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Effects of Glucagon and Dibutyryl Cyclic AMP on Phosphorylase Activity and Gluconeogenesis in Rat Liver Slices[1] (38066)

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Although glucagon has been found to give small, but significant, increase in ketogenesis when added to liver slices *in vitro* (1, 2) it does not increase gluconeogenesis. In contrast, the hormone does stimulate both ketogenesis and gluconeogenesis in isolated perfused liver (3, 4). Phosphorylase activation and glycogenolysis are readily initiated by glucagon in both slices and perfused liver. To investigate further the lack of glucagon effect on gluconeogenesis in liver slices, the hormone was studied in combination with theophylline in order to further increase tissue cyclic AMP levels (5). Attempts were made to correlate changes in phosphorylase activity, ketogenesis and gluconeogenesis with tissue levels of cyclic AMP. The results of these studies are summarized in this report.

Materials and Methods. Animals. Male, Cox rats weighing between 180 and 200 g were used for all studies. All animals were fasted overnight and sacrificed by a blow on the head and exsanguination. Livers were rapidly removed and placed in chilled petri dishes, cut into small pieces and sliced with a Stadie-Riggs hand microtome.

Metabolic Studies. Liver cyclic AMP levels and phosphorylase activity. Rat liver slices (300–350 mg wet weight for cyclic AMP and 350–500 mg for phosphorylase) were preincubated in 5 ml of a Ringer bicarbonate buffer (6), gassed with 95% O_2–5% CO_2. After 30 min the slices were transferred to fresh media containing glucagon (10^{-6} *M*) and/or theophylline (10^{-3} *M*) and incubated for an additional 5 min.

Cyclic AMP Levels. To measure cyclic AMP, the tissue was removed from the incubation flasks and homogenized in 3 ml of cold 5% trichloroacetic acid containing 0.1 ml of 1 *N* HCl. The samples were centrifuged in a clinical centrifuge at full speed for 5 min and the supernatant was extracted 3 times with 2.5 volumes of water saturated diethyl ether. The samples were lyophylized and reconstituted in 200 m*M* sodium acetate buffer pH 4.0 to give a final concentration of 50 mg (wet wt of tissue)/ml. Cyclic AMP was measured by a modification of the competitive protein binding assay of Gilman (7). The binding reaction was carried out in final volume of 0.2 ml in 50 m*M* sodium acetate, pH 4.0. The incubation mixture contained sufficient binding protein obtained from rabbit skeletal muscle to bind 30% of the added cyclic AMP, and ^{3}H-cyclic AMP (5 pmoles:0.1 μCi). The reaction was initiated by the addition of binding protein and was incubated for 60 min at 0°C. Upon reaching equilibrium, the mixture was diluted to 1 ml with cold 20 m*M* sodium phosphate buffer pH 6.0 and filtered through a Millipore filter (0.45 μm). The filter was washed with 10 ml of the same buffer and dissolved in toluene-triton scintillation fluid.

Phosphorylase Activity. After the 5 min incubation period in the presence of glucagon and/or theophylline, the tissue was

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removed from the incubation flasks and homogenized in 3 ml of cold 0.154 *M* KF (adjusted to pH 6.7 with 0.2 *M* Tris). The assay involved the incorporation of glucose-1-phosphate into a primer of glycogen with the release of inorganic phosphate. The reaction mixture contained 0.1 ml of the homogenate, glucose-1-phosphate (7.5 mg/ml) and glycogen (3 mg/ml) in a final volume of 1 ml (8). This mixture was incubated 10 min at 38°C and 1 ml of 5% trichloroacetic acid was added. Following centrifugation 1 ml of the supernatant was taken for determination of P_i (9).

Gluconeogenesis and Ketogenesis. Liver slices (300–400 mg) were placed in 5 ml of lactate (10 m*M*) Ringers solution and gassed with 95% O_2–5% CO_2 for 5 min. After addition of 0.1 ml of 0.1 *M* $NaH^{14}CO_3$ (5 μCi), the samples were incubated for 90 min at 37°C (10). Incubation media was then decanted, heated to 95°C for 3–5 min to precipitate protein, centrifuged and samples removed for glucose and ketone body determinations. Glucose was isolated as the phenylosazone (11), plated on preweighed plaques and counted in a Nuclear Chicago gas flow counter as previously described (12). Results are expressed as observed counts/min and % of added ^{14}C/mg wet weight. Glucose production was measured by the glucose oxidase method as described by Huggett and Nixon (13). Samples were deproteinized by the addition of 0.25 ml each of 5% $ZnSO_4$ and 0.3 *N* $Ba(OH)_2$ (14). Ketone bodies were measured in 0.5 ml of deproteinized sample by an enzymatic method (15).

Results and Discussion. Rat liver phosphorylase is converted almost entirely to the active form during the process of killing the animal and removing the liver (8). During the 30 min preincubation period values rapidly fall to a mean activity of 65 ± 3.5 μmoles P_i/g/10 min incubation. At this stage phosphorylase activation may be readily demonstrated by addition of glucagon, epinephrine or dibutyryl cyclic AMP (N^6,O^2 dibutyryl adenosine 3′,5′ cyclic monophosphate). Maximum activation is achieved in 3–5 min. For convenience, all values have been recorded as precent of control for each experiment and the results are summarized in Table I.

Phosphorylase activity was stimulated by glucagon which was not further augmented by the addition of theophylline. Theophylline alone or in the presence of glucagon seemed to reduce phosphorylase activity.

Rall *et al.* (16) have suggested that methylxanthines reduce phosphorylase activity by activation of phosphorylase phosphatase. A direct inhibition of phosphorylase by theophylline has also been reported (17). In our own experience, purified dog liver phosphorylase (kindly supplied by Dr. Joel Hardman, Dept. Physiology, Vanderbilt University) is inhibited 45% by the presence of 10^{-3} *M* theophylline.

Glucagon produced a small increase in tissue cyclic AMP levels and this was potentiated by theophylline although the methylxanthine alone produced no change in tissue cyclic AMP levels. No correlation between tissue cyclic AMP levels and phosphorylase activity was observed.

Previous studies (10) have demonstrated that incorporation of ^{14}C from labeled CO_2

TABLE I. Effects of Glucagon and Theophylline on Phosphorylase Activity and Cyclic 3′,5′ AMP Levels in Rat Liver Slices.

	Phosphorylase %[a]	cAMP (pmoles/mg)
Control (6)	100 ± 11	0.74 ± 0.13
Glucagon 10^{-6} *M* (6)	180 ± 15	1.01 ± 0.16
Theophylline 10^{-3} *M* (6)	82 ± 9	0.66 ± 0.16
Theophylline + Glucagon (6)	130 ± 14	2.37 ± 0.42

[a] Control phosphorylase activity was 65 ± 3.5 μmoles P_i/g wet liver/10 min assay. All values are the mean ± SEM for 6 experiments.

TABLE II. Effect of Theophylline and Glucagon on Glucose and Ketone Body Production in Rat Liver Slices. (All Values are Mean ± S.E. and are Expressed/g Wet Tissue for a 90 min Incubation.)

		Glucose			Ketones		
	n	μmoles/g	cpm/g	% added ^{14}C	AcAc μmoles/g	B-OH μmoles/g	Total μmoles/g
Control	(6)	66 ± 2.3[a]	17,000 ± 1,200	1.13 ± 0.08	5.2 ± 0.27	6.1 ± 0.64	11.3 ± 0.74
Glucagon 1 μ*M*	(6)	71 ± 3.5	19,300 ± 2,600	1.29 ± 0.17	5.2 ± 0.23	6.1 ± 0.61	11.4 ± 0.72
Theophylline 1 m*M*	(6)	66 ± 4.3	19,000 ± 1,500	1.27 ± 0.11	4.4 ± 0.20	6.0 ± 0.56	10.4 ± 0.48
Theophylline 1 m*M* + Glucagon 1 μ*M*	(6)	62 ± 4.9	19,300 ± 1,700	1.29 ± 0.11	4.2 ± 0.24	6.0 ± 0.57	10.1 ± 0.62

[a] All values are the mean ± SEM for 6 experiments.

into glucose is a good index of gluconeogenesis. This method has two advantages: (1) the specific activity of the label does not change significantly during the course of incubation and (2) addition of the tracer does not alter levels of intracellular metabolites. CO_2 is incorporated by carboxylation of pyruvate to form oxaloacetate (18). Incorporation of CO_2 into glucose is increased in alloxan diabetes (19) and following stimulation with glucocorticoids (20).

Studies on gluconeogenesis and ketogenesis are summarized in Table II. No demonstrable effect by glucagon or theophylline on gluconeogenesis or ketogenesis was observed. On the other hand, dibutyryl cyclic AMP at 5×10^{-4} *M* increased the rate of gluconeogenesis, net glucose production, and ketogenesis (Table III). Since butyrate is released from dibutyryl-cyclic AMP and since 3 m*M* sodium butyrate stimulates both ^{14}C glucose (21) formation and ketogenesis, effects of 5×10^{-4} *M* dibutyryl cyclic AMP have been compared with those of 1 m*M* butyrate. Butyrate at this concentration appears to cause a slight, but statistically insignificant, increase in ^{14}C glucose and ketone body formation. As a further control, 5′ AMP was tested and this too was without affect.

Since glucagon stimulation of both gluconeogenesis and ketogenesis is readily demonstrated in isolated perfused livers, the present studies suggest that some defect in

TABLE III. Effects of 5′ AMP, Butyrate and Dibutyryl Cyclic 3′,5′ AMP on Glucose and Ketone Body Production in Rat Liver Slices.

	Glucose			Ketones		
	μmoles/g	cpm/g	% added	AcAc μmoles/g	B-OH μmoles/g	Total μmoles/g
Control (6)	52 ± 4.6[a]	14,600 ± 1,200	1.01 ± 0.83	6.1 ± 0.53	4.8 ± 0.40	10.9 ± 0.87
5′ AMP (6) 5×10^{-4} *M*	54 ± 3.2	15,600 ± 1,100	1.08 ± 0.076	5.9 ± 0.62	5.4 ± 0.42	11.3 ± 0.93
Butyrate (6) 10^{-3} *M*	51 ± 5.2	17,800 ± 1,380	1.22 ± 0.094	7.6 ± 0.41	5.6 ± 0.54	13.2 ± 1.02
Dibutyryl cAMP (6) 5×10^{-4} *M*	70 ± 6.1	25,600 ± 2,100	1.77 ± 0.14	7.3 ± 0.80	9.3 ± 0.62	16.6 ± 0.94

[a] All values are the mean ± SEM for 6 experiments.

the response must exist in the liver slice preparation. The accepted mode of action of glucagon is *via* cyclic 3′,5′-AMP. The hormone activates adenyl cyclase and thus increases tissue cyclic 3′,5′-AMP levels. Cyclic 3′,5′-AMP activates a protein kinase which in turn initiates a series of protein phosphorylations resulting in the activation of glycogen phosphorylase. With the activation of glycogen phosphorylase glycogen is metabolized to glucose. It has been suggested that stimulation of gluconeogenesis and ketogenesis by glucagon also involves increased cyclic 3′,5′-AMP production and activation of protein kinase. Since glucagon readily elevates cyclic AMP levels, and activates phosphorylase in liver slices, it would appear that no defect exists in the cyclic AMP protein kinase system. The stimulation of gluconeogenesis by dibutyryl cyclic AMP and by glucocorticoids (20) suggests that the pathway for gluconeogenesis is intact in this preparation. It may be, however, that the protein kinase in the sequence of reactions leading to activation of phosphorylase is not the same protein kinase which is part of the pathway leading to activation of gluconeogenesis and that there is some defect in the latter enzyme. An alternate explanation is that compartmentalization of cyclic AMP exists and that the elevated level of cyclic AMP seen with glucagon was in a compartment which was related to phosphorylase activation but not activation of gluconeogenesis. It also must be considered that dibutyryl cyclic AMP and glucocorticoids activate gluconeogenesis in some manner independent of protein kinases.

Summary. Although glucagon alone or in combination with theophylline increased cyclic 3′,5′-AMP levels and activated glycogen phosphorylase in rat liver slices, gluconeogenesis and ketogenesis were not increased under these conditions.

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