RESEARCH COMMUNICATION

Cell shrinkage follows, rather than mediates, the short-term effects of glucagon on carbohydrate metabolism

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The initial effects of glucagon on glycogen breakdown in isolated hepatocytes were found to be independent of cell volume and, when it occurred, cell shrinkage followed rather than mediated the glycogenolytic effect of glucagon. Similar conclusions could be drawn for the effect of glucagon on glycolysis/gluconeogenesis and for the antagonistic effect of insulin on glucagon action.

INTRODUCTION

Cell swelling induced by several amino acids or by hypotonicity stimulated glycogen synthesis and lipogenesis in isolated rat liver preparations [1,2]. Many other effects of cell swelling, e.g. on the metabolism of amino acids and proteins, have also been reported (for a review see [3]). The stimulation of glycogen synthesis and lipogenesis results from an activation of glycogen synthase and acetyl-CoA carboxylase [1,4], the two key enzymes in glycogen synthesis and lipogenesis respectively. The mechanism linking cell swelling to glycogen synthase activation has been elucidated recently [5]. The fall in intracellular Cl^- concentration as well as the increase in intracellular glutamate and aspartate are responsible, at least in part, for the stimulation of the phosphatase that activates glycogen synthase.

Since the metabolic effects of cell swelling in the liver favour anabolism, it was tempting to investigate whether cell swelling could be part of, or even mediate, the action of anabolic hormones, such as insulin. Indeed, it has been reported recently that the stimulation of glycogen synthesis by insulin in cultured hepatocytes [6,7] and the inhibition of proteolysis by the same hormone in perfused liver [8,9] were mediated by cell swelling. On the other hand, cell shrinkage could be implicated in the action of catabolic hormones, such as glucagon. Indeed, the stimulation of proteolysis by glucagon in perfused liver was shown to be mediated by cell shrinkage [8,9]. Moreover, hypertonic cell shrinkage was found to stimulate glycogen breakdown [10]. Accordingly, Häussinger & Lang [3] have speculated that 'the hormone-induced alterations of cell volume may contribute not only to the known effects of glucagon and insulin on hepatic protein turnover, but also on carbohydrate metabolism'.

The present work was undertaken to test whether cell shrinkage mediates the known glycogenolytic and gluconeogenic actions of glucagon, and whether cell swelling is implicated in the antagonistic effect of insulin on glucagon action in isolated hepatocytes.

MATERIALS AND METHODS

Glucagon and insulin (Novo Nordisk), radiochemicals (Amersham, New England and Nuclear Research Products) and

other biochemical reagents (Sigma or Boehringer Mannheim) were purchased as indicated.

Hepatocytes from male Wistar rats were prepared and incubated as described [11]. The standard incubation medium was a Krebs–Henseleit bicarbonate buffer at pH 7.4 [12]. The medium was made hypotonic by decreasing the concentration of NaCl in the buffer from 118 mM to 58 mM. All media were in equilibrium with a gas phase of O_2/CO_2 (19:1). For the measurement of enzymes and metabolites, samples were withdrawn at the appropriate times and immediately frozen in tubes kept in a cooling mixture (solid CO_2 in acetone).

Cyclic AMP was measured with the assay kit from Amersham. Glycogen [13] and fructose 2,6-bisphosphate [14] contents were measured as described in the references. Glycogen content is expressed as glucose equivalent.

The active form of phosphorylase was assayed as described in [15], in extracts prepared by thawing the cellular suspension in $\frac{1}{5}$ vol. of a solution containing 0.45 M-NaF, 90 mM-EDTA, 2.25 % glycogen and 45 mM-glycylglycine adjusted to pH 7.4. The activity of pyruvate kinase was measured at low [0.15 mM ($v_{0.15}$)] and saturating [5 mM (v_5)] concentrations of phosphoenolpyruvate in extracts prepared as described in [16]. The $v_{0.15}/v_5$ ratio gives an indication of the proportion of pyruvate kinase in the active form [16,17]. One unit of enzyme activity corresponds to 1 μmol of product formed/min under the assay conditions.

The cell volume was either estimated by the cell weight as described previously [1] or calculated by the difference between the total and the extracellular volumes corresponding to the $^3\text{H}_2\text{O}$ and the [^{14}C]carboxydextran-accessible spaces respectively. To do this, $^3\text{H}_2\text{O}$ (0.5 μCi) and [^{14}C]carboxydextran (0.15 μCi) were added to 2 ml of cell suspension 30 s before taking a 1 ml sample for centrifugation (Eppendorf Microfuge for 5 s). The radioactivity in the cell pellet and supernatant was measured by liquid-scintillation counting. The values were corrected for quenching and expressed as a function of the protein content of the cell suspension. The intracellular water space was $2.69 \pm 0.09 \mu\text{l}$ of water/mg of protein (Fig. 1a, 0 min, $n = 7$) and corresponds to 0.54 ml of water/g of cells, taking into account the protein content per g wet weight of hepatocytes (about 200 mg of protein/g wet wt. [15,18]). The values are similar to those reported for hepatocytes in suspension [1,19] and for the perfused rat liver [8,9]. The values reported for hepatocytes in

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Table 1. Effects of glucagon on cell weight, cyclic AMP content, phosphorylase *a* activity and glycogen content in hepatocytes from fed rats

After incubation for 15 min in the presence of 25 mM-glucose, hepatocytes from fed rats were incubated without or with 0.1 μ M-glucagon for the indicated periods of time. The values are means $\pm$ S.E.M. for three (5 min) or four (30 min) different cell preparations: *significantly different ($P < 0.05$) from the control values. n.d., not determined.

Time of incubation (min)	Conditions	Cell weight (% of control)	Cyclic AMP content (nmol/g of cells)	Phosphorylase <i>a</i> activity (units/g of cells)	Glycogen content (μ mol/g of cells)
5	Control	100	0.7 $\pm$ 0.1	1.0 $\pm$ 0.6	145 $\pm$ 24
	Glucagon	98.8 $\pm$ 1.5	5.1 $\pm$ 0.3*	9.7 $\pm$ 1.6*	139 $\pm$ 26
30	Control	100	n.d.	3.0 $\pm$ 1.2	157 $\pm$ 7
	Glucagon	92.1 $\pm$ 0.9*	n.d.	8.6 $\pm$ 0.9*	114 $\pm$ 10*

Fig. 1. Time course of the effects of glucagon on intracellular water space (a) and on phosphorylase *a* activity (b)

After incubation for 15 min in the presence of 25 mM-glucose, hepatocytes from fed rats were incubated without (○; control) or with (●) 0.1 μ M-glucagon for the indicated periods of time. The values are means $\pm$ S.E.M. for seven different cell preparations; *significantly different ($P < 0.05$) from the corresponding control values.

culture range from 1.44–1.86 μ l of water/mg of protein [6] to 2.48 μ l of water/mg of protein [20].

The statistical significance of differences was calculated by Student's *t* test (paired data).

RESULTS AND DISCUSSION

It is generally accepted that the second messenger of glucagon in liver is cyclic AMP, which activates the cyclic AMP-dependent protein kinase, which in turn activates (or inactivates) key enzymes by phosphorylation. This applies to several metabolic pathways, and in particular to the glycogenolytic cascade, in

which the activation of phosphorylase is the final key step. If we assume that cell shrinkage mediates the glycogenolytic action of glucagon, it should precede the activation of phosphorylase, or even the increase in the concentration of cyclic AMP. Therefore we studied the time course of phosphorylase activation, cyclic AMP accumulation, volume changes and glycogen depletion in isolated rat hepatocytes from fed and fasted rats. In these experiments, 25 mM-glucose was added to prevent glycogen breakdown in control hepatocytes. Table 1 shows that, in hepatocytes from fed rats treated for 5 min with saturating concentration of glucagon, cell weight was not affected, whereas the concentration of cyclic AMP and the activity of phosphorylase were greatly increased. A decrease (8%) in cell weight was detectable after 30 min of incubation. These results were confirmed in other experiments designed to detail the time course of the early effects of glucagon. In these experiments, the cell volume was measured by the intracellular water space (see the Materials and methods section). Fig. 1 shows that maximal activation of phosphorylase was reached within 1 min after addition of glucagon, without significant change in cell volume. A decrease in the intracellular water space was detected after 30 min of incubation, thus confirming the data shown in Table 1.

The cell shrinkage after 30 min of incubation in glucagon-treated cells (Table 1 and Fig. 1) coincided with a 27% decrease in glycogen content (Table 1). This suggests that the disappearance of glycogen by itself could contribute to cell shrinkage. Indeed, if one takes into account the amount of water accumulated per g of glycogen, i.e. about 2.4 ml [21,22], the amount of glycogen lost after 30 min represents about 40% of

Table 2. Effects of glucagon on cell weight, phosphorylase *a* activity and glycogen content in hepatocytes from fasted rats

After incubation for 15 min in the presence of 25 mM-glucose, hepatocytes from 24 h-fasted rats were incubated without or with 0.1 μ M-glucagon for 3 min (phosphorylase *a*) or 30 min (cell weight and glycogen). The values are means $\pm$ S.E.M. for two (phosphorylase *a*) or three (others) different cell preparations: *significantly different ($P < 0.05$) from the control values.

Conditions	Cell weight (% of control)	Phosphorylase <i>a</i> activity (units/g of cells)	Glycogen content (μ mol/g of cells)
Control	100	0.9	1.9 $\pm$ 0.6
Glucagon	97.3 $\pm$ 1.4	7.0*	0.9 $\pm$ 0.1

Table 3. Effect of glucagon on cell weight, cyclic AMP content and phosphorylase *a* activity in hepatocytes incubated in a hypotonic medium

After incubation for 15 min in the presence of 25 mM-glucose in an iso- (*a*, *b*) or hypo- (*c*, *d*) tonic medium, hepatocytes from fed rats were incubated without (*a*, *c*) or with (*b*, *d*) 0.1 μ M-glucagon for the indicated periods of time. The values are means $\pm$ S.E.M. for (*n*) different cell preparations. The significance of the difference ($P < 0.05$) is indicated by * for (*a*) versus (*b*), † for (*c*) versus (*d*), ‡ for (*a*) versus (*c*), and § for (*b*) versus (*d*).

Conditions	Cell weight (% of control)		Cyclic AMP content (nmol/g of cells) 5 min	Phosphorylase <i>a</i> activity (units/g of cells) 5 min
	5 min	30 min		
(<i>a</i>) Isotonic medium	100 (3)	100 (5)	0.5 $\pm$ 0.1 (4)	3.2 $\pm$ 1.1 (3)
(<i>b</i>) Isotonic medium + glucagon	98.9 $\pm$ 2.3 (3)	94.7 $\pm$ 0.8* (5)	6.5 $\pm$ 1.7* (4)	14.7 $\pm$ 2.5* (3)
(<i>c</i>) Hypotonic medium	111.0 $\pm$ 1.4† (3)	114.7 $\pm$ 0.8† (5)	0.5 $\pm$ 0.2 (4)	2.2 $\pm$ 0.6 (3)
(<i>d</i>) Hypotonic medium + glucagon	110.6 $\pm$ 1.6§ (3)	108.3 $\pm$ 1.0†§ (5)	5.8 $\pm$ 0.9† (4)	12.8 $\pm$ 2.9† (3)

Table 4. Effect of insulin and glucagon on intracellular water space, phosphorylase *a* and pyruvate kinase activities and fructose 2,6-bisphosphate content

After incubation for 25 min in the presence of 10 mM-glucose, hepatocytes from fed rats were incubated without or with 0.1 μ M-insulin for 5 min; they were then further incubated for 2 min without or with 0.1 nM-glucagon. The values are means $\pm$ S.E.M. for four different cell preparations: *significantly different ($P < 0.05$) from the control values; †significantly different ($P < 0.05$) from the values in the presence of glucagon.

Conditions	Intracellular water space (μ l/mg of protein)	Phosphorylase <i>a</i> activity (units/g of cells)	Pyruvate kinase (<i>v</i> 0.15/ <i>v</i> 5 ratio)	Fructose 2,6-bisphosphate content (nmol/g of cells)
Control	2.31 $\pm$ 0.14	6.3 $\pm$ 1.0	0.64 $\pm$ 0.10	11.3 $\pm$ 1.1
Insulin	2.30 $\pm$ 0.14	5.4 $\pm$ 0.7	0.70 $\pm$ 0.09*	10.4 $\pm$ 1.1
Glucagon	2.26 $\pm$ 0.19	11.6 $\pm$ 1.8*	0.43 $\pm$ 0.07*	6.5 $\pm$ 1.1*
Insulin + glucagon	2.24 $\pm$ 0.15	7.9 $\pm$ 1.1†	0.49 $\pm$ 0.07*†	8.7 $\pm$ 1.0*†

the volume lost, which is in agreement with data published recently [8]. The importance of glycogen breakdown in the change of volume is further documented by the results obtained with hepatocytes from fasted rats (Table 2). In these hepatocytes, which contain little or no glycogen, glucagon caused an activation of phosphorylase similar to that observed in hepatocytes from fed rats, but with no detectable change in cell volume at 30 min.

If cell shrinkage is essential for glucagon action, cell swelling should counteract the glycogenolytic effect of this hormone. Therefore, we tested the glycogenolytic effect of glucagon in hepatocytes, prepared from fed rats, and which were allowed to swell in a hypotonic medium. Table 3 shows that, despite the 11–15% increase in cell volume due to the hypotonic medium, glucagon was able to cause an accumulation of cyclic AMP and an activation of phosphorylase at 5 min that were not different from those observed in hepatocytes incubated in an isotonic medium. Although glucagon had no effect on volume at 5 min, it decreased in the same proportion the cell volume at 30 min in hepatocytes incubated in either iso- or hypo-tonic medium.

Finally, we tested the possibility that the action of glucagon on glycolysis/gluconeogenesis could be mediated by a change in volume. The inactivation of pyruvate kinase and the decrease in the concentration of fructose 2,6-bisphosphate were taken as an index of the effect of glucagon on glycolysis/gluconeogenesis [23,24]. Table 4 shows that a sub-optimal (0.1 nM) concentration of glucagon could indeed affect these two parameters without change in cell volume. In these experiments, we also tested the antagonistic effect of insulin towards sub-optimal doses of glucagon on pyruvate kinase inactivation, fructose 2,6-bisphosphate concentration and phosphorylase activation. The data shown that 0.1 μ M-insulin could indeed antagonize the effect of 0.1 nM-glucagon without detectable change in cell volume.

Conclusion

The early events responsible for glucagon action on glycogen breakdown, namely cyclic AMP accumulation and phosphorylase activation, are independent of cell volume. These results rule out a mediating effect of cell shrinkage in glucagon action on glycogen breakdown. Moreover, the effect of glucagon on glycolysis/gluconeogenesis and the antagonistic effect of insulin on glucagon action in hepatocytes of fed rats are not mediated by a significant change in cell volume. Therefore these results question the general idea that glucagon action is mediated by volume changes [3]. This conclusion applies to the initial effect of glucagon and is consistent with our previous finding, that swelling did not decrease cyclic AMP, but on the contrary slightly increased its concentration [25]. However, we cannot rule out the possibility that in the long term the glucagon-induced cell shrinkage in hepatocytes from fed rats, which results in part from glycogen breakdown, could re-inforce the glycogenolytic action of this hormone. This action might involve the cytoskeleton, since stimulation of glycogen degradation by glucagon could be inhibited by cytochalasins. The inhibition of the glucagon response occurred at a site beyond the activation of cyclic AMP-dependent protein kinase [26]. Similarly, cell swelling might well be involved in the long-term effect of insulin on glycogen synthesis in hepatocytes in culture [6,7].

Our results show that hepatocytes from fed rats eventually shrink after a glucagon treatment. They do not allow any conclusion to be drawn for the mediating role of cell volume in the hormonal control of liver protein metabolism in fed animals. In hepatocytes from fasted rats, however, glucagon did not change the cell volume after 30 min of incubation, and, if glucagon controls the metabolism of protein under these con-

ditions, it should do so without changing the cell volume. Finally, it is worth noting that the time course of decrease in cell volume induced by glucagon differs in isolated hepatocytes from that in perfused liver. Indeed, a change in cell volume was readily detectable within a few minutes in perfused liver [8], whereas in isolated hepatocytes it could not be observed within 15 min of incubation, but became significant at 30 and 45 min (the present paper; [19,27]).

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