP-^{3}H for the purpose of correcting for cyclic nucleotide recovery through the purification procedures, aliquots of urine containing approximately 2 mg of creatinine were chromatographed as described previously (3), except that both cyclic GMP and cyclic AMP fractions were usually collected from each urine specimen.

Plasma. A convenient means of obtaining cyclic nucleotide recoveries from plasma and avoiding the potential source of error posed by metabolism has been adopted for routine use in nonisotopic studies. The samples of whole blood were added to chilled tubes containing 0.005 μCi of cyclic AMP-^{3}H and/or cyclic GMP-^{3}H, well mixed, and handled rapidly as described previously (3), except that the plasma aliquots were pipetted directly into $HClO_4$. The protein-free $HClO_4$ extracts could be stored at 4°C for at least 3 months without detectable loss of the nucleotides. Calculation of the cyclic nucleotide-^{3}H recoveries involved use of the volume of blood withdrawn, the hematocrit, and the volume of the plasma aliquot taken for cyclic nucleotide determinations. Distribution of the tracers into the cellular elements of blood was not detectable. Pilot studies have demonstrated excellent agreement between the present method and the method described previously (3).

Ancillary analyses[2]

Plasma glucose was analyzed with a Technicon Auto Analyzer (Technicon Co., Tarrytown, N. Y.) using the ferricyanide method. Glycerol assays performed by the method of Wieland (32) and lactate assays performed by the method of Hohorst (33) were accomplished in the same reaction mixture. Urinary catecholamine levels were determined by use of a modification of the method of von Euler and Floding (34). Plasma insulin concentrations were measured by radioimmunoassay (35, 36).

[1] We are indebted to Doctors J. Exton and S. Lewis for carrying out the rat liver perfusions and hepatic glucose production determinations.

[2] We wish to thank Mr. D. Island for the catecholamine determinations and Dr. O. Crofford for the insulin determinations.

RESULTS

The effects of glucagon on plasma and urinary levels of cyclic AMP and cyclic GMP. The intravenous infusion of glucagon, 200 ng/kg per min, into a fasting, well-hydrated, normal subject caused striking increases in the plasma content and urinary excretion of cyclic AMP (Fig. 1). The pattern of the urinary cyclic AMP response curve was very similar to that of the plasma cyclic AMP response curve.

The dose-response relationship between glucagon and plasma and urinary cyclic AMP content was investigated by serial infusions of the hormone into a single subject at approximately weekly intervals. Greater than 2-fold elevations in cyclic AMP were seen with as little as 50 ng of glucagon per kg per min, and the largest dose tested in this subject brought about approximately 30-fold increases over control levels (Fig. 2). The dose-related increments in the excretion of cyclic AMP in these studies mirrored the elevated concentrations of the nucleotide in plasma (Fig. 2). Increases in plasma glucose and immunoreactive insulin were seen at all doses of glucagon tested.

The relationship between plasma content and urinary excretion of cyclic AMP is developed more fully in Table I which contains data on the simultaneous renal clearances of cyclic AMP and inulin. During the control periods, the clearance of cyclic AMP exceeded inulin clearance by a factor of about 1.3. Thus, the nephrogenous component (3) was small in these two subjects, averaging approximately 23% of the total amount of cyclic AMP excreted. During glucagon infusion, the quantity of nephrogenous cyclic AMP was negligible in comparison to the greatly elevated filtered load of the nucleotide; and the renal clearance of cyclic AMP approximated inulin clearance, except in those clearance periods in which the cyclic AMP levels were undergoing rapid change. As

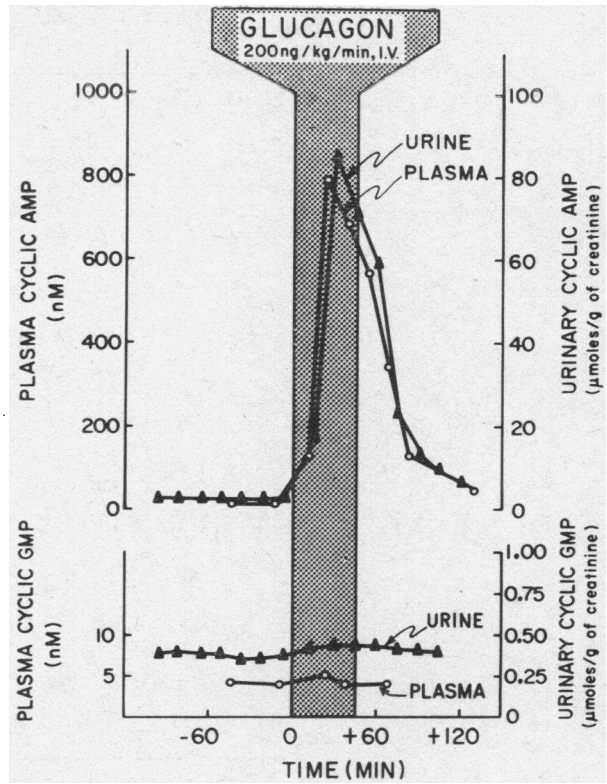

FIGURE 1 The effects of an intravenous infusion of glucagon on plasma and urinary cyclic AMP and cyclic GMP in a fasting, well hydrated, normal subject. Time is expressed in relation to the beginning of the glucagon infusion.

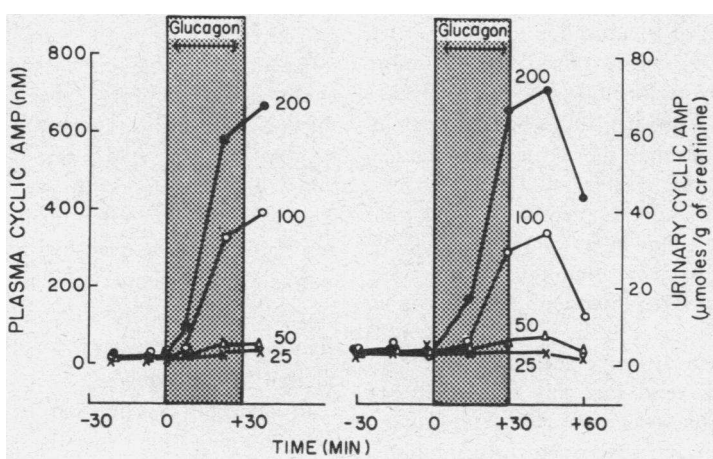

FIGURE 2 The dose-response relationship between infused glucagon and plasma and urinary levels of cyclic AMP in a fasting, well-hydrated, normal subject. The doses are indicated beside each curve in nanograms per kilogram per minute. Time is expressed in relation to the beginning of the glucagon infusion.

TABLE I

Comparison of the Renal Clearances on Inulin and Cyclic

Subject	Dosage of glucagon	Clearance period	Time*	Creatinine excretion	Urinary cyclic AMP
	ng/kg per min		*min*	*mg/min*	*nmoles/min*
W. C.	200	I	−45 to −30	1.34	3.14
		II	−30 to −15	1.21	3.08
		III	−15 to 0	1.32	3.30
	0 to +45 min	IV	0 to +15	1.28	23.0
		V	+15 to +30	1.28	107.8
		VI	+30 to +45	1.43	99.1
		VII	+45 to +60	1.29	74.9
		VIII	+60 to +75	1.31	30.4
		IX	+75 to +90	1.31	16.68
H. H.	75	I	−45 to −30	1.69	2.74
		II	−30 to −15	1.69	2.72
		III	−15 to 0	1.58	2.50
	0 to +75 min	IV	0 to +15	1.89	4.58
		V	+15 to +30	1.61	19.0
		VI	+30 to +45	1.72	70.0
		VII	+45 to +60	1.49	61.0
		VIII	+60 to +75	1.61	41.8

* Time is expressed in relation to the beginning of the hormone infusion ($t = 0$).

‡ Blood samples were drawn at the midpoints of the clearance periods.

noted by others (18), glucagon induced a slight increase in inulin clearance. Thus, the increase in cyclic AMP excretion elicited by glucagon could be accounted for by glomerular filtration of the elevated plasma levels of the nucleotide.

No appreciable change in plasma or urinary cyclic GMP was seen in response to glucagon (Fig. 1), and the clearance of cyclic GMP approximated that of inulin during both control and experimental periods (Table I).

Glucagon inactivation. Experiments were carried out in rats to verify the point that the active principle in these studies was glucagon. Glucagon (0.25 mg/ml) was inactivated by incubation with trypsin (1.0 mg/ml) for 2 hr at 37°C, and the digestion was terminated by the addition of soybean trypsin inhibitor (3 mg/ml). Rats receiving subcutaneous injections of glucagon (25 μg/100 g at 0, 4, and 8 hr of 12-hr urine collections) exhibited a 6-fold increase in cyclic AMP excretion as compared to control rats receiving only diluent injections. Trypsin-treated glucagon was completely inactive. A mixture of trypsin and soybean trypsin inhibitor had no influence on cyclic AMP excretion.

Parathyroid hormone. The possible interrelationship between glucagon and parathyroid hormone (19, 20) and the well-documented effects of the latter hormone on urinary cyclic AMP (4, 7, 21) prompted experiments designed to determine whether or not parathyroid hormone was involved in the glucagon-induced cyclic AMP response.

Fig. 3 illustrates results of experiments in which cyclic AMP excretion was studied in control and glucagon-injected groups of normal and thyroparathyroidectomized rats. The animals lacking parathyroid glands excreted only slightly lower quantities of cyclic AMP than did the intact controls and demonstrated at least as great a cyclic AMP response to subcutaneous injections of glucagon as did the normal rats. In a single study, the intravenous infusion of glucagon (150 ng/kg per min) into a well-hydrated, parathyroidectomized man elicited large increases in plasma and urinary levels of cyclic AMP. Thus, the integrity of the parathyroid glands is not necessary for the cyclic AMP response elicited by glucagon.

In addition, as detailed in an accompanying paper (4), we have found that the pattern of the cyclic AMP response to parathyroid hormone differs markedly from that described here for glucagon. That is, in contrast to glucagon, parathyroid hormone causes a great increase in nephrogenous cyclic AMP (4).

AMP before, during, and after Glucagon Infusion in Man

Plasma cyclic AMP‡	Clearance cyclic AMP	Clearance inulin	Clearance ratio: cyclic AMP / inulin	Urinary cyclic GMP	Plasma cyclic GMP	Clearance cyclic GMP	Clearance ratio: cyclic GMP / inulin
nmoles/liter	*ml/min*	*ml/min*		*nmoles/min*	*nmoles/liter*	*ml/min*	
20.8	150	119	1.26	0.45	4.5	101	0.85
23.9	129	110	1.17	0.43	4.0	107	0.98
		119		0.51			
121	190	125	1.52	0.53			
798	135	119	1.13	0.55	5.1	108	0.91
689	144	139	1.03	0.63	4.0	158	1.14
578	129	128	1.01	0.56			
342	89	132	0.67	0.55	4.2	130	0.99
137	122	133	0.92	0.54			
17.5	156	121	1.29				
18.6	146	118	1.25				
17.9	140	112	1.26				
		137					
		140					
490	143	150	0.95				
428	143	139	1.03				
328	156	146	1.07				

Cyclic GMP excretion by the rats (Fig. 3) was unaffected by any of the variables studied in these experiments.

Catecholamines. The reports that large doses of glucagon release catecholamines into the circulation (22–25) and the effects of these hormones on cyclic AMP formation in many tissues (1) prompted consideration of a possible role of the catecholamines in the cyclic AMP response to glucagon. An intravenous infusion of a rather large dose of epinephrine in a normal subject caused modest increases in plasma and urinary levels of cyclic AMP (Fig. 4), and further studies have shown the effects of epinephrine on cyclic AMP levels to be dose related. The epinephrine-induced increments in urinary cyclic AMP were entirely attributable to increases in filtered load of the nucleotide; nephrogenous cyclic AMP was, if anything, decreased (five experiments).

The effects of separate infusions of glucagon and epinephrine into the same subject are depicted in Fig. 5. Characteristic effects of epinephrine on the cardiovascular system, plasma glycerol, lactate and insulin, and urinary catecholamines were observed. In contrast, glucagon did not produce detectable sympathomimetic effects or an increase in catecholamine excretion, yet glucagon was associated with a far greater cyclic AMP response than epinephrine. Therefore, nothing was found to indicate that epinephrine plays a role in the glucagon-induced cyclic AMP response.

The epinephrine infusions led to small elevations in plasma and urinary cyclic GMP (five studies). The clearance of cyclic GMP approximated inulin clearance during both control and experimental clearance periods (Fig. 4).

Insulin and glucose. The marked hyperglycemia and hyperinsulinemia which accompanied the glucagon infusions prompted consideration of what effect, if any, these factors might have on plasma and urinary cyclic AMP. Fig. 6 illustrates a study in which oral glucose induced large increases in plasma glucose and insulin without causing significant changes in urinary cyclic AMP. These findings have been confirmed in other studies, including experiments in which glucose was administered intravenously.

A slight increase in cyclic AMP excretion occurred during the hypoglycemic phase of each of four glucose tolerance experiments. Greater than a 2-fold increase in cyclic AMP excretion was observed during the hypoglycemia of a single insulin tolerance test in a normal subject.

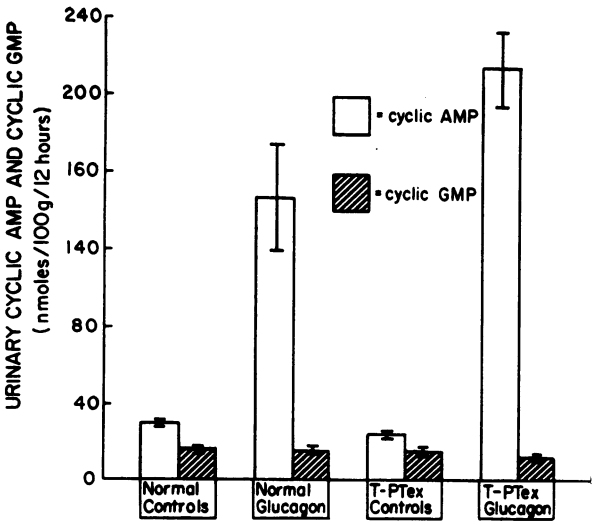

FIGURE 3 The effect of subcutaneous injections of glucagon on cyclic AMP and cyclic GMP excretion by normal and thyroparathyroidectomized (T-PTex) rats. The T-PTex rats were maintained on a 1% calcium lactate solution in place of drinking water and daily intraperitoneal injections of thyroxine (5 μg/100 g). During study, all rats were housed in pairs in metabolic cages and were fed only a 10% dextrose and 0.5% calcium lactate solution. The urine issuing from each cage was collected in a vessel over dry ice for 12 hr. Four pairs of animals were studied for each condition, and the injected groups received glucagon (25 μg/100 g) in 0.9% saline at 0, 4, and 8 hr. Each bar represents the mean of four observations, and the vertical lines represent 2 SEM. Results are expressed as nmoles of cyclic nucleotide excreted per 100 g rat weight per 12 hr. The differences between the control groups of normal and T-PTex rats and between the glucagon-injected groups of normal and T-PTex rats were not statistically significant by the Student's t distribution ($P > 0.10$).

Cyclic GMP excretion was uninfluenced by the hyperglycemia and hyperinsulinemia in these studies (Fig. 6).

Source of the glucagon-induced elevations in cyclic AMP levels. The incubation of glucagon (0.6 μmoles/liter or about 150 times the maximum infused levels achieved) with whole human blood in vitro for 10 min at 37°C did not influence the plasma content of the cyclic nucleotides as compared with appropriate controls. Thus, the elevations in plasma cyclic AMP levels induced by glucagon in vivo must have been reflections of the action of the hormone on one or more of its target tissues.

Table II contains the results of a representative experiment in which glucagon was infused into the isolated rat liver perfused in situ. Cyclic AMP concentrations in the perfusate increased in dose-related fashion with highest levels being approximately 125 times the control values. The increments in perfusate cyclic AMP concentration appeared to provide a sensitive correlation between cyclic AMP production and physiological effect (e.g., glucose production). Cyclic GMP was undetectable in the liver perfusates (less than about 1 nmole/liter).

DISCUSSION

Glucagon effects on plasma and urinary cyclic AMP. Glucagon is a potent stimulus in elevating plasma and urinary cyclic AMP in man. The increase in cyclic AMP excretion after glucagon administration was entirely a consequence of the increase in filtered load of the nucleotide, which was due principally to greatly elevated plasma levels of the compound and, to a slight extent, to an increase in glomerular filtration rate.

During extended infusions of large doses of glucagon, plasma and urinary cyclic AMP levels rose precipitously and then fell slightly, well before termination of the infusions (Fig. 1 and Table I). There is no clear explanation for this fall in cyclic AMP, but a similar

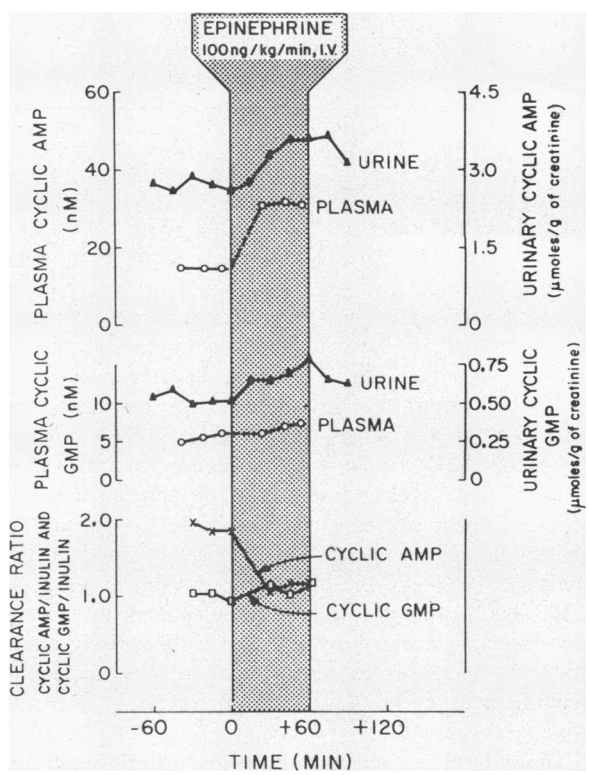

FIGURE 4 The effects of an intravenous infusion of epinephrine on plasma and urinary cyclic AMP and cyclic GMP in a fasting, well hydrated, normal subject. The clearance ratios (cyclic nucleotide clearance/inulin clearance) of cyclic AMP and cyclic GMP are shown in the lower panel. Time is expressed in relation to the beginning of the epinephrine infusion.

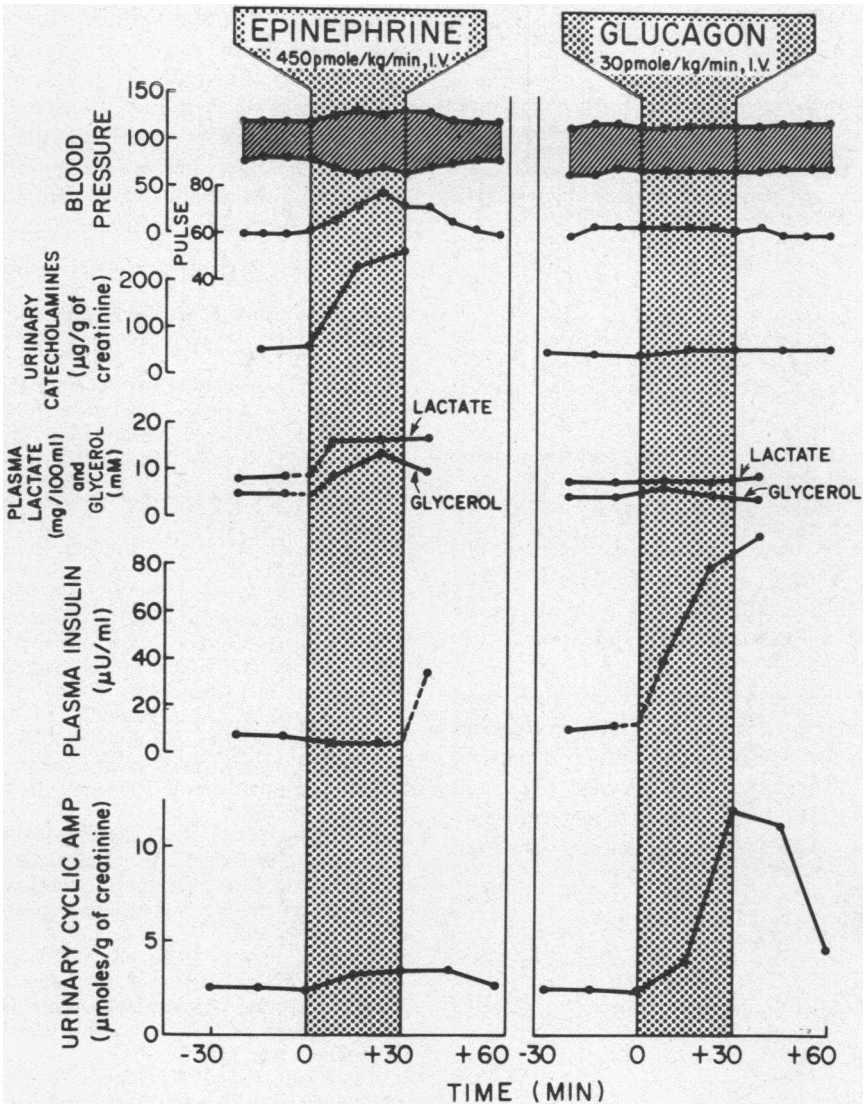

FIGURE 5 The comparative effects of epinephrine (left panel) and glucagon (right panel) infusions into the same subject on blood pressure, heart rate, urinary catecholamine excretion, plasma lactate, glycerol and insulin concentrations, and the excretion of cyclic AMP. The doses of epinephrine and glucagon are expressed on a molar basis (450 and 30 pmoles/kg per min, respectively). Time is expressed in relation to the initiation of the hormone infusions.

temporal response of the nucleotide to other hormones has been demonstrated (4, 37, 38).

Specificity of the glucagon-induced cyclic AMP response. The various specificity studies presented above indicated that the elevations in extracellular fluid levels of cyclic AMP elicited by glucagon infusion were direct effects of the intact peptide and were not mediated by parathyroid hormone, epinephrine, hyperinsulinemia, or hyperglycemia.

Tissue source of the increased extracellular cyclic AMP produced by glucagon. The perfused rat liver released large quantities of cyclic AMP into the medium in response to glucagon. In a preliminary study in a hepatectomized dog with a portacaval shunt,[3] an infusion of glucagon produced no increase in plasma or urinary cyclic AMP levels, whereas the same dose of the

[3] We are indebted to Dr. J. Threlkel and Mr. A. Menton for the surgery.

TABLE II

The Effects of Glucagon in the Isolated Rat Liver Perfused in Situ

Dose of glucagon	Perfusate cyclic AMP	Hepatic cyclic AMP	Hepatic glucose production
ng/min	*nmoles/liter*	*nmoles/g**	*μmoles/100 g per hr‡*
0	8.0	0.50	143
0.025	11.7	0.55	176
0.05	14.0	0.56	234
0.10	16.7	0.71	189
0.25	23.6	0.65	318
2.5	191	2.10	845
25.0	997	7.00	693

* nmoles/g of liver, wet weight.
‡ μmoles/100 g rat weight per hr.

hormone administered before hepatectomy elicited 60-fold elevations in plasma and urinary levels of the compound. Thus, the liver appears to be a major source of the cyclic AMP released into the circulation in response to glucagon.

Cyclic nucleotide transport. Because of their size, charge, and molecular structure, the cyclic nucleotides would not be expected to diffuse freely across plasma membranes. Studies in avian erythrocytes (39) and microorganisms (40, 41) have indicated that transport systems for cyclic AMP exist in nature, and the nucleotide has an extracellular function as a chemotactic agent in at least one species of cellular slime mold (42–44). Currently, only indirect evidence for the possible occurrence of such transport systems in mammalian tissues exists, including the release of cyclic AMP by the liver and the kidney (4) and the miscibility of an intracellular pool with the plasma pool of the cyclic nucleotides in intact human subjects (3).

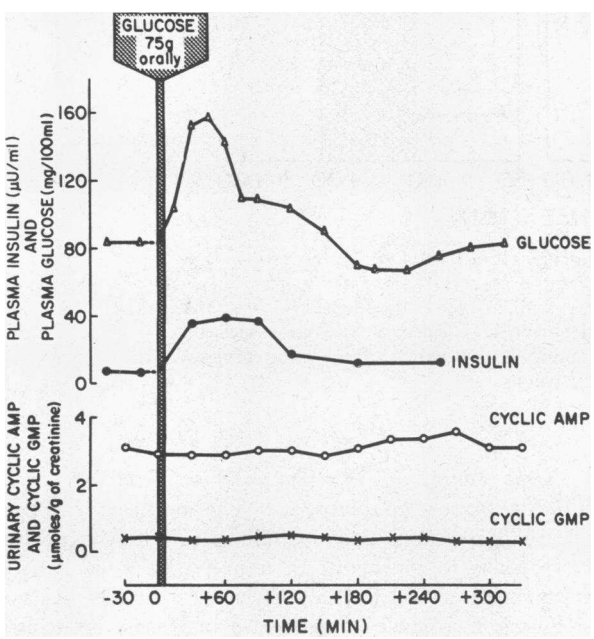

FIGURE 6 Plasma glucose and insulin concentrations and cyclic AMP and cyclic GMP excretion of a fasting, well hydrated, normal subject after a 75 g oral glucose load at zero time.

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