Biochem. J. (1977) **162**, 135–142
Printed in Great Britain

Hormonal and Ionic Control of the Glycogenolytic Cascade in Rat Liver

GERALD VAN DE WERVE, LOUIS HUE and HENRI-GÉRY HERS
Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, 1200 Brussels, Belgium

(*Received* 26 *July* 1976)

1. A parallel dose-dependent activation of histone kinase, phosphorylase kinase and phosphorylase was observed in isolated hepatocytes incubated in the presence of glucagon; the effect of suboptimal concentrations of glucagon was antagonized by insulin. 2. An activation of phosphorylase which was not accompanied by a stable change in the activity of phosphorylase kinase was observed in hepatocytes incubated with phenylephrine, isoproterenol or vasopressin as well as on decapitation of unanaesthetized animals. A dissociation of the two enzymic activities was also observed in hepatocytes incubated in the presence of a high concentration of glucose, in which phosphorylase was strongly inactivated with no change in the activity of phosphorylase kinase. 3. The activation of phosphorylase by phenylephrine in isolated hepatocytes was counteracted by insulin, greatly decreased by the absence of Ca^{2+} from the incubation medium, and completely suppressed by the replacement of Na^+ by K^+. 4. In a liver extract, phosphorylase kinase could also be activated by trypsin. Control, glucagon-activated or trypsin-activated phosphorylase kinase was inhibited by about 70% by EGTA and the activity was restored by the addition of Ca^{2+}. 5. The mechanisms that control the activity of phosphorylase kinase and of phosphorylase are discussed.

The antagonistic action of glucagon and insulin on glucose production and phosphorylase interconversion as well as on the formation of cyclic AMP and ion movements in the perfused rat liver is well established (for a review see Exton & Park, 1972). The role of cyclic AMP as an intermediary in the insulin effect on the liver is, however, still a matter of dispute (see, e.g., Claus & Pilkis, 1976).

On the other hand, the activation of liver glycogenolysis and gluconeogenesis by α-adrenergic agonists (Sherline *et al.*, 1972; Tolbert *et al.*, 1973; Exton & Harper, 1975), vasopressin (Kirk & Hems, 1974; Keppens & De Wulf, 1975) and angiotensin (Keppens & De Wulf, 1976) appears to be independent of a change in cyclic AMP concentration. It has also been shown that increasing the concentration of K^+, as well as that of glucose, in the incubation medium causes an inactivation of phosphorylase and a secondary activation of glycogen synthase in isolated hepatocytes (Hue *et al.*, 1975) and that the absence of Ca^{2+} affects the action of several agonists on both gluconeogenesis (Tolbert & Fain, 1974; Pilkis *et al.*, 1975) and phosphorylase activation (De Wulf & Keppens, 1976). Cations appear therefore to be possible substitutes for cyclic AMP in acting as intracellular messengers of the hormonal action.

The purpose of the present work was to investigate the mechanism by which phosphorylase is activated in the liver under various experimental conditions, and, more precisely, to check to what extent it could, in each case, be explained by the classical cascade of reactions which involves the activation of protein kinase by cyclic AMP, of phosphorylase kinase by protein kinase and of phosphorylase by phosphorylase kinase (Walsh & Krebs, 1973).

This cascade has been little studied in the liver, and it is only in recent years that an activation of protein kinase by glucagon *in vivo* (Sudilovsky, 1974) of phosphorylase kinase by cyclic AMP in liver extract (van de Werve *et al.*, 1974) and by glucagon *in vivo* (Shimazu & Amakawa, 1975) or in isolated hepatocytes (Vandenheede *et al.*, 1976) has been reported.

In the present paper we show that, whereas concerted activation of protein kinase (measured by the phosphorylation of histones), phosphorylase kinase and phosphorylase occurs by the action of glucagon, the activation of the three enzymes can be dissociated under other experimental conditions. Some properties of liver phosphorylase kinase are also described.

Materials and Methods

Proteins

Phosphorylase *b* was purified from rabbit muscle by the method of Fischer & Krebs (1962), and from pig liver as described by Stalmans & Hers (1975). The heat-stable protein inhibitor of cyclic AMP-

dependent protein kinase was purified from rabbit muscle, up to the step of trichloroacetic acid precipitation (Walsh *et al.*, 1971). It was verified that the inhibitor suppressed the activity of liver histone kinase and glycogen synthase kinase in the presence of cyclic AMP, and that purified muscle phosphorylase kinase (Brostrom *et al.*, 1971) was not inhibited. Collagenase (type I), bovine albumin (fraction V), calf thymus histone II-A, trypsin (type 1) and soya-bean trypsin inhibitor (type IS) were obtained from Sigma Chemical Co., St Louis, MO, U.S.A.

Chemicals

Glucose 1-phosphate was from Fluka A.G., Buchs, Switzerland. Sodium pentobarbital was from Serva, Heidelberg, Germany. 3′:5′-Cyclic AMP was obtained from Calbiochem, San Diego, CA, U.S.A., and was further purified by paper chromatography (van de Werve *et al.*, 1974). [γ-^{32}P]ATP came from The Radiochemical Centre, Amersham, Bucks., U.K. Vasopressin, phenylephrine, isoproterenol, glucose and shellfish glycogen (type II) were purchased from Sigma. Glucagon and insulin were obtained from Novo-Insutri A/S, Copenhagen, Denmark.

Experiments with rats in vivo

Well-fed male Wistar rats (200–250g) were anaesthetized by intraperitoneal injection of pentobarbital (0.1mg/g body wt). The abdomen was opened and the liver exposed. A loose ligature was placed around the base of the caudate lobe of the liver and a first biopsy was taken from this lobe 20min after the beginning of anaesthesia. To avoid bleeding, the ligature was tied soon after cutting the liver sample. At 5min after the first biopsy was taken, glucagon (0.1μg/g body wt.) was injected intravenously and a second biopsy was taken from the left lateral lobe 2min after the injection. Decapitation was performed after the same duration of anaesthesia (20min) and the liver was frozen within a few seconds. Non-anaesthetized animals were decapitated and the liver was quick-frozen within 12s. Biopsies were quick-frozen by the method of Wollenberger *et al.* (1960) and kept in liquid N_2 until further processing.

Isolation and incubation of hepatocytes

Hepatocytes were isolated from livers of fed Wistar rats as previously described (Hue *et al.*, 1975), except that (1) the cells were suspended and incubated in a Krebs–Henseleit (1932) bicarbonate buffer (Na^+ medium) or in a K^+ medium containing 118mM-KCl, 4.75mM-NaCl, 2.5mM-$CaCl_2$, 1.18mM-KH_2PO_4, 1.18mM-$MgSO_4$, 25mM-$KHCO_3$ [both media were in equilibrium with O_2+CO_2 (95:5) at pH7.4] and (2) the cell suspensions were centrifuged for 30s at 50*g*. Packed cells contained 220mg of protein/g (Hue *et al.*, 1975), as measured by the method of Lowry *et al.* (1951), with bovine albumin as a standard. Cell suspension (1ml, containing about 50mg of cells) was shaken (120strokes/min) in stoppered 20ml vials at 37°C in the presence of 10mM-glucose, bacitracin (1mg/ml), and, when added, 10nM-insulin. Bacitracin was added to prevent the degradation of the hormones (Desbuquois *et al.*, 1974). The gas phase was O_2+CO_2 (95:5). Other hormones or agonists dissolved in 0.9% NaCl were added after 30min of incubation; 2min later, 0.1ml samples of the cell suspension were pipetted and immediately frozen in tubes kept at the temperature of solid CO_2 in acetone until further processing.

Measurement of enzyme activities

Frozen liver biopsies were homogenized in a Potter–Elvehjem tube in an ice-cold solution of which the composition and volume varied according to the enzyme to be measured. Frozen samples of the liver-cell suspension were thawed by shaking them with the same solution and used for enzymic determinations without further homogenization of the cells, as indicated by Vandenheede *et al.* (1976).

Histone kinase. Protein kinase is known to catalyse the phosphorylation of several proteins, including histones, phosphorylase kinase and glycogen synthase. Its activity is currently measured by the phosphorylation of purified histones, and is referred to here as histone kinase. The solution used to prepare the liver extracts contained 0.25M-sucrose, 4mM-EDTA, 10mM-theophylline and 10mM-sodium phosphate buffer, pH7.4; 50μl of a 1% (w/v) liver extract was incubated at 30°C in polypropylene tubes containing 100μl of a solution of 0.3mM-[γ^{32}P]ATP (0.35μCi/test), 15mM-magnesium acetate, 1.5mM-theophylline, 0.2mM-dithiothreitol, 2.25mg of histone II-A/ml, 22mM-NaF, with or without 5μM-cyclic AMP. At various times of incubation up to 6min, 25μl portions were spotted on Whatman P81 paper (2cm×2cm) and the procedure was continued as described by Witt & Roskoski (1975). Each sample was run in duplicate or triplicate. Activity ratio is expressed as the activity in the absence of cyclic AMP divided by the total activity in the presence of cyclic AMP. Within one experiment, this latter activity was constant.

Phosphorylase kinase. The solution used to prepare the liver extracts contained 75mM-KCl, 125mM-KF, 2.5mM-EGTA, 12.5mM-glycylglycine, pH7.4, and 0.75mg of the heat-stable protein inhibitor/ml; 50μl of a 2.5% extract was added to 100μl of a solution containing 60 units of purified muscle phosphorylase *b* (except where otherwise stated)/ml, measured at

30°C, 0.15M-KCl, 5mM-EGTA and 25mM-glycylglycine at pH 7.4. The mixture was preincubated for 3min at 37°C and the reaction was started by the addition of 10μl of 32mM-ATP and 20mM-$MgSO_4$. At various times afterwards and up to 9min, 25μl samples were taken to measure the activity of phosphorylase *a* as described by Hue *et al.* (1975), except that the temperature was 30°C, the final concentration of caffeine was 5mM, and that EDTA was added at a final concentration of 10mM. This high caffeine concentration was required in order to counteract the stimulation of muscle phosphorylase *b* by small concentrations of AMP. EDTA was added to stop the ATP/Mg-dependent activation of phosphorylase. One unit of phosphorylase kinase is the amount of enzyme that catalyses the transformation of 1 unit of phosphorylase *b* into phosphorylase *a* per min under the conditions of the assay.

Phosphorylase a. The method used for the determination of glycogen phosphorylase *a* at 20°C and the homogenization medium used were previously described (Hue *et al.*, 1975). The unit of phosphorylase is the amount of enzyme that converts 1μmol of substrate/min in the conditions of the assay.

Results

Activation of phosphorylase and phosphorylase kinase in the liver of rats in vivo

Stalmans *et al.* (1974) had shown that the decapitation of non-anaesthesized mice caused an activation of phosphorylase and that this effect was greatly decreased under general anaesthesia. We show in Table 1 that a similar effect was obtained in the rat, but that the activation of phosphorylase caused by decapitation of non-anaesthetized rats was not accompanied by activation of phosphorylase kinase, whereas glucagon administered to anaesthetized animals activated both enzymes.

Experiments with isolated rat hepatocytes

The results shown in Figs. 1–3 are illustrative of a series of experiments which gave consistently the same type of results with, however, some quantitative variation with regard to the sensitivity of phosphorylase towards the effector. Fig. 1 shows that the addition of 0.3nM-glucagon to a suspension of isolated hepatocytes caused a transient activation of histone kinase, phosphorylase kinase and phosphorylase. This effect was already maximal within 2min of the addition of the hormone and then decreased progressively during the following 30min. Fig. 2 illustrated the dose-dependence of the glucagon effect, measured 2min after the addition of the hormone, and its antagonism by insulin; it is shown that glucagon caused a parallel activation of histone kinase, phosphorylase kinase and phosphorylase and incubation of the cells in the presence of 10nM-

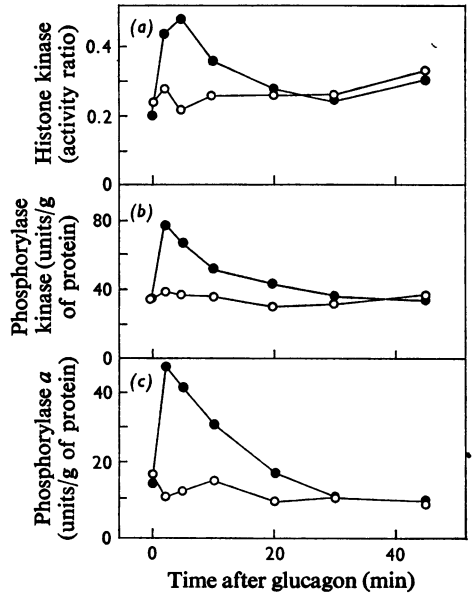

Fig. 1. *Time-course of the effect of glucagon on the activity of histone kinase, phosphorylase kinase and phosphorylase of isolated hepatocytes*
●, Glucagon (0.3nM); ○, control.

Table 1. *Activation of phosphorylase and phosphorylase kinase in vivo*
Experimental details are given in the Materials and Methods section. Values are means ± S.E.M. for three animals.

Experimental conditions	Phosphorylase *a* (units/g of protein)	Phosphorylase kinase (units/g of protein)
Anaesthesia	43.4±15.9*	46.4± 7.2*
Anaesthesia, intravenous glucagon	83.2± 9.7*	71.6±12.1*
Anaesthesia, decapitation	43.0±15.7	41.2± 4.1
No anaesthesia, decapitation	82.4±11.4	49.9± 8.1

* Samples were taken from the same livers, before and after administration of glucagon

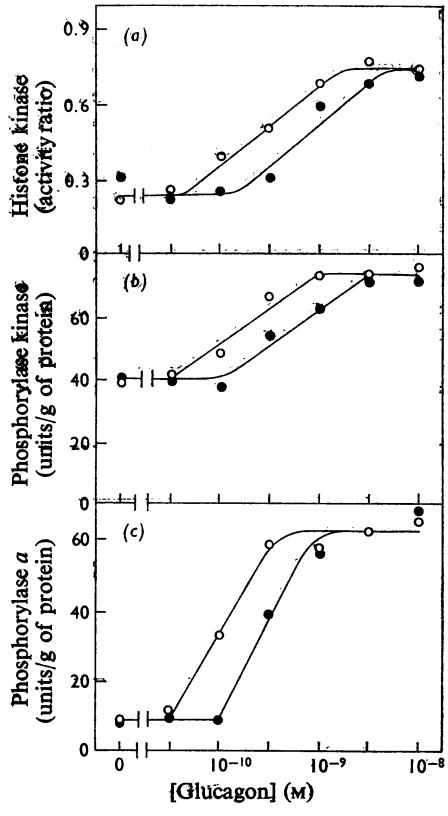

Fig. 2. *Effect of various concentrations of glucagon on the activity of histone kinase, phosphorylase kinase and phosphorylase of hepatocytes incubated with* (●) *or without* (○) *insulin*

insulin decreased their sensitivity to glucagon by a factor of 3.

Table 2 shows that phosphorylase could also be activated by phenylephrine, vasopressin and by a high concentration of isoproterenol and that these agonists did not cause an activation of phosphorylase kinase. Insulin had no effect on the activation of phosphorylase by isoproterenol. The sensitivity of the cells to phenylephrine varied from one experiment to another and, as illustrated in Fig. 3, was decreased by the presence of insulin. Further, the ability of phenylephrine to activate phosphorylase was not observed in a K^+ medium and was greatly decreased in a Na^+ medium without added Ca^{2+}. In all four experimental conditions, the activity of phosphorylase at 10nM-phenylephrine was the same as in the absence of phenylephrine.

The concentration of glucose in the incubation medium is another factor known to influence the proportion of phosphorylase that is in the active form

Table 2. *Activities of phosphorylase and phosphorylase kinase in isolated hepatocytes incubated with hormones or other agonists*

When indicated, values are means ± S.E.M., for four or 11 cell preparations. Experimental details are given in the Materials and Methods section.

Addition	Phosphorylase kinase activity (% of control)	Phosphorylase activity (% of control)
Phenylephrine		
10nM	102	81
0.1μM	111	119
1μM	111	305
10μM	89	348
0.1mM	120	319
Isoproterenol		
10nM	86	100
0.1μM	102	88
1μM	104	84
10μM	105	152
0.1mM	106	470
Isoproterenol + insulin (10nM)		
10nM	111	71
0.1μM	106	74
1μM	95	68
10μM	107	152
0.1mM	92	420
Vasopressin		
1munit/ml	104 ± 3 (4)	541 ± 20 (4)
Glucagon		
10nM	215 ± 9 (11)	657 ± 24 (11)

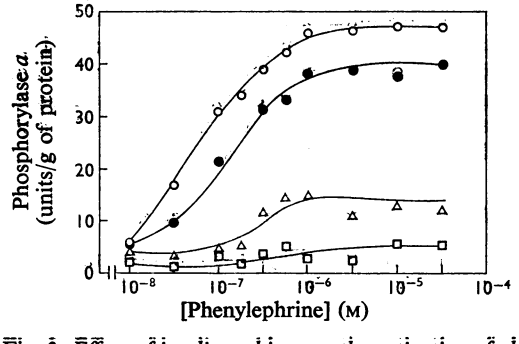

Fig. 3. *Effect of insulin and ions on the activation of phosphorylase by phenylephrine*

○, Control; ●, 10nM-insulin; △, incubation in a Na^+ medium without Ca^{2+}; □, incubation in a K^+ medium.

in isolated hepatocytes (Hue *et al.*, 1975). Table 3 shows that this glucose effect is not related to a change in the degree of activation of phosphorylase kinase.

Sensitivity of liver phosphorylase kinase to EGTA, Ca^{2+} and trypsin

Whereas muscle phosphorylase kinase is completely inactive in the presence of EGTA and is reactivated by Ca^{2+} (Ozawa *et al.*, 1971; Brostrom *et al.*, 1971), results for the liver enzyme are contradictory; indeed, in contrast with negative results reported by Krebs *et al.* (1973), several groups of workers have described a partial inactivation of liver phosphorylase kinase by EGTA (Shimazu & Amakawa, 1975; Khoo & Steinberg, 1975). Fig. 4 shows the effect of EGTA and various concentrations of Ca^{2+} on the activity of phosphorylase kinase of rat liver before and after glucagon treatment: both preparations were inhibited by about 70% in the presence of 7mM-EGTA and displayed an optimal activity at concentrations of Ca^{2+} close to 0.1 μM.

Trypsin is also known to cause an activation of muscle phosphorylase kinase (Krebs *et al.*, 1964). We show in Fig. 5 that a similar effect can be obtained on phosphorylase kinase of isolated hepatocytes incubated with or without glucagon. After trypsin activation, the activities of both preparations were about equal. The enzyme treated by trypsin was also inhibited about 60% by 7mM-EGTA and the activity was restored by the addition of very small (10nM) concentrations of Ca^{2+} (not shown).

Table 3. *Activities of phosphorylase and phosphorylase kinase in isolated hepatocytes incubated with various concentrations of glucose*

Isolated hepatocytes were incubated for 30min at 37°C as described in the Materials and Methods section in the presence of various concentrations of glucose. Pig liver phosphorylase *b* (35units/ml) was used as a substrate. Values are means ± S.E.M., for six cell preparations.

Glucose (mM)	Phosphorylase *a* (units/g of protein)	Phosphorylase kinase (units/g of protein)
10	7.3±0.6	14±0.2
40	2.9±0.3	15±0.5

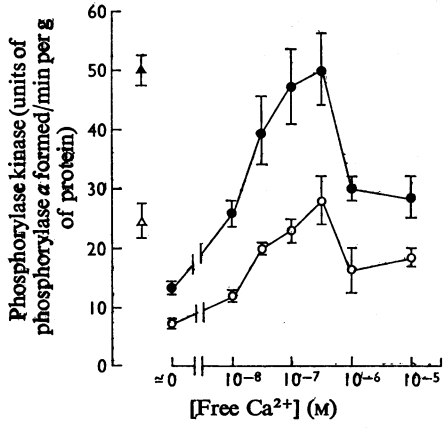

Fig. 4. *Effect of EGTA and Ca^{2+} on phosphorylase kinase activity*

Liver biopsies were taken before (○, △) and after (●, ▲) treatment with glucagon. Phosphorylase kinase activity was assayed in the presence (○, ●) or absence (△, ▲) of 6.6mM-EGTA and with increasing concentrations of free Ca^{2+}, calculated as described by Portzehl *et al.* (1964). Pig liver phosphorylase *b* (35units/ml) was used as a substrate. Values are means ± S.E.M., for three liver biopsies.

Discussion

In the following discussion, the words 'activation' and 'inactivation' are used to designate a stable

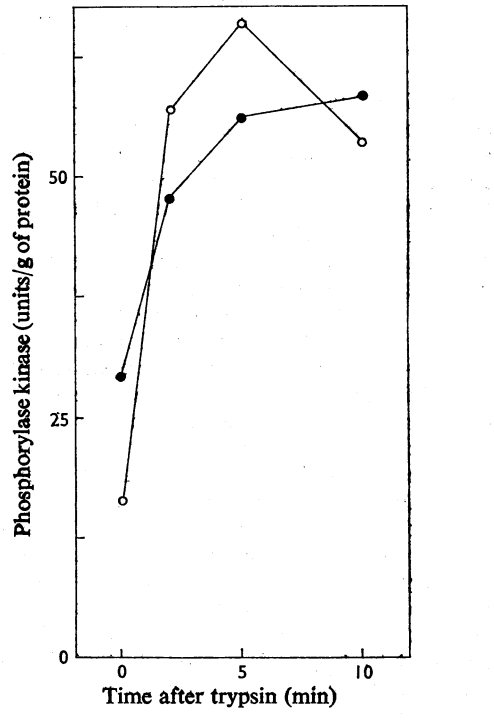

Fig. 5. *Time-course of the activation of phosphorylase kinase by trypsin*

A 1% extract of hepatocytes that had been incubated with (●) or without (○) 10nM-glucagon was incubated at 30°C in the presence of 13mM-glycylglycine, pH7.4, 133mM-KF, 80mM-KCl, 2.7mM-EGTA and 27 μg of trypsin/ml. At the time indicated, 1mg of soya-bean trypsin inhibitor/ml was added and phosphorylase kinase was assayed with pig liver phosphorylase *b* (35units/ml) as a substrate.

change in the activity of an enzyme as occurs, for instance, on phosphorylation or dephosphorylation of phosphorylase, phosphorylase kinase or glycogen synthase. In contrast, the words 'stimulation' and 'inhibition' designate changes in activity that are secondary to the freely reversible fixation of ligands, for instance ions or glucose, on the enzyme protein. The action of cyclic AMP on protein kinase is an intermediary situation, since, as reported by Corbin *et al.* (1975), the fixation of the nucleotide on only one of the two types of protein kinase existing in the liver is rapidly reversible. We will use the word 'activation' to designate an augmentation of the activity of histone kinase measured in the absence of added cyclic AMP.

Properties of liver phosphorylase kinase

The data presented in this paper confirm that the activity of phosphorylase kinase in the liver can be modulated by two mechanisms.

(1) The conversion of a less-active form into a more-active form. This conversion occurs under the action of glucagon and is presumably catalysed by cyclic AMP-dependent protein kinase (see below). The change in activity that we have registered under the action of glucagon varied from two- to three-fold, and it is not possible to say at the present time if the activity registered in the absence of glucagon is due to a partial activity of the less-active form or to the presence of a certain percentage of more-active form. A similar activation of phosphorylase kinase by glucagon has been described by Shimazu & Amakawa (1975) and by Vandenheede *et al.* (1976).

(2) Changes in Ca^{2+} concentration may also affect the activity of the enzyme; since there was an optimum of activity at 0.1–0.3 μM-Ca^{2+}, an increase in Ca^{2+} concentration may either stimulate or inhibit the enzymic activity. Stimulation that can be expected from an increase in Ca^{2+} concentration seems to be maximally three- to four-fold. The fact that the two forms of liver phosphorylase kinase displayed the same sensitivity towards Ca^{2+} is in contrast with the property of muscle phosporylase kinase, of which the active form is more sensitive to Ca^{2+} than the less active form (Brostrom *et al.*, 1971). The ability to be activated by trypsin is a property that liver phosphorylase kinase has in common with its muscle homologue.

Two mechanisms of phosphorylase activation

Our data indicate that there are at least two mechanisms by which phosphorylase can be activated in the liver.

(1) The activation of phosphorylase by glucagon is clearly related to an activation of phosphorylase kinase and histone kinase. The latter activation may reflect an increase in cyclic AMP concentration in the liver. The same doses of glucagon caused the activation of the three enzymes, and these doses were also very similar to those reported to stimulate cyclic AMP production, glycogenolysis, gluconeogenesis (Exton *et al.*, 1970) and pyruvate kinase inactivation (Felíu *et al.*, 1976). It therefore seems reasonable to assume that the cascade known to operate in muscle (Walsh & Krebs, 1973) also works in the liver.

(2) Phosphorylase can also be activated without a change in the proportion of the two forms of phosphorylase kinase in the liver. This was the case on decapitation of non-anaesthetized rats as well as on incubation of isolated hepatocytes in the presence of phenylephrine, vasopressin and isoproterenol. A likely explanation for the activation of phosphorylase is a stimulation of phosphorylase kinase by a change (presumably an increase) in Ca^{2+} concentration. This hypothesis is supported by the observation that activation of phosphorylase by phenylephrine is markedly decreased when the hepatocytes were incubated in the absence of Ca^{2+} (see also De Wulf & Keppens, 1976).

A complete suppression of the phenylephrine effect in a K^+ medium may be related to the known influence of univalent cations on the change in the intracellular Ca^{2+} concentration (for a review, see Rasmussen *et al.*, 1975). A change in the activity of phosphorylase phosphatase is another mechanism by which phenylephrine and other agonists might modify the concentration of phosphorylase *a* in the liver. The glucose effect, which, as shown in the present paper, is also not related to a stable change in the activity of phosphorylase kinase, has been previously explained by a stimulation of phosphorylase phosphatase (Hers, 1976; Stalmans, 1976).

Effect of insulin

Insulin seems to prevent the activation of phosphorylase by interfering at several steps of the glycogenolytic cascade.

(1) The property of insulin that prevents the activation of histone kinase, phosphorylase kinase and phosphorylase in the presence of low concentrations of glucagon is presumably related to the known effect of this hormone of preventing the accumulation of cyclic AMP under these experimental conditions (Exton & Park, 1972).

(2) Insulin also counteracted the activation of phosphorylase by phenylephrine, suggesting that it might affect Ca^{2+} concentration, which, as indicated above, seems to play a role in the action of the α-adrenergic agonist. It is of interest to recall that hepatic mitochondrial Ca^{2+} transport is stimulated in rats treated with insulin (Dormann *et al.*, 1975).

(3) We show in the following paper (van de Werve *et al.*, 1977) that the administration of insulin to rabbits *in vivo* decreases the activity of phosphorylase

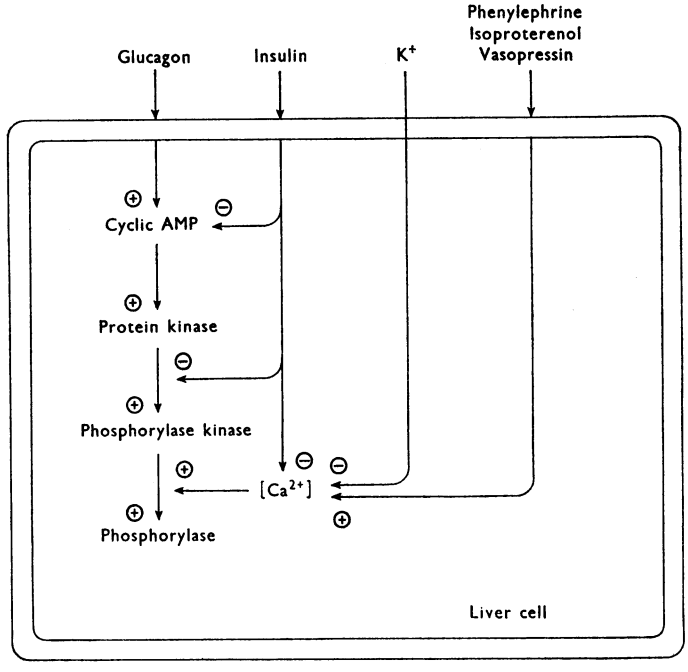

Fig. 6. *Mechanism of action of hormones and ions on the glycogenolytic cascade in the liver*

kinase and phosphorylase, without changing cyclic AMP concentration or the detectable activity of cyclic AMP-dependent histone kinase, suggesting an inhibition of phosphorylase kinase kinase or a stimulation of phosphorylase kinase phosphatase.

The different mechanisms discussed above are summarized in Fig. 6.

This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale and by the U.S. Public Health Service (grant AM 9235). L. H. is Chargé de Recherches of the Fonds National de la Recherche Scientifique.

References

Brostrom, C. O., Hunkeler, F. L. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1961–1967

Claus, T. H. & Pilkis, S. J. (1976) *Biochim. Biophys. Acta* **421**, 246–262

Corbin, J. D., Keely, S. L. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 218–225

Desbuquois, B., Krug, F. & Cuatrecasas, P. (1974) *Biochim. Biophys. Acta* **343**, 101–120

De Wulf, H. & Keppens, S. (1976) *Arch. Int. Physiol. Biochim.* **84**, 159

Dormann, D. M., Barritt, G. J. & Bygrave, F. L. (1975) *Biochem. J.* **150**, 389–395

Exton, J. H. & Harper, S. C. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 519–532

Exton, J. H. & Park, C. R. (1972) *Handb. Physiol. Section 7*, **1**, 431–455

Exton, J. H., Mallette, L. E., Jefferson, L. S., Wong, E. H. A., Friedmann, N., Miller, T. B., Jr. & Park, C. R. (1970) *Recent Prog. Horm. Res.* **26**, 411–461

Felíu, J. E., Hue, L. & Hers, H. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2762–2766

Fischer, E. H. & Krebs, E. G. (1962) *Methods Enzymol.* **5**, 369–373

Hers, H. G. (1976) *Annu. Rev. Biochem.* **45**, 167–189

Hue, L., Bontemps, F. & Hers, H. G. (1975) *Biochem. J.* **152**, 105–114

Keppens, S. & De Wulf, H. (1975) *FEBS Lett.* **51**, 29–32

Keppens, S. & De Wulf, H. (1976) *Arch. Int. Physiol. Biochim.* **84**, 167

Khoo, J. C. & Steinberg, D. (1975) *FEBS Lett.* **57**, 68–72

Kirk, C. J. & Hems, D. A. (1974) *FEBS Lett.* **47**, 128–131

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L. & Fischer, E. H. (1964) *Biochemistry* **3**, 1022–1033

Krebs, E. G., Stull, J. T., England, P. J., Huang, T. S., Brostrom, C. O. & Vandenheede, J. R. (1973) in *Protein Phosphorylation in Control Mechanism* (Huijing, F. & Lee, E. Y. C., eds.), pp. 31–45, Academic Press, New York

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Ozawa, E., Hosoi, K. & Ebashi, S. (1971) *J. Biochem. (Tokyo)* **61**, 531–533
Pilkis, S. J., Claus, T. H., Johnson, R. A. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 6328–6336
Portzehl, H., Caldwell, P. C. & Rügg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
Rasmussen, H., Jensen, P., Lake, W., Friedmann, N. & Goodmann, D. B. P. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 375–394
Sherline, P., Lynch, A. & Glinsmann, W. H. (1972) *Endocrinology* **91**, 680–690
Shimazu, T. & Amakawa, A. (1975) *Biochim. Biophys. Acta* **385**, 242–256
Stalmans, W. (1976) *Curr. Top. Cell. Regul.* **11**, 51–97
Stalmans, W. & Hers, H. G. (1975) *Eur. J. Biochem.* **54**, 341–350
Stalmans, W., De Wulf, H., Hue, L. & Hers, H. G. (1974) *Eur. J. Biochem.* **41**, 127–134
Sudilovsky, O. (1974) *Biochem. Biophys. Res. Commun.* **58**, 85–91
Tolbert, M. E. M. & Fain, J. N. (1974) *J. Biol. Chem.* **249**, 1162–1166
Tolbert, M. E. M., Butcher, F. R. & Fain, J. N. (1973) *J. Biol. Chem.* **248**, 5686–5692
Vandenheede, J. R., Keppens, S. & De Wulf, H. (1976) *FEBS Lett.* **61**, 213–217
van de Werve, G., Van Den Berghe, G & Hers, H. G. (1974) *Eur. J. Biochem.* **41**, 97–102
van de Werve, G., Stalmans, W. & Hers, H. G. (1977) *Biochem. J.* **162**, 143–146
Walsh, D. A. & Krebs, E. G. (1973) *Enzymes 3rd Ed.* **8**, 555–581
Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1977–1985
Witt, J. J. & Roskoski, R., Jr. (1975) *Anal. Biochem.* **66**, 253–258
Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol. Menschen Tieren* **270**, 399–412