THE ACTIVATION OF LIVER PHOSPHORYLASE *b* KINASE BY GLUCAGON

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1. Introduction

Although cyclic AMP was discovered in studies of the mode of action of glucagon in promoting liver glycogenolysis (see [1]), the entire sequence of events initiated by the cyclic nucleotide is less well known in liver than in muscle. Indeed, in this tissue, as has been worked out very well by Krebs and co-workers (see [2]) cyclic AMP brings about a sequential activation of protein kinase, phosphorylase kinase and glycogen phosphorylase, leading ultimately to an enhanced rate of glycogen breakdown. In liver, the activation of glycogen phosphorylase by glucagon also involves a rise in the cyclic AMP content (see [1]) and an activation of protein kinase [3,4] but up till now, there has only been one report describing an enhanced activity of phosphorylase kinase [5].

In the present report, we show that glucagon does indeed cause a substantial increase in the liver phosphorylase kinase activity; we show furthermore that this activation is caused by the action of the cyclic AMP dependent protein kinase.

2. Materials and methods

2.1. *Materials*

Glucagon was obtained from Novo-laboratories (Copenhagen), collagenase type I, bacitracin, histone f_{2b}, ATP and cyclic AMP from Sigma Chemical Co. (St. Louis), and glycogen from Merck A. G. (Darmstadt). [γ-^{32}P]ATP was purchased from the Radiochemical Centre (Amersham). All other chemicals were of reagent grade. Crystalline rabbit muscle phosphorylase *b*, isolated according to [6], was a gift of Dr G. Defreyn.

Protein kinase inhibitor was isolated up to the TCA precipitation step, as described by Walsh et al. [7]. After dialysis against 5 mM potassium phosphate, 1 mM EDTA, pH 7.0, the preparation was clarified by centrifugation at 34 000 *g* for 20 min [7] and made 100 mM in NaF, 250 mM in sucrose and 5% in glycogen.

2.2. *Preparation and incubation of rat liver cells*

Liver cells were isolated essentially as described by Hue et al. [8] with the following modifications: the liver was perfused after its removal from the animal; a Krebs–Henseleit bicarbonate buffer at pH 7.4, gassed with 95% O_2, 5% CO_2 was used; the inital cell suspension was passed through a nylon filter (100 mesh). Before incubation, the cells were stored at 0°C in a large Erlenmeyer containing the O_2/CO_2 mixture.

The incubation was started with 0.6 ml of a cell suspension in Krebs–Henseleit bicarbonate buffer (about 10^7 cells/ml) containing bacitracin (1 mg/ml); the cells were incubated at 37°C in closed plastic 20 ml vials with continuous shaking (120 oscillations/min) in the presence of the O_2/CO_2 mixture. A preincubation of up to 45 min in the presence of 20 mM glucose was routinely performed in order to bring phosphorylase *a* to a low level [8]. After this preincubation, one or several aliquots of the cell suspension were frozen in tubes kept in liquid N_2; 10–20 μl of the hormone solution were then added, the vial gassed again with the O_2/CO_2 mixture, stoppered and further incubated; at 1 min intervals, one or more aliquots were taken out and frozen, while the vial was each time gassed with O_2/CO_2 and closed. At the end of the incubation more than 85–90% of the cells excluded trypan blue; the frozen cells were kept at −20°C until they were processed for enzyme assays.

2.3. *Enzyme assays*

For the assay of the endogenous liver phosphorylase *a* (EC 2.4.1.1) the frozen cells were thawed out by shaking them in 3 vol of an ice-cold 100 mM glycyl–glycine buffer, pH 7.4, containing 0.5% glycogen, 100 mM NaF and 20 mM EDTA; liver phosphorylase *a* was measured as described in detail [8] except that temperature was raised to 25°C. It was checked that this method of homogenization yielded the same enzyme activities as those obtained with the conventional homogenization in a Potter–Elvehjem tube.

For the assay of protein kinase (EC 2.7,1.37) the cells were thawed out by shaking them in 9 vol of an ice-cold medium, containing 250 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4, 4 mM EDTA and 2 mM dithiotreitol. Protein kinase was assayed as described before [4] with histone f_{2b} as the substrate.

The amount of protein kinase inhibitor required to abolish completely the protein kinase activity (as assayed with 10^{-6} M cyclic AMP) was determined and was then used in those experiments where it was decided to block protein kinase activity. The protein kinase inhibitor preparation had no effect on the phosphorylase kinase activity.

For the assay of phosphorylase kinase (EC 2.7.1.38), the cells were thawed out directly in 4 vol of the protein kinase inhibitor preparation. For some experiments, where preincubation with the endogenous protein kinase was desirable, the same phosphate buffer containing EDTA, NaF, sucrose and glycogen (see under 2.1.) was used without protein kinase inhibitor. In standard phosphorylase kinase assays, 50 μl of the thawed out cell suspension were mixed with 100 μl of a solution containing 0.2 mg phosphorylase *b*, 1 mM Tris pH 7.0 and, whenever necessary, protein kinase inhibitor. (The final concentration of the inhibitor in the assay of phosphorylase kinase was the same whether it was added in the thawing of the cells or only in the phosphorylase kinase assay). This mixture was then equilibrated at 30°C for 2 min after which the reaction was started by the addition of 30 μl of an ATP-Mg acetate solution. The final concentration of ATP and Mg was 0.3 mM and 1.5 mM respectively. To stop the phosphorylase kinase reaction, a 20 μl aliquot was withdrawn at various times of incubation and diluted with 0.2 ml of cold 10 mM β-glycerolphosphate buffer, pH 6.8, containing 40 mM β-mercaptoethanol and 20 mM NaF. 100 μl of these diluted samples were then used for the determination of phosphorylase *a* activity at 30°C [9]. One mU of phosphorylase kinase catalyzes the formation of 1 mU of phosphorylase *a* per min under these conditions.

3. Results

As shown in fig.1, glucagon (2×10^{-9} M) caused a rapid activation of the endogenous glycogen phosphorylase as previously observed with intact anaesthetized rats [4]. Since maximal activation is obtained within 1 min, in subsequent experiments cells stimulated by glucagon for 1 min were used.

Glucagon, known to activate liver protein kinase in vivo [3,4], also activated this enzyme in isolated liver cells (table 1). When assayed without added

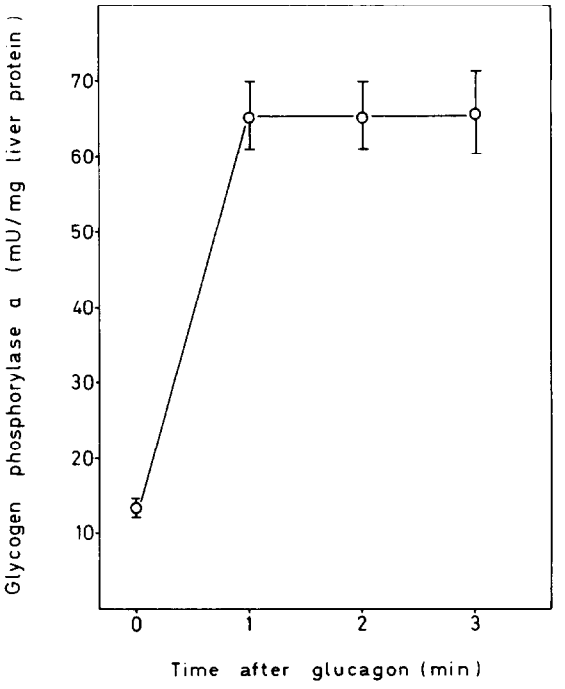

Fig.1. Kinetics of the activation of liver glycogen phosphorylase by glucagon in isolated liver cells. Liver cells (10^7/ml) were preincubated for 30–45 min with 20 mM glucose; phosphorylase *a* level was measured in aliquots taken immediately before and at various time intervals after the addition of glucagon (2×10^{-9} M). Results shown are the means of 10 experiments ± S.E.M.

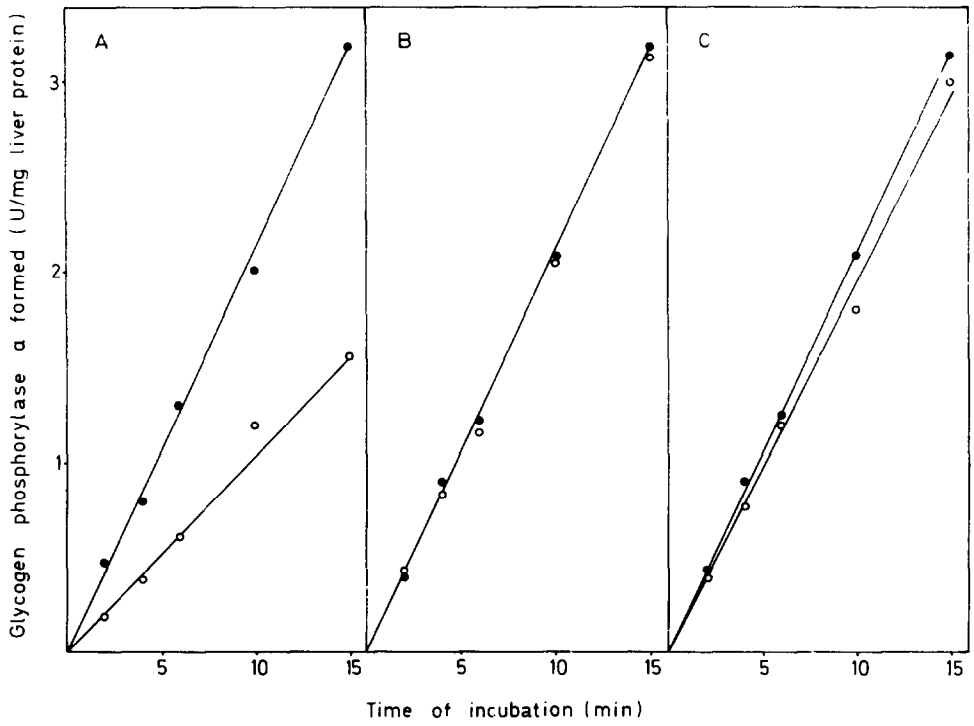

Fig.2. Effect of glucagon on the activity of liver phosphorylase kinase. Liver cells (5×10^6/ml) were preincubated for 45 min with 20 mM glucose; phosphorylase *b* kinase was assayed in samples taken immediately before and 1 min after the addition of 2×10^{-9} M glucagon, which raised endogenous phosphorylase *a* from 15 to 65, and protein kinase from 0.1 to 0.2 mU/mg liver protein. (A) homogenization of the liver cells in the buffer containing the protein kinase inhibitor; (B) homogenization in buffer only; (C) homogenization as in (B) but protein kinase inhibitor present in the assay medium for phosphorylase kinase (see also under 2.3.). (○) Before and (●) after glucagon.

cyclic AMP, the protein kinase activity of the glucagon (2×10^{-9} M) stimulated cells was twice that of the non stimulated cells. Higher concentrations of glucagon (10^{-6} M) caused a four- to five-fold increase (not shown). Total protein kinase activity, as measured in the presence of 10^{-6} M cyclic AMP, was not significantly affected.

When the activity of the phosphorylase kinase was determined in the presence of the protein kinase inhibitor, a clearcut activation of this enzyme was observed after glucagon (fig.2A). However, when the protein kinase inhibitor was not included in the dilution buffer to thaw out the liver cells, no difference was observed and the non-stimulated cells displayed now as much activity as the stimulated cells (fig.2B). About the same result was obtained when the cells were thawed out in the absence of the protein kinase inhibitor and then assayed for phosphorylase kinase in the presence of added inhibitor (fig.2C).

The average values of phosphorylase kinase recorded before and after the glucagon stimulation are presented in table 1. A nearly 2-fold stimulation by glucagon was observed. We checked that the increase in phosphorylase *a* formation induced by the hormone was not due to an enhanced AMP production leading to an erroneous overestimation of phosphorylase *a* activity due to a stimulation of the exogenous phosphorylase *b*.

4. Discussion

The results presented here clearly indicate that the activation of glycogen phosphorylase by glucagon

Table 1
Effect of glucagon on liver glycogen phosphorylase, protein kinase and phosphorylase *b* kinase

Enzyme	cAMP	Glucagon	
(mU/mg liver protein)	(10^{-6} M)	Before	After
Phosphorylase *a*		15.4 ± 1.4	61.2 ± 2.0[a]
Protein kinase	–	0.15 ± 0.02	0.33 ± 0.05[a]
	+	0.78 ± 0.05	0.79 ± 0.05
Phosphorylase *b* Kinase		110 ± 3	194 ± 7[a]

Enzyme activities were measured in liver cells (5×10^6/ml) before and after 1 min stimulation by 2×10^{-9} M glucagon. The cells had been preincubated with 20 mM glucose for 45 min. It was verified that no activity changes occurred when glucagon was not added to the cells. Values shown are means (± S.E.M.) for 6 experiments. Statistical significance was calculated from paired data.

[a] $P<0.005$

involves a substantial increase in the activity of liver phosphorylase kinase. This activation of phosphorylase kinase completes for the liver the cascade of kinase reactions leading to the formation of phosphorylase *a* as known to proceed in the muscle. Glucagon initiates glycogenolysis in the liver by stimulating the adenyl cyclase; the increase in intracellular cyclic AMP activates the protein kinase resulting in the activation of the phosphorylase kinase responsible for the increased production of glycogen phosphorylase *a*.

It should be noted that when no precaution was taken to add the protein kinase inhibitor when thawing out the liver cells, the phosphorylase kinase activity was already high without the addition of glucagon and was not further increased by the hormone. Furthermore, high activities of phosphorylase kinase were also recorded without hormonal stimulation when the protein kinase inhibitor was included too late, i.e. in the assay mixture of phosphorylase kinase, but after the cells had already been thawed out (fig.2). It seems therefore that the time it takes to thaw out the cell suspension is enough for protein kinase, even at O°C, to insure activation of the phosphorylase kinase.

These results show that the liver phosphorylase kinase, as the muscle enzyme, can be activated under the action of protein kinase, presumably in a phosphorylation step. This early blocking of protein kinase by its inhibitor seems to be necessary to show a glucagon promoted activation of phosphorylase kinase under our working conditions. This may not be an absolute requirement when using other experimental conditions as has been reported by others [5].

We conclude that glucagon causes a substantial activation of the liver phosphorylase kinase and that this activation is catalyzed by the cyclic AMP dependent protein kinase.

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