Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 912-918

HORMONAL CONTROL OF PHENYLALANINE HYDROXYLASE ACTIVITY IN ISOLATED RAT HEPATOCYTES

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Received November 10,1979

SUMMARY:Glucagon addition to isolated rat hepatocytes increases the level of Cyclic AMP inside the cells and the activity of the enzyme phenylalanine hydroxylase. These effects of glucagon are time and dose dependent and are detectable at hormone concentration as low as 0.02nM. The glucagon concentrations causing half-maximal increases in Cyclic AMP production and phenylalanine hydroxylase activity are 0.2 nM and 0.1 nM respectively. When hepatocytes are incubated with norepinephrine or the ionophore A23187, at concentrations between 1 nM and 10 μM, a slight increase in enzyme activity is seen only at the highest dose of either drug. The effect of norepinephrine can be completely antagonized by 20 μM propranolol but not by 20 μM ergocryptine. These results suggest that the activity of phenylalanine hydroxylase can be hormonally regulated, in vivo, through a phosphorylation mechanism catalyzed by a Cyclic AMP-dependent protein kinase.

INTRODUCTION

Rat hepatic phenylalanine hydroxylase has been shown to be a phosphoprotein containing 0.3 moles of phosphate per mole of hydroxylase subunit, MW=50,000 (1). Hydroxylase activity can be stimulated 2 to 3 fold by phosphorylation in vitro with an exogenous Cyclic AMP-dependent protein kinase. This effect was seen only when activity was determined with the natural cofactor tetrahydrobiopterin. The activation was accompanied by the incorporation of 0.7 moles of phosphate per subunit of hydroxylase (1). Moreover it had been shown (2) that the two major forms (3) of rat liver phenylalanine hydroxylase differed in their degree of phosphorylation and that they could be converted to a chromatographically distinct form by enzymatic phosphorylation in vitro. These results suggested that the hydroxylase might be regulated in vivo by hor-

0006-291X/80/030912-07$01.00/0

mones known to increase the levels of hepatic Cyclic AMP. Although it has been demonstrated that a pharmacological dose of glucagon could induce the stimulation of rat hepatic phenylalanine hydroxylase in vivo (4), no direct evidence of hormone effect on the enzyme activity had appeared up to now.

In this paper we report that, in isolated rat hepatocytes, addition of glucagon concentrations within the physiological range results in a time and dose dependent increase of phenylalanine hydroxylase activity correlated with an increase in the level of Cyclic AMP inside the cells.

MATERIALS AND METHODS

Hepatocytes were isolated by a modification (5) of the procedure described by Seglen involving a perfusion of liver with a calcium-free buffer followed by another one containing collagenase (6,7). Hepatocytes were incubated in Krebs-Ringer phosphate buffer, pH 7.5, containing 3% bovine serum albumin, 2,000 IU/ml of kallikrein inhibitor, 100 µg/ml of bacitracin, 0.2 mM of 3-isobutyl-1-methyl xanthine, without or with hormones at concentrations indicated in the figures legends. At the end of the incubation periods the cells were collected by centrifugation and either processed for Cyclic AMP measurement as already described (8), or resuspended in 0.5 ml of Tris-HCl buffer, pH 7.4, containing 100 mM NaF and sonicated for 20 sec with a sonifier cell disruptor Model B.12 from Branson Sonic Power Company. The homogenate was centrifuged at 16,000 g for 10 min in the cold. The supernatant was assayed for phenylalanine hydroxylase activity, in the presence of tetrahydrobiopterin, by following the conversion of [^{14}C] phenylalanine to [^{14}C] tyrosine at 25° (9).

Protein concentration was determined by the method of Lowry et al. (10) using bovine serum albumin as a standard.

Purified "monocomponent" porcine glucagon lot n° G 501575 was generously supplied by Dr. Schlichtkrull, The Novo Research Institute, Copenhagen, Denmark. Tetrahydrobiopterin was obtained by catalytic hydrogenation of biopterin (Regis Chemical Co., Morton Grove, Ill. U.S.A.). Bovine serum albumin (fraction V) was from Miles Laboratories and was extensively dialyzed against Krebs-phosphate buffer before use. Crude collagenase (type I, 140 IU/mg), 3-isobutyl-1-methyl xanthine, bacitracin, norepinephrine were from SIGMA. Kallikrein inhibitor (Trasylol 6,600 KIU/mg) was from Bayer AG and the ionophore A 23187 was from Calbiochem.

RESULTS

Fig.1A shows that incubation of hepatocytes, at 20°, in the presence of 1 nM glucagon, results in the accumulation of Cyclic AMP inside the cells which is maximum by 30 min. Fig.1B shows that this increase in Cyclic AMP is accompanied by an increase in phenylalanine hydroxylase activity. This activity is multiplied by a factor of two after 10 min of incubation of hepatocytes with 1 nM glucagon and remains constant thereafter.

Fig.2 presents the results of experiments in which the effect of the hormone on the increase in Cyclic AMP production was correlated with the activity of phenylalanine hydroxylase. These experiments were performed by incubating he-

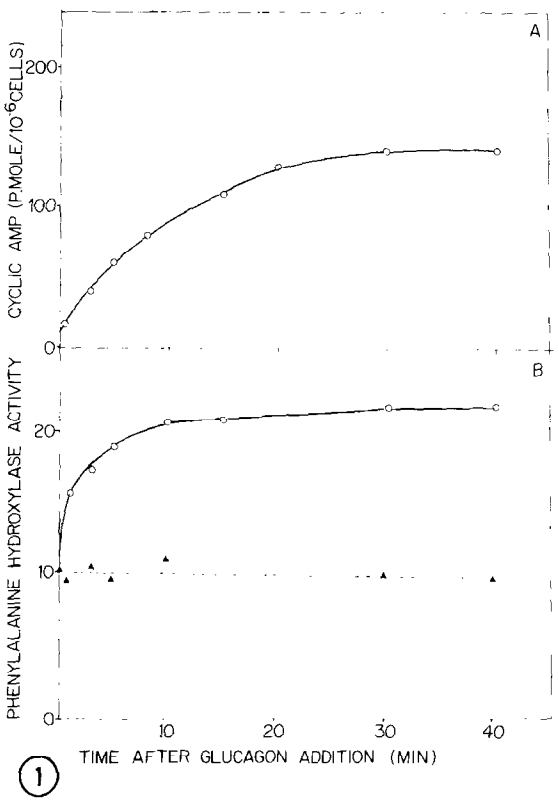

Fig 1. A: Time-course of glucagon stimulated Cyclic AMP accumulation in isolated rat hepatocytes. Cells were incubated, at 20°, in the presence of glucagon 1 nM. Each point is the mean of four values. In the absence of glucagon the level of Cyclic AMP inside the cells was 5.7±0.7 pmoles/10^6 cells.
B: Time-course of the effect of glucagon on phenylalanine hydroxylase activity . Hepatocytes were incubated without (▲- - -▲) or with (O——O) glucagon 1 nM at 20°. At intervals, aliquots were removed and cells extracts prepared. Phenylalanine hydroxylase activity was measured at 25° in the presence of tetrahydrobiopterin 20 µM and phenylalanine 0.1 mM and is expressed as nmoles of tyrosine formed per 15 min per mg of protein.

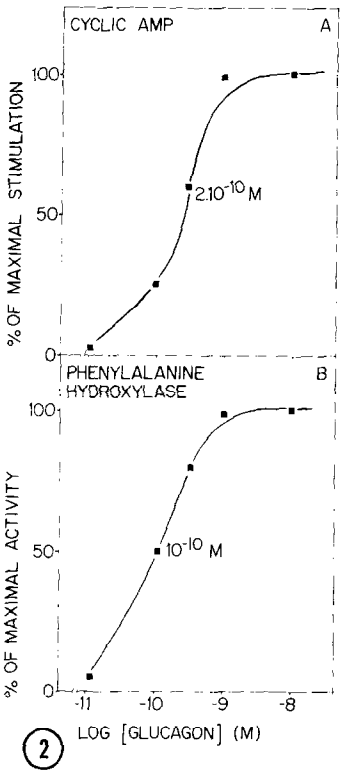

Fig 2. Correlation of the increase in Cyclic AMP production and the increase in phenylalanine hydroxylase activity. Hepatocytes were incubated at 20° for 30 min with graded doses of glucagon then processed for Cyclic AMP measurement and hydroxylase activity determination. All points were normalyzed to percentage of maximal stimulation to allow comparison of the data.

patocytes with graded doses of glucagon, at 20°, for 30 min, then preparing the cells extracts for Cyclic AMP measurement or for the hydroxylase assay. It can be seen that the hormone causes parallel increases in Cyclic AMP production and in hydroxylase activity. The glucagon concentrations causing half-maximal stimulation of Cyclic AMP production and phenylalanine hydroxylase activity are $2X10^{-10}$M and 10^{-10}M respectively.

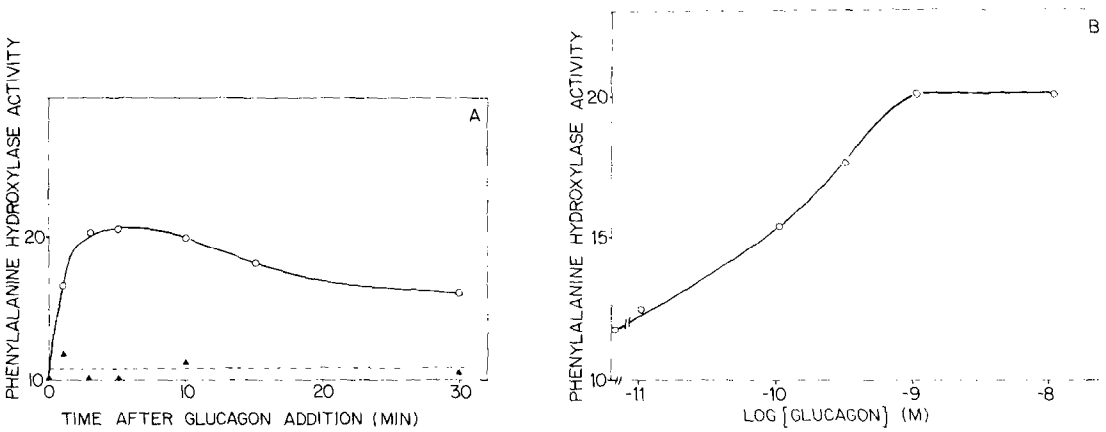

Fig 3. A: Time-course of glucagon stimulation of phenylalanine hydroxylase activity in hepatocytes incubated at 37°. Isolated cells were incubated without (▲- - -▲) or with (○—○) glucagon 1nM. Enzyme activity was measured and expressed as described in the legend of Fig 1B. B: Dose-response curve of the effect of glucagon addition to hepacytes, at 37°, on the activity of phenylalanine hydroxylase. Cells were incubated for 10 min with graded doses of glucagon before being processed for enzyme activity determination.

Fig 3A shows that 1 nM glucagon stimulates phenylalanine hydroxylase activity, to the same extent, when hepatocytes are incubated with the hormone at 37° instead of 20°. In these conditions the kinetics of activation is much more rapid and the maximum of activation is reached after 3 min. The activity of the hydroxylase decayed after 10min of incubation presumably because of a decrease in the level of Cyclic AMP (8,12) and also in response to protein phosphatase action more pronounced at this temperature (13). Fig 3B shows the effect of different concentrations of glucagon on hydroxylase activity in hepatocytes incubated at 37°. The half-maximally effective concentration of the hormone is, as it was at 20°, about 10^{-10}M.

Table I shows the effect of norepinephrine and of the calcium ionophore A 23187 on the activity of phenylalanine hydroxylase in hepatocytes incubated at 37°. Norepinephrine has a small activating effect only at the highest dose tested 10^{-5}M. It has been shown (8,14) that, at this concentration, norepinephrine can cause a modest increase in the level of Cyclic AMP in hepatocytes via activation of its β-adrenergic receptor. The data in Table I show that, indeed, the activation of phenylalanine hydroxylase, seen when hepatocytes were incubated for 10 min with norepinephrine 10^{-5}M, was abolished by propranolol but not by the α-blocker ergocryptine. The calcium ionophore A 23187 has no effect at concentrations from 10^{-9}M to 10^{-6}M and, like norepinephrine, a slight activating effect at 10^{-5}M.

Table I. Isolated rat hepatocytes were incubated , at 37°, for 10 min without or with different concentrations of hormones or drugs and extracts were prepared as described in Materials and Methods. Phenylalanine hydroxylase activity was measured in the presence of the natural cofactor tetrahydrobiopterin 20 µM and the substrate phenylalanine 0.1 mM, at 25°.

Hormone added	Hydroxylase activity nmoles Tyr formed/15 min/mg Protein
None	11,8
Glucagon 10^{-10}M	15,3
Norepinephrine 10^{-8}M	12,7
Norepinephrine 10^{-7}M	12,3
Norepinephrine 10^{-6}M	12
Norepinephrine 10^{-5}M	17,6
Norepinephrine 10^{-5}M+Ergocryptine 20 µM	16,8
Norepinephrine 10^{-5}M+Propranolol 20 µM	12,4
A 23187 10^{-9}M	12,3
A 23187 10^{-8}M	11,3
A 23187 10^{-7}M	12,4
A 23187 10^{-6}M	12,2
A 23187 10^{-5}M	15,2

D I S C U S S I O N

This report clearly demonstrates that addition of glucagon, at concentrations which are within the physiological range, to isolated rat hepatocytes results in a time and dose dependent activation of the enzyme phenylalanine hydroxylase. The half-maximally effective concentration of glucagon was 10^{-10}M. This concentration of the hormone is similar to that which is necessary for half-maximal stimulation of gluconeogenesis in hepatocytes (15) as well as for half- maximal inactivation of pyruvate kinase (16,17) and phosphofructokinase (18). It is noticeable that maximum enzymatic activation occurs at sub-maximal concentrations of Cyclic AMP (see Fig.1) and that glucagon is twice more potent in stimulating the enzyme activity than in promoting a rise in Cyclic AMP. Such a result has also been observed in the glucagon stimulation of the activity of another liver enzyme, phosphorylase (14).

These results extend the finding by Donlon and Kaufman (4) that phenylalanine hydroxylase activities, in extracts of liver of rats which received an intraperitoneal injection of 300 µg of glucagon, are higher than in controls. These authors had demonstrated that, in these conditions, there is an incorporation of $[^{32}P]$ into the enzyme protein. On the other hand, Abita et al. (1), had shown that pure rat liver phenylalanine hydroxylase is partly phosphorylated and can be fully phosphorylated

in the presence of ATP, Cyclic AMP and an exogenous protein kinase with a concomitant increase in the activity of the enzyme. Consequently, it is highly probable that the augmentation of the activity of phenylalanine hydroxylase seen after incubation of isolated rat hepatocytes with glucagon is due to its phosphorylation by an endogenous protein kinase.

It had been established that, in rat liver, catecholamines regulate glycogenolysis and gluconeogenesis mainly through an α-adrenergic mechanism that does not involve changes in the level of Cyclic AMP (19-22). On the other hand, results published recently (14,23) suggested that norepinephrine acted on isolated rat hepatocytes by promoting the phosphorylation of some of the cytoplasmic proteins that were also phosphorylated when hepatocytes were stimulated with glucagon. Among these proteins, only two were positively identified: phosphorylase and pyruvate kinase (23). The activity of phosphorylase was stimulated by 30%, 80% and 100% at norepinephrine concentrations of 10^{-7} M, 10^{-6} M and 10^{-5} M respectively (14). The phosphorylation and the activation of the enzyme were totaly abolished when hepatocytes were incubated with norepinephrine in the presence of the α-blocker ergotamine but not when the cells were incubated in the presence of the β-blocker propranolol (14).

On the contrary, under our conditions, the activity of phenylalanine hydroxylase was increased (60% over control) only when hepatocytes were incubated with the highest concentration of norepinephrine, 10^{-5} M. Moreover, this increase in activity was abolished in the presence of propranolol but not in the presence of the α-blocker ergocryptine. Thus it is highly probable that the increase in the activity of phenylalanine hydroxylase seen after incubation of rat hepatocytes with norepinephrine is not mediated by an α-adrenergic mechanism. This activation is certainly due to the increase in the level of Cyclic AMP (5 times the basal level) provoked by norepinephrine 10^{-5} M through activation of its β-receptor (8).

In conclusion, the data presented in this paper suggest that, at least in the rat, phenylalanine hydroxylase is regulated, in vivo, through a phosphorylation mechanism catalysed exclusively by a Cyclic AMP-dependent protein kinase.

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