INSULIN INACTIVATION OF RAT HEPATOCYTE CYCLIC AMP-DEPENDENT PROTEIN KINASE

M. Angels MOR, Jordi VILA, Carlos J. CIUDAD and Joan J. GUINOVART
Department de Bioquímica, Facultat de Farmacia, Universitat de Barcelona, Barcelona 28, Spain

Received 22 October 1981

1. Introduction

Insulin can activate liver glycogen synthase from a basal state (in the absence of glucose) [1]. Since glycogen synthase is regulated by covalent modification through phosphorylation, it could be expected that insulin would exert its action either by increasing the dephosphorylation catalyzed by protein phosphatases or by decreasing the phosphorylation catalyzed by protein kinases. Following the latter hypothesis, the hormone was shown to decrease synthase I kinase in perfused livers of fed rats [2] while in [3] no variation in the detectable activity of cyclic-AMP dependent histone kinase from liver was observed when insulin was injected into rabbits in vivo. In the liver, several enzymes are able to phosphorylate glycogen synthase [4]. The first to be discovered was cyclic AMP-dependent protein kinase. Subsequently cyclic AMP-independent glycogen synthase kinases have been described and studied [5,6].

Here, we have determined that insulin is able to inactivate the cyclic AMP-dependent protein kinase in liver cells, a process that would explain the mechanism producing the observed activation of glycogen synthase by insulin. We show that by employing a new low cAMP/high cAMP activity ratio assay, an inactivation of cyclic AMP-dependent protein kinase from rat hepatocyte after incubation with insulin can be detected. The inactivation is caused by a decrease in the sensitivity of protein kinase to its positive modulator cyclic AMP.

2. Materials and methods

2.1. *Preparation of hepatocytes homogenates*

Male Sprague-Dawley rats (200–220 g) were starved 24 h before hepatocyte isolation as in [7]. Cells were finally resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) pre-gassed with O_2/CO_2 (19:1). Aliquots (5 ml, 6–8 × 10^6 cells/ml) were poured into stoppered 30 ml vials and incubated at 37°C with shaking (100 strokes/min). Hepatocytes were allowed to equilibrate with the medium for 30 min before insulin addition. Immediately after incubation the contents of each vial were centrifuged (1000 × *g*, 20 s) and the cell pellet was homogenized at 2°C with the extraction buffer using a motor-driven Teflon–glass Potter-Elvehjem homogenizer. The cell homogenates were centrifuged at 10 000 × *g* for 15 min at 4°C and the supernatants were used for determination of enzymic activities.

2.2. *Enzyme assays*

For protein kinase activity assays, cell pellets were homogenized with 320 μl buffer containing 150 mM potassium fluoride, 10 mM EDTA, at pH 7.0. Protein kinase activity was assayed using histone as a substrate by the method in [8] in the presence of 1 mM theophylline to inhibit phosphodiesterase. Kinase activity was expressed as the ratio of activities measured:

(i) In the absence of added cAMP and in the presence of 2 × 10^{-5} M cAMP (−cAMP/+cAMP);
(ii) In the presence of 10^{-7} M cAMP and in the presence of 2 × 10^{-5} M (low cAMP/high cAMP).

To determine the degree of stimulation of protein kinase in control and insulin extracts at different concentrations of cAMP, the extracts were filtered through Ultragel AcA 202 columns (1 cm × 15 cm) equilibrated with the extraction buffer at 4°C, recovering only the peak of protein. Protein determinations were immediately performed on the filtered extracts and they were diluted to equal their protein concen-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

trations. Then, activity was measured in the presence of increasing concentrations of cyclic AMP. In experiments designed to separate cyclic AMP-dependent protein kinase from cyclic AMP-independent protein kinases, 500 μl extracts were applied to 1 cm × 2 cm phosphocellulose columns equilibrated with buffer A described in [6], and then washed with 1 ml same buffer A. Cyclic AMP-independent protein kinases were retained in the column, while cyclic AMP-dependent protein kinase was not. Cyclic AMP-dependent protein kinase activities were assayed in the eluates.

2.3. *Analytical methods*

When cellular cyclic AMP was to be determined, incubations were terminated by rapid centrifugation (1000 × *g*, 20 s) and 1 ml 5% (w/v) trichloroacetic acid was rapidly added to the resulting pellet. After centrifugation (3000 × *g*, 10 min), the supernatants were added with 10 μl 10 N HCl, then extracted 4 times with 5 ml water-saturated diethyl ether. After lyophylization, cyclic AMP was dissolved in 0.2 ml 100 mM sodium acetate (pH 4) and determined by the method in [9]. Protein was determined in cell homogenates by the biuret method in [10].

2.4. *Materials*

Collagenase, cyclic AMP, theophylline and histone II A were purchased from Sigma Chemical Co. Phosphocellulose P-11 was obtained from Whatman. Insulin proceeded from Eli Lilly Co. Cyclic[^{3}H]AMP and [γ-^{32}P]ATP came from New England Nuclear.

3. Results

When rat hepatocytes were incubated with insulin (10^{-8} M) and the activation state of the cyclic AMP-dependent protein kinase measured in the extracts by the standard –cAMP/+cAMP activity ratio assay, the activity ratio decreases were so small as to be of no statistical significance ($p > 0.5$). However, when the activation state of the enzyme was measured by determining the activity ratio at two concentrations of cAMP, one low (10^{-7} M) and one saturating (2 × 10^{-5} M), a clear-cut inactivation of the enzyme could be observed. The new activity ratio decreased by ~0.185 upon insulin incubation.

This effect of insulin was dependent on both the concentration of insulin and the time of incubation with the hormone. Fig.1 shows the time course of the

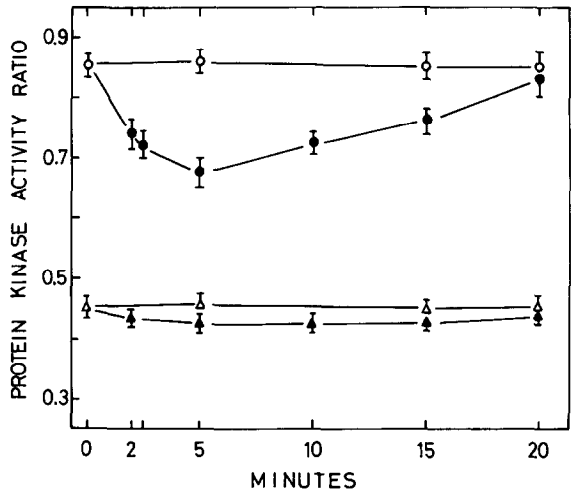

Fig.1. Changes in the standard –cAMP/+cAMP (△,▲) and low cAMP/high cAMP (○,●) activity ratios of protein kinase in rat hepatocytes incubated with 10^{-8} M insulin (▲,●) or saline (△,○) for the indicated times. Results are mean ± SD of ⩾4 expt. performed on different days.

effects of the hormone, maximal effects being observed at 5 min, and the low cAMP/high cAMP activity ratio returning to basal levels after 20 min. Fig.2 shows that the effect was already observed at 5 × 10^{-10} M

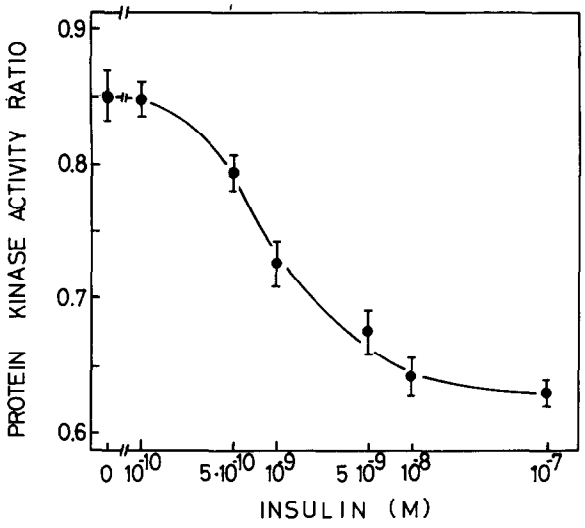

Fig.2. Dose-dependence of the effect of insulin in the low cAMP/high cAMP (10^{-7} M/2 × 10^{-5} M) (●) activity ratio of cyclic AMP-dependent protein kinase in liver cells. Hepatocytes were incubated for 5 min at the indicated concentrations of the hormone. Results are mean ± SD of ⩾4 expt. performed on different days.

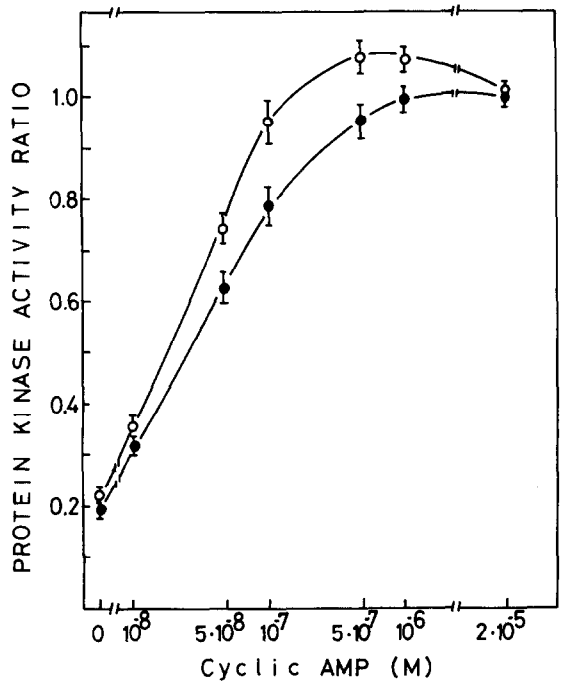

Fig.3. Cyclic AMP stimulation of protein kinase activity in control and insulin treated hepatocyte extracts. Liver cells were incubated with 10^{-8} M insulin (•) or saline (○) for 5 min. After gel filtration, the activity was determined at increasing concentrations of cyclic AMP. Results are expressed as ratios referred to the activity measured in the presence of 2×10^{-5} M cyclic AMP. Results are mean ± SD of ⩾4 expt. performed on different days.

insulin. The half-maximal dose was $\sim 10^{-9}$ M and maximal effect was reached at 10^{-8} M of the hormone.

These effects cannot be attributed to a decrease in the cAMP levels after insulin incubation, as measurements of the cell cyclic AMP content showed that there was no significant difference ($p > 0.4$) between saline (0.61 ± 0.05 nmol/g wet wt) and 10^{-8} M (5 min) insulin-treated hepatocytes (0.58 ± 0.04 nmol/g wet wt).

The kinetic basis of this effect was studied by assaying at several concentrations of cAMP the protein kinase activity of insulin and control extracts previously gel-filtered to remove endogenous cAMP (fig.3). Neither in the absence of added cAMP nor in the presence of a high concentration of the nucleotide, could any differences be observed between control and insulin extracts.

However, at intermediate concentrations of cAMP significant differences between insulin and control extracts were observed, maximal differences taking place at $\sim 10^{-7}$ M cAMP. That is, insulin increased $M_{0.5}$ for cAMP of rat hepatocyte protein kinase. This effect persisted after gel filtration and was maintained by freezing of the extracts. Extracts were passed through phosphocellulose columns to remove cyclic AMP-independent protein kinase also present in the liver extracts [6]. Cyclic AMP-dependent protein kinase was recovered in the first fraction and activity was measured. The low cAMP/high cAMP activity ratio significantly decreased ($p < 0.0005$) from 0.85 ± 0.03 to 0.69 ± 0.03 upon insulin treatment whereas the −cAMP/+cAMP activity ratio did not ($p > 0.45$) (saline 0.56 ± 0.03 and insulin 0.52 ± 0.03).

4. Discussion

These results demonstrate that insulin produces a lack of sensitivity of cyclic AMP-dependent protein kinase to its positive modulator cyclic AMP. This effect should result in an inactivation of the enzyme at non-saturating concentrations of cyclic AMP. This inactivation in fact is exerted on cyclic AMP-dependent protein kinase as it persists even when the cyclic AMP-independent protein kinases are separated from the extracts by absorption on phosphocellulose. It should be stressed that the inactivation of protein kinase by insulin is only observed when the low cAMP/high cAMP activity ratio or more sophisticated kinetic measurements are used. Almost no effect is observed when using the −cAMP/+cAMP assay.

In the high assay, the concentration of cAMP is 2×10^{-5} M equal to that generally used in the +cAMP assay. However, by adjusting the concentration of cAMP in the low assay, in accordance with the kinetic properties of protein kinase (as shown in fig.3), maximum sensitivity to the effect of insulin can be obtained. In our conditions, cAMP at 3×10^{-8} M to 10^{-7} M for the low assay were satisfactory. It could be argued that the cAMP used in the low assay was degraded by phosphodiesterase, known to be activated after insulin treatment [11]. This effect would lead to an artificial decrease in the protein kinase activity of insulin extracts measured at low cAMP as compared with controls. To avoid this situation, assays were performed in the presence of 1 mM theophylline to inhibit phosphodiesterase activity.

The effect of insulin is reflected in an increase in the $M_{0.5}$ for cAMP of the enzyme. It is worth noting

that no differences between control and insulin-treated cells could be observed at saturating concentrations of cAMP or in the absence of cAMP. That would explain why no effects of insulin on cAMP-dependent protein kinase had been observed using the –cAMP/+cAMP activity ratio.

This decrease in sensitivity of cyclic AMP-dependent protein kinase to cAMP from hepatocytes treated with insulin is similar to that in rat hemidiaphragms [12, 13], which led them to propose that a specific mediator of insulin might be bound to cyclic AMP-dependent protein kinase, decreasing its response to cyclic AMP. The possibility that glycogen synthase phosphatases may also play a role in the mechanism of glycogen synthase activation by insulin cannot be precluded. However, our results on cyclic AMP-dependent protein kinase could account for the observed effects of the hormone. To affirm that the inactivation of protein kinase is responsible for glycogen synthase activation, a determined sequence of events must be observed, i.e., the inactivation of protein kinase must precede the activation of glycogen synthase. This requirement is fulfilled as maximum inactivation of protein kinase takes place at 5 min cell incubation with the hormone, while glycogen synthase is maximally activated at 10 min [1].

In conclusion, the present results demonstrate the inactivating effect of insulin on cyclic AMP-dependent protein kinase in liver cells, which represents a further step in the elucidation of the mechanism by which insulin activates glycogen synthase even in the absence of glucose.

Acknowledgements

We thank Dr Emilio Itarte (U. A. B., Barcelona) for helpful discussions. This work has been sponsored by grants from the University of Barcelona (Ajut a la investigació) and M. Francisca de Roviralta Foundation. We are indebted to Mrs Mary Ann Newman for her valuable assistance in the preparation of the English manuscript.

References

[1] Ciudad, C. J., Bosch, F. and Guinovart, J. J. (1981) FEBS Lett. 129, 123–126.
[2] Miller, T. B. and Larner, J. (1973) J. Biol. Chem. 248, 3483–3488.
[3] Van de Werve, G., Stalmans, W. and Hers, H. G. (1977) Biochem. J. 162, 143–146.
[4] Schlender, K. K. and Reimann, E. M. (1977) J. Biol. Chem. 252, 2384–2389.
[5] Itarte, E., Mor, M. A., Pena, J. M., Salavert, A., Cusso, R. and Guinovart, J. J. (1979) FEBS Lett. 101, 347–350.
[6] Itarte, E., Mor, M. A., Salavert, A., Pena, J. M., Bertomeu, J. F. and Guinovart, J. J. (1981) Biochim. Biophys. Acta 658, 334–347.
[7] Massagué, J. and Guinovart, J. J. (1978) Biochim. Biophys. Acta 543, 269–272.
[8] Itarte, E., Robinson, J. C. and Huang, K. P. (1977) J. Biol. Chem. 252, 1231–1234.
[9] Gilman, A. G. (1970) Proc. Natl. Acad. Sci. USA 67, 305–312.
[10] Layne, E. (1957) Biochim. Biophys. Acta 540, 151–161.
[11] House, P. D. R., Poulis, P. and Werdemann, M. J. (1972) Eur. J. Biochem. 24, 429–437.
[12] Walkenbach, R. J., Hazen, R. and Larner, J. (1977) Mol. Cell. Biochem. 19, 31–41.
[13] Walkenbach, R. J., Hazen, R. and Larner, J. (1980) Biochim. Biophys. Acta 629, 421–430.