Calcium ions and glycogen act synergistically as inhibitors of hepatic glycogen-synthase phosphatase

Lelo MVUMBI, Mathieu BOLLEN and Willy STALMANS

Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

1. We investigated the inhibitory effect of Ca^{2+} in the micromolar range on the activation of glycogen synthase in crude gel-filtered liver extracts [van de Werve (1981) *Biochem. Biophys. Res. Commun.* **102**, 1323–1329]. The magnitude of the inhibition was highly dependent on the glycogen concentration in the final liver extract. 2. Ca^{2+} inhibited the activation of purified hepatic synthase *b* by the G-component of synthase phosphatase, as present in the isolated glycogen–protein complex. The cytosolic S-component was not inhibited. Maximal inhibition of the crude G-component occurred at $0.3 \mu\text{M}\text{-Ca}^{2+}$. The inhibition was not influenced by the addition of either calmodulin or calmodulin antagonists, or by various proteinase inhibitors. 3. The use of purified G-component revealed that the inhibition by $0.3 \mu\text{M}\text{-Ca}^{2+}$ increased from 45% to 85% when the concentration of glycogen was raised from 1.5 to 20 mg/ml. 4. Muscle glycogen synthase, extensively phosphorylated *in vitro*, was also used as substrate for purified G-component. Activation and dephosphorylation were similarly inhibited by $0.3 \mu\text{M}\text{-Ca}^{2+}$, but the magnitude of the inhibition was much greater with the hepatic substrate. 5. No effect of $0.3 \mu\text{M}\text{-Ca}^{2+}$ was found on the activity of phosphorylase phosphatase in various liver preparations. 6. We conclude that the inhibition of synthase activation by Ca^{2+} is one of the mechanisms by which cyclic AMP-independent glycogenolytic hormones promote the inactivation of glycogen synthase in the liver, especially in the fed state.

INTRODUCTION

Several hormones (noradrenaline, vasopressin and angiotensin) cause glycogenolysis in rat liver by a mechanism that does not involve cyclic AMP. These agents generate two intracellular messengers: Ca^{2+} and diacylglycerol (Garrison *et al.*, 1984). The initial event in the generation of both messengers appears to be the breakdown of phosphatidylinositol bisphosphate (Michell, 1983; Berridge, 1984; Fain, 1984). The stimulation of phosphorylase kinase by Ca^{2+} explains to a large extent the activation of phosphorylase and hence the increased glycogenolysis (De Wulf *et al.*, 1980; Whitton, 1981; Exton, 1982). However, the activation of protein kinase C by diacylglycerol (Nishizuka, 1984) appears to potentiate the action of Ca^{2+} through an as yet unknown mechanism (Fain *et al.*, 1984; Kimura *et al.*, 1984).

These cyclic-AMP-independent hormones also cause a substantial inactivation of glycogen synthase (Hutson *et al.*, 1976; Hue *et al.*, 1978; Garrison *et al.*, 1979; De Wulf *et al.*, 1980). This hormonal effect has to be attributed, at least in part, to the increased intracellular concentration of Ca^{2+} , since it is mimicked by the Ca ionophore A23187 (Strickland *et al.*, 1980). Conversely, incubation of hepatocytes in the presence of EGTA causes a partial activation of glycogen synthase (Oron *et al.*, 1984) and impairs the inactivation of the enzyme by vasopressin and angiotensin (Garrison *et al.*, 1979; Strickland *et al.*, 1980). Several mechanisms have been proposed to account for the effect of Ca^{2+} on the activity of glycogen synthase (see the Discussion section). One proposal involves a direct inhibition of the conversion of glycogen synthase *b* into *a*. van de Werve (1981) reported on such an inhibition by micromolar concentrations of Ca^{2+} in gel-filtered mouse liver extracts.

The activity of glycogen-synthase phosphatase in the

liver results from the action of at least two enzymes: a G-component, which binds tightly to glycogen particles, and a cytosolic S-component (Doperé *et al.*, 1980). These two enzymes are regulated independently (Vanstapel *et al.*, 1980; Mvumbi *et al.*, 1983; Bollen & Stalmans, 1984; Bollen *et al.*, 1984). In the present paper we report on the characteristics of the inhibition by Ca^{2+} of the purified G-component of synthase phosphatase. Parts of the present work have been published in a preliminary form (Stalmans & Mvumbi, 1985; Mvumbi *et al.*, 1985).

EXPERIMENTAL

Materials and buffers

Leupeptin, chymostatin, pepstatin and soya-bean trypsin inhibitor were purchased from Sigma Chemical Co. Phenylmethanesulphonyl fluoride and calmidazolium (R 24571) were obtained from Janssen Chimica (Beerse, Belgium). Trifluoperazine was from Rhône-Poulenc. Calmodulin was prepared as described by Gopalakrishna & Anderson (1982). Particulate glycogen was purified from dog liver by the phenol method (Laskov & Margoliash, 1963). Na^{125}I , $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{C}^{14}]\text{glucose}$ 1-phosphate were obtained from Amersham Belgium. UDP- $[\text{C}^{14}]\text{glucose}$ was prepared as described by Tan (1979). ^{125}I -labelled casein was prepared by the general procedure of Markwell (1982), with the use of Iodo-beads (Pierce Chemical Co.), and isolated by successive filtrations on Sephadex G-25 and G-100.

Casein kinase-1 was purified as described by Itarte *et al.* (1983). Purified glycogen synthase *b* from dog liver was a pool of the forms termed *b*₂ and *b*₃ by Doperé *et al.* (1980). Glycogen synthase *a* was purified from rabbit muscle as described by Takeda *et al.* (1975). The enzyme was phosphorylated *in vitro* by casein kinase-1 as

described by Vila *et al.* (1982), except for the use of an up to 3-fold higher concentration of the kinase. Each phosphorylation was done in parallel with labelled and unlabelled ATP. After incubation for 90 min at 30 °C, the synthase was precipitated with $(\text{NH}_4)_2\text{SO}_4$, dissolved in standard buffer, and dialysed against the same buffer before its use as substrate for the phosphatase. Three such preparations of muscle synthase *b* contained 4–7 phosphate groups per subunit.

The standard buffer contained 0.25 M-sucrose, 0.5 mM-dithiothreitol and 50 mM-imidazole, adjusted at room temperature to pH 7.4.

Animals

Unless otherwise specified, male Wistar rats weighing about 250 g and fed *ad libitum* were used. When animals with depleted hepatic glycogen stores were required, they were fasted overnight and given 0.35 mg of glucagon intraperitoneally 30 min before death. Homozygous *gsd* rats (Malthus *et al.*, 1980) of both sexes were used without further treatment. All rats were killed by decapitation.

Gel-filtered liver extracts

The livers were rinsed in ice-cold 0.15 M-NaCl and homogenized in a Potter–Elvehjem device in 2.5 vol. of standard buffer. The homogenates were centrifuged for 10 min at 8000 *g* in the cold, and 1 ml of the supernatant ('extract') was filtered through a column of Sephadex G-25 (5 cm $\times$ 1.5 cm) equilibrated in cold standard buffer. The gel-filtered extracts were diluted to a final concentration of about 20% with respect to the liver homogenate from which they were derived. The additions included either 5 mM- $(\text{NH}_4)_2\text{SO}_4$ or, when explicitly mentioned, 3 mM-AMP and 5 mM-magnesium acetate; further additions were standard buffer ('control'), or standard buffer plus EGTA (final concn. 3 mM) ('EGTA'), or standard buffer with a mixture of 3 mM-EGTA and various concentrations of CaCl_2 (' Ca^{2+} '). The concentration of Ca^{2+} in the last condition was calculated as described by Portzehl *et al.* (1964), without correction for the chelation of calcium by other components of the incubation medium (van de Werve, 1981). The calculated concentration of Ca^{2+} was 0.3 μM , unless otherwise stated. The gel-filtered extracts were incubated at 25 °C, and samples were periodically withdrawn for assays of glycogen synthase and phosphorylase.

Assays of fractions of glycogen-synthase phosphatase

The two components of synthase phosphatase were separated from glycogen-depleted rat livers (see above) with the use of added particulate glycogen as described by Doperé *et al.* (1980). Unless otherwise stated, they were incubated in the presence of purified synthase *b* at a final concentration of 5% (S-component) or 20% (G-component) with respect to the homogenate from which they were derived. The G-component was partially purified from the protein–glycogen complex by chromatography on phosphocellulose and fractionation with polyethylene glycol as previously described (Mvumbi *et al.*, 1983).

The activity of synthase phosphatase was routinely determined by the rate of activation of purified glycogen synthase. The assay mixture (0.1 ml) contained liver synthase *b* (0.5 unit/ml), a preparation of synthase phosphatase, standard buffer, 500 μg of bovine serum albumin, and either 5 mM- $(\text{NH}_4)_2\text{SO}_4$ or, when explicitly

mentioned, 3 mM-AMP and 5 mM-magnesium acetate; buffer without or with $\text{EGTA} \pm \text{CaCl}_2$ was added as described above. During incubation of the mixture at 25 °C, samples (10 μl) were regularly withdrawn for the assay of glycogen synthase.

The synthase-phosphatase activity of the purified G-component was also assayed by the rate of activation and dephosphorylation of glycogen synthase *b* from muscle (1.3 unit/ml) in assay mixtures that were otherwise identical with those described in the preceding paragraph. During incubation at 25 °C samples were withdrawn for the determination of acid-soluble radioactivity.

Other assays and methods

Phosphorylase *a* was assayed as described by Stalmans & Hers (1975) and glycogen synthase (total and *a* form) as described by Doperé *et al.* (1980). One unit of these enzymes converts 1 μmol of substrate into product/min at 25 °C. Phosphorylase phosphatase was assayed in dilute liver preparations as described by Doperé & Stalmans (1982). Protein was determined as described by Bradford (1976), with reagent from Bio-Rad Laboratories.

Proteinase activity was measured in an assay mixture (50 μl) containing 16 μg of radioiodinated casein, 50 mM-imidazole, pH 7.4, and a proteinase preparation. The mixture was incubated at 25 °C, and samples were withdrawn periodically until 40 min for the determination of acid-soluble radioactivity.

Before the incubations, the purified glycogen-bound synthase was equilibrated with standard buffer by drop-dialysis for 45 min on a Millipore membrane type VSWP (pore size 25 nm) as described by Marusyk & Sergeant (1980). The same procedure was used in another experiment (Fig. 3) to lower the concentration of Ca^{2+} .

RESULTS

Effects of Ca^{2+} in crude extracts

Gel-filtered liver extracts from Wistar rats were incubated in the conditions described by van de Werve (1981). The inactivation of phosphorylase during incubation of these extracts was not affected by the addition of 3 mM-EGTA with or without CaCl_2 (Fig. 1*a*). The activation of glycogen synthase was preceded by an absolute lag that corresponds to the time required to inactivate phosphorylase. Thereafter, Ca^{2+} decreased the rate of activation of glycogen synthase, whereas 3 mM-EGTA alone had no consistent effect (Fig. 1*a*). The results in Fig. 1(*b*) show that the inhibitory effect of Ca^{2+} is equally well expressed in the presence of phosphorylase *a*; in the latter experiment, the addition of AMP and Mg^{2+} served to eliminate the inhibitory effect of phosphorylase *a* on synthase phosphatase, and to inhibit the inactivation of phosphorylase (Stalmans *et al.*, 1971; Mvumbi *et al.*, 1983).

The inhibitory effect of Ca^{2+} on the activation of glycogen synthase was variable, and in some experiments it was barely noticeable, at first without apparent reason. An extensive inhibitory effect of Ca^{2+} was, however, reproducibly obtained when gel-filtered extracts from *gsd* rats were used, whether in the presence of sulphate (Fig. 2*a*) or of AMP and Mg^{2+} (Fig. 2*b*). Livers from the latter animals contain about 13% glycogen, as a result of a genetically defective phosphorylase kinase (Malthus *et al.*, 1980). These gel-filtered liver extracts therefore

Fig. 1. Effects of Ca^{2+} on the inactivation of phosphorylase and on the activation of glycogen synthase in a gel-filtered liver extract from a Wistar rat

The gel-filtered liver extract of a rat killed at 09:00 h was incubated in the presence of sulphate (a) or AMP and Mg^{2+} (b) as such (control: ○, ●), or plus 3 mM-EGTA (EGTA: △, ▲) or 3 mM-EGTA and 2.7 mM- CaCl_2 (0.3 μM - Ca^{2+} : □, ■). Open symbols, synthesase a; filled symbols, phosphorylase a.

Fig. 2. Effects of Ca^{2+} on the inactivation of phosphorylase and on the activation of glycogen synthase in a gel-filtered liver extract from a gsd rat

Experimental conditions and symbols are as in the legend to Fig. 1.

contain about 25 mg of glycogen/ml. In glycogen-free gel-filtered liver extracts, obtained from fasted glucagon-treated Wistar rats, Ca^{2+} was almost without effect; however, when the homogenate was made in buffer containing glycogen to give a final concentration of

24 mg/ml after gel filtration, 0.3 μM - Ca^{2+} inhibited the activation of glycogen synthase by 70% (results not shown). A corollary for this synergism between Ca^{2+} and glycogen was found at the level of the G-component of synthase phosphatase (see below).

Effects of Ca^{2+} on synthase phosphatase in subcellular fractions

The activation of purified hepatic glycogen synthase *b* by the S-component, as present in the cytosolic fraction, was not influenced by 3 mM-EGTA with or without CaCl_2 for at least 45 min incubation (results not shown). In contrast, activation by the G-component, as present in the enzyme-glycogen complex, was strongly and reproducibly inhibited by $0.3 \mu\text{M}\text{-Ca}^{2+}$ (Fig. 3a). The reversibility of the Ca^{2+} effect was investigated in an experiment where either synthase *b* (Fig. 3b) or the crude G-component (Fig. 3c) was preincubated in the presence of either EGTA alone or EGTA plus CaCl_2 . After drop-dialysis, the assay of synthase phosphatase was initiated. In spite of the dialysis, the rate of activation in both preparations preincubated with EGTA plus CaCl_2 (Figs. 3b and 3c) was as low as before dialysis (Fig. 3a). This reflects probably the exquisite Ca^{2+} -sensitivity of the activation (see below); indeed, addition of 3 mM-EGTA after dialysis restored the activation rate either completely (Fig. 3b) or to 60% of the initial value (Fig. 3c).

When the concentration of CaCl_2 was varied in the presence of 3 mM-EGTA, the degree of inhibition observed at $0.3 \mu\text{M}\text{-Ca}^{2+}$ exceeded that occurring at $0.03 \mu\text{M}\text{-Ca}^{2+}$ and at $3 \mu\text{M}\text{-Ca}^{2+}$ (Fig. 4).

Other experiments (not shown) showed that the inhibitory effect of $0.3 \mu\text{M}\text{-Ca}^{2+}$ on the activation of hepatic synthase *b* by the crude G-component was not reinforced by the addition of calmodulin (up to $12 \mu\text{M}$). Neither was the inhibitory effect of Ca^{2+} decreased by calmodulin antagonists (up to $10 \mu\text{M}$ -trifluoperazine or 50 nM -calmidazolium).

Interference by a Ca^{2+} -dependent proteinase?

Some observations in the previous experiments prompted an investigation into the possible involvement of a Ca^{2+} -dependent proteinase: the observation that the inhibitory effect of Ca^{2+} was sometimes progressive during the incubation (Figs. 2 and 3a); the lack of effect of calmodulin and of calmodulin antagonists; and the possibly incomplete reversibility of the inhibition in one condition (Fig. 3c). Two Ca^{2+} -dependent proteinases have been characterized in several tissues, including liver; both act on casein, but they differ about 100-fold in their affinity for Ca^{2+} (Mellgren, 1980; Murachi *et al.*, 1981; Kishimoto *et al.*, 1983; Malik *et al.*, 1983; Yoshimura *et al.*, 1983).

With radioiodinated casein as substrate we were unable to detect a proteinase in the crude G-component, but several preparations of purified hepatic glycogen synthase *b* did hydrolyse casein to a measurable extent. However, several subsequent experiments (results not shown) led to the conclusion that this proteinase is not involved in the effect of Ca^{2+} on the activation of the synthase.

(i) An identical Ca^{2+} effect was observed on the activation by crude G-component of two preparations of glycogen synthase which differed at least 10-fold in proteinase content.

(ii) The properties of the proteinase that contaminated synthase *b* were not those of the proteinase that is activated by micromolar concentrations of Ca^{2+} . Indeed, the latter proteinase is completely inhibited by leupeptin and by chymostatin, at $10\text{--}50 \mu\text{g/ml}$, whereas soya-bean trypsin inhibitor ($0.1\text{--}2 \text{ mg/ml}$) has no effect (Mellgren, 1980; Kishimoto *et al.*, 1983; Malik *et al.*, 1983). In

Fig. 3. Effect of $0.3 \mu\text{M}\text{-Ca}^{2+}$ on the activation of purified hepatic synthase *b* by crude G-component

In (a) glycogen synthase was incubated in the presence of 5 mM- $(\text{NH}_4)_2\text{SO}_4$ and an enzyme-glycogen complex (crude G-component) at a concentration of 40% (with respect to the liver from which it was derived); buffer with or without EGTA $\pm \text{CaCl}_2$ was added as indicated in the legend to Fig. 1. In (b) the synthase *b* was preincubated for 10 min in the presence of 3 mM-EGTA, without (Δ) or with ($\square$, $\blacksquare$) $2.7 \text{ mM}\text{-CaCl}_2$. The mixture was then drop-dialysed for 45 min. Subsequently the crude G-component (at a final concentration of 20%) and 5 mM- $(\text{NH}_4)_2\text{SO}_4$ were added, without ($\square$) or with (Δ , $\blacksquare$) an additional 3 mM-EGTA. In (c) the enzyme-glycogen complex was preincubated and dialysed as described in (b), and then incubated with synthase *b* and sulphate $\pm$ EGTA as indicated in (b).

Fig. 4. Effect of the Ca^{2+} concentration on the activation of purified synthase *b* by crude G-component

The activation of synthase *b* by crude G-component (20% with respect to the liver from which it was derived) was monitored as in Fig. 3(a). The concentration of glycogen was about 3 mg/ml. Initial phosphatase activities were determined during incubation for 15 min and are expressed with respect to the phosphatase activity in the absence of EGTA and CaCl_2 . Zero Ca^{2+} represents 3 mM-EGTA only.

contrast, the casein-proteinase activity present in a preparation of synthase *b* was not influenced by either EGTA or Ca^{2+} . Neither was it influenced by leupeptin, chymostatin or pepstatin at 1 mg/ml, but more than 50% inhibition was exerted by 0.2 mg of soya-bean trypsin inhibitor/ml.

(iii) The effect of proteinase inhibitors was investigated on the activation of the proteinase-contaminated glycogen synthase *b* [see (ii) above]; the activation by G-component, in the presence of EGTA with or without CaCl_2 , was not influenced by any tested proteinase inhibitor, namely 1 mg of leupeptin, chymostatin or pepstatin/ml, 2 mg of soya-bean trypsin inhibitor/ml, or 4 mM-phenyl-methanesulphonyl fluoride.

(iv) As shown below, experiments with purified G-component argue against the involvement of a proteinase that attacks either synthase *b* or synthase phosphatase.

Experiments with purified G-component

The time-dependent activation of glycogen synthase by purified G-component proceeded linearly in the presence or absence of Ca^{2+} (Fig. 5a). The extent of inhibition was, however, smaller than in the experiments with crude G-component. This difference could be attributed to the near-complete removal of glycogen during the purification of the G-component, since an important inhibitory effect

of Ca^{2+} re-appeared on addition of glycogen to the incubation mixture (Fig. 5a). The dependence of the Ca^{2+} effect on the concentration of glycogen is illustrated in Fig. 5(b); when the glycogen concentration was increased in the range from 1.5 to 20 mg/ml, the inhibitory effect of $0.3 \mu\text{M-Ca}^{2+}$ rose from 45 to 85%. These glycogen concentrations did not affect the activation of synthase *b* in the absence of Ca^{2+} .

Synthase from skeletal muscle was phosphorylated *in vitro* and used as substrate in a few experiments. Ca^{2+} inhibited the dephosphorylation of the synthase by purified G-component (Fig. 6a), and this effect was matched by an equivalent inhibition of the activation of the enzyme (Fig. 6b). Similar patterns were obtained when the incubation mixture contained AMP and Mg^{2+} instead of sulphate (results not shown). The inhibitory effect of Ca^{2+} was reproducibly obtained with two preparations of purified G-component and three preparations of labelled synthase, but the magnitude of the Ca^{2+} effect on the activation of muscle synthase was considerably smaller than that observed with synthase from liver.

Phosphorylase phosphatase

Dilute gel-filtered liver extracts from fed and fasted Wistar rats and from *gsd* rats were incubated in the presence of ^{32}P -labelled phosphorylase *a*. EGTA with or without CaCl_2 did not influence the rate of dephosphorylation, whether or not 0.5% glycogen was added to the incubation medium. Neither was the phosphorylase-phosphatase activity in preparations of crude S- and G-component affected by Ca^{2+} (not shown).

DISCUSSION

Inhibition of synthase phosphatase by Ca^{2+}

Role of glycogen. The inhibitory effect of Ca^{2+} on the activation of glycogen synthase was discovered by van de Werve (1981) in a study of gel-filtered mouse liver extracts. However, during subsequent work on similar preparations from rat liver, Strickland *et al.* (1983) were unable to observe an effect of Ca^{2+} unless MgATP was added as a substrate for phosphorylase kinase. Our results show that the magnitude of the Ca^{2+} effect depends on the glycogen concentration in the range investigated (1.5–20 mg/ml). They indicate that the discrepant results may well be explained by the actual glycogen concentration in the liver extracts. One factor that can account for some variability in the experimental results is the nutritional state of the animals. Rats and mice feed at night and have maximal hepatic glycogen stores (50–60 mg/g) early in the morning. Subsequently their liver glycogen is being degraded at an average rate of 6 mg/g per h (Ray *et al.*, 1964). Another factor of possible significance is the dilution of the liver extracts. Strickland *et al.* (1983) used a final tissue concentration of 10%. We used a 2-fold higher concentration, as did van de Werve (1981), who furthermore added glycogen (5 mg/ml) to the final preparation.

Other characteristics. The basic findings of van de Werve (1981) were reproduced in our study with purified enzymes, including the biphasic Ca^{2+} -dependence with an optimal effect around $0.3 \mu\text{M-Ca}^{2+}$. The major difference was that in our experiments the inhibitory effect of Ca^{2+} was expressed in the presence as well as in the absence

Fig. 5. Interaction of glycogen and Ca^{2+} in the activation of hepatic synthase *b* by purified G-component

In (a) the incubation medium contained 5 mM- $(\text{NH}_4)_2\text{SO}_4$ and 3 mM-EGTA without (Δ , $\blacktriangle$) or with ($\square$, $\blacksquare$) 2.7 mM- CaCl_2 , and glycogen at either 1.5 mg/ml (Δ , $\square$) or 20 mg/ml ($\blacktriangle$, $\blacksquare$). In (b) the rate of activation is shown at the indicated glycogen concentrations in the presence of EGTA $\pm$ CaCl_2 .

Fig. 6. Effect of Ca^{2+} on the dephosphorylation (a) and activation (b) of muscle synthase *b* by purified G-component

Muscle synthase, phosphorylated to 6 phosphate groups per subunit, was incubated with purified G-component in the presence of 5 mM- $(\text{NH}_4)_2\text{SO}_4$, 20 mg of glycogen/ml and 3 mM-EGTA without (Δ) or with ($\square$) 2.7 mM- CaCl_2 .

of phosphorylase *a*. Since Ca^{2+} and phosphorylase *a* are both generated in response to cyclic-AMP-independent glycogenolytic agents, a particularly strong inhibition of synthase phosphatase can be expected in these conditions (see below).

Mechanism of the inhibition. The effect of Ca^{2+} was not influenced by calmodulin or calmodulin antagonists.

However, the latter findings should not necessarily exclude any implication of calmodulin. Indeed, Strickland *et al.* (1983) have reported an analogous insensitivity of phosphorylase kinase in liver extracts, and yet there is little doubt that calmodulin is a subunit of liver phosphorylase kinase and confers Ca^{2+} -sensitivity to the enzyme (Chrisman *et al.*, 1982). The involvement of a Ca^{2+} -stimulated proteinase in the present results was

investigated with care, but this hypothesis had to be discarded.

Clear Ca^{2+} effects were only observed with the G-component of synthase phosphatase. This would favour the view that Ca^{2+} acts on the phosphatase, rather than on the substrate. However, the substrate seems to be important as well, since (in otherwise identical conditions) the activation of muscle synthase was considerably less inhibited than was the activation of the liver enzyme.

Effects of Ca^{2+} on protein phosphatases have previously been described. An extensive inhibition of phosphorylase phosphatase activity has been reported during the Ca^{2+} -triggered 'flash activation' of phosphorylase in a concentrated glycogen-enzyme complex from skeletal muscle (Haschke *et al.*, 1970), but the exact mechanism was not clarified. Ca^{2+} in the micromolar range caused also a profound inhibition of a protein phosphatase (acting on troponin T and on phosphorylase *a*) associated with the membranes of the sarcoplasmic reticulum from skeletal muscle (Varsányi & Heilmeyer, 1979). In both studies the effect of Ca^{2+} was lost on purification of the enzyme. Indirect evidence has been adduced in favour of a Ca^{2+} -inhibited synthase phosphatase in polymorphonuclear leucocytes (Juhl & Borregaard, 1981). However, it is difficult to reconcile this hypothesis with a stimulation of synthase phosphatase by Ca^{2+} , as proposed soon afterwards by the same research group (Juhl *et al.*, 1982). Calcineurin is a Ca^{2+} -dependent protein phosphatase which, however, does not act on glycogen synthase or phosphorylase (Stewart *et al.*, 1983).

Mechanism of the inactivation of glycogen synthase by cyclic-AMP-independent hormones

Current evidence indicates that the cyclic-AMP-independent glycogenolytic hormones can use either branch of the phosphatidylinositol pathway to achieve the inactivation of glycogen synthase, and the mechanisms are known to some extent. One part of the ultimate effect is mimicked by phorbol esters (Roach & Goldman, 1983). It involves presumably the liberated diacylglycerol, which stimulates protein kinase C. Kishimoto *et al.* (1978) reported initially on the phosphorylation and inactivation of muscle glycogen synthase by a proteolytically activated form of protein kinase C. The native kinase also phosphorylates glycogen synthase (Takai *et al.*, 1979), and the topography of the phosphorylation sites has been investigated (Ahmad *et al.*, 1984; Imazu *et al.*, 1984b). However, it remains unclear whether phosphorylation by the native protein kinase C does cause a substantial inactivation of glycogen synthase from muscle and liver (Ahmad *et al.*, 1984; Imazu *et al.*, 1984a).

The other part of the effect of cyclic-AMP-independent hormones depends on an intracellular increase of Ca^{2+} . The search for an enzyme that mediates the latter signal has initially focused on Ca^{2+} -stimulated synthase kinases. Two such kinases have been identified. A calmodulin-dependent protein kinase was isolated from rabbit liver (Payne *et al.*, 1983). However, phosphorylation of hepatic glycogen synthase by the latter kinase hardly caused any inactivation (Camici *et al.*, 1984; Imazu *et al.*, 1984a). Phosphorylase kinase is also able to phosphorylate liver glycogen synthase, without inactivation (Akatsuka *et al.*, 1984) or with a mild decrease of activity (Camici *et al.*, 1984; Imazu *et al.*, 1984a). It seems therefore that these Ca^{2+} -dependent protein kinases have only a minor

role in the inactivation of glycogen synthase by cyclic-AMP-independent glycogenolytic hormones.

Another Ca^{2+} -dependent mechanism has been proposed some years ago by Keppens & De Wulf (1977). In their hypothesis the target enzyme is again phosphorylase kinase, which is stimulated by Ca^{2+} , but it would operate exclusively by converting phosphorylase *b* into *a*. Since phosphorylase *a* is a potent inhibitor of glycogen-synthase phosphatase (Stalmans *et al.*, 1971; Mvumbi *et al.*, 1983), the unopposed action of synthase kinase(s) should lead to a lower activity state of glycogen synthase. Observations on isolated hepatocytes (De Wulf *et al.*, 1980) were in agreement with this hypothesis: the inactivation of glycogen synthase by glucagon started immediately, but the inactivation by various cyclic-AMP-independent glycogenolytic agents was preceded by a lag during which phosphorylase was activated. Strickland *et al.* (1983) confirmed the operation of such an indirect mechanism in crude liver extracts. They studied the stimulatory effect of Ca^{2+} on the inactivation of glycogen synthase in the presence of MgATP. Making use of rats deficient in phosphorylase kinase, of purified enzymes, and of antibodies, they were able to show that the effect of Ca^{2+} had an absolute requirement for both phosphorylase kinase and its substrate, phosphorylase *b*.

It thus appears at present that the inactivation of glycogen synthase by cyclic-AMP-independent glycogenolytic agents is the result of several mechanisms acting in a concerted way. Evidence *in vivo* (Roach & Goldman, 1983) indicates that a direct phosphorylation of glycogen synthase by protein kinase C is likely to be involved. Two mechanisms should furthermore inhibit the antagonistic action of the G-component of synthase phosphatase: one is a direct inhibition of the enzyme by Ca^{2+} (van de Werve, 1981; the present work); the other is an inhibition of synthase phosphatase by phosphorylase *a*, which in turn has been produced by the Ca^{2+} -stimulated phosphorylase kinase.

One can at present only speculate on the relative importance of these three mechanisms. However, the nutritional state of the animal could be important. Indeed, the inhibition of synthase phosphatase by phosphorylase *a* as well as by Ca^{2+} is rather poor in glycogen-depleted livers (Hue *et al.*, 1975; the present work). On the other hand, the activity of protein kinase C on glycogen synthase is drastically inhibited by small concentrations of glycogen (Ahmad *et al.*, 1984). Thus, in the fed state cyclic-AMP-independent hormones may cause the inactivation of liver glycogen synthase chiefly through the inhibition of synthase phosphatase; an enhanced kinase activity may be an important mechanism in the fasted state.

This work was supported by the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Grant 3.0051.82). We are grateful to Dr J. G. T. Sneyd and Dr R. Malthus (Dunedin), who donated *gsd* rats, to Dr E. Itarte (Barcelona) for gifts of muscle glycogen synthase *a* and of casein kinase-1, to Dr F. Wuytack (Leuven) for a gift of calmodulin, and to Dr M. Van den Berghe (Rhône-Poulenc Belgique) for a sample of trifluoperazine. Ms N. Sente provided expert technical assistance.

REFERENCES

- Ahmad, Z., Lee, F.-T., DePaoli-Roach, A. & Roach, P. J. (1984) *J. Biol. Chem.* **259**, 8743-8747

- Akatsuka, A., Singh, T. J. & Huang, K.-P. (1984) *J. Biol. Chem.* **259**, 7878–7883
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Bollen, M. & Stalmans, W. (1984) *Biochem. J.* **217**, 427–434
- Bollen, M., Doperé, F., Goris, J., Merlevede, W. & Stalmans, W. (1984) *Eur. J. Biochem.* **144**, 57–63
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Camici, M., Ahmad, Z., DePaoli-Roach, A. A. & Roach, P. J. (1984) *J. Biol. Chem.* **259**, 2466–2473
- Chrisman, T. D., Jordan, J. E. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 10798–10804
- De Wulf, H., Keppens, S., Vandenheede, J. R., Haustraete, F., Proost, C. & Carton, H. (1980) *Horm. Cell Regul.* **4**, 47–71
- Doperé, F. & Stalmans, W. (1982) *Biochem. Biophys. Res. Commun.* **104**, 443–450
- Doperé, F., Vanstapel, F. & Stalmans, W. (1980) *Eur. J. Biochem.* **104**, 137–146
- Exton, J. H. (1982) *Trends Pharmacol. Sci.* **3**, 111–115
- Fain, J. N. (1984) *Vitam. Horm. (N.Y.)* **41**, 117–160
- Fain, J. N., Li, S.-Y., Litosch, I. & Wallace, M. (1984) *Biochem. Biophys. Res. Commun.* **119**, 88–94
- Garrison, J. C., Borland, M. K., Florio, V. A. & Twible, D. A. (1979) *J. Biol. Chem.* **254**, 7147–7156
- Garrison, J. C., Johnsen, D. E. & Campanile, C. P. (1984) *J. Biol. Chem.* **259**, 3283–3292
- Gopalakrishna, R. & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun.* **104**, 830–836
- Haschke, R. H., Heilmeyer, L. M. G., Jr., Meyer, F. & Fischer, E. H. (1970) *J. Biol. Chem.* **245**, 6657–6663
- Hue, L., Bontemps, F. & Hers, H.-G. (1975) *Biochem. J.* **152**, 105–114
- Hue, L., Feliu, J. E. & Hers, H.-G. (1978) *Biochem. J.* **176**, 791–797
- Hutson, N. J., Brumley, F. T., Assimacopoulos, F. D., Harper, S. C. & Exton, J. H. (1976) *J. Biol. Chem.* **251**, 5200–5208
- Imazu, M., Strickland, W. G., Chrisman, T. D. & Exton, J. H. (1984a) *J. Biol. Chem.* **259**, 1813–1821
- Imazu, M., Strickland, W. G. & Exton, J. H. (1984b) *Biochim. Biophys. Acta* **789**, 285–293
- Itarte, E., Plana, M., Guasch, M. D. & Martos, C. (1983) *Biochem. Biophys. Res. Commun.* **117**, 631–636
- Juhl, H. & Borregaard, N. (1981) *Biochim. Biophys. Acta* **675**, 101–109
- Juhl, H., Kristoffersen, P. & Esmann, V. (1982) *Biochem. Biophys. Res. Commun.* **106**, 210–216
- Keppens, S. & De Wulf, H. (1977) *Abstr. FEBS Meet.* 11th A1–9/107
- Kimura, S., Nagasaki, K., Adachi, I., Yamaguchi, K., Fujiki, H. & Abe, K. (1984) *Biochem. Biophys. Res. Commun.* **122**, 1057–1064
- Kishimoto, A., Mori, T., Takai, Y. & Nishizuka, Y. (1978) *J. Biochem. (Tokyo)* **84**, 47–53
- Kishimoto, A., Kajikawa, N., Shiota, M. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 1156–1164
- Laskov, R. & Margoliash, E. (1963) *Bull. Res. Counc. Isr. Sect. A* **11**, 351–362
- Malik, M. N., Fenko, M. D., Iqbal, K. & Wisniewski, H. M. (1983) *J. Biol. Chem.* **258**, 8955–8962
- Malthus, R., Clark, D. G., Watts, C. & Sneyd, J. G. T. (1980) *Biochem. J.* **188**, 99–106
- Markwell, M. A. K. (1982) *Anal. Biochem.* **125**, 427–432
- Marusyk, R. & Sergeant, A. (1980) *Anal. Biochem.* **105**, 403–404
- Mellgren, R. L. (1980) *FEBS Lett.* **109**, 129–133
- Michell, B. (1983) *Trends Biochem. Sci.* **8**, 263–265
- Murachi, T., Tanaka, K., Hatanaka, M. & Murakami, T. (1981) *Adv. Enzyme Regul.* **19**, 407–424
- Mvumbi, L., Doperé, F. & Stalmans, W. (1983) *Biochem. J.* **212**, 407–416
- Mvumbi, L., Bollen, M. & Stalmans, W. (1985) *Abstr. Spec. FEBS Meet. Metal Ions, Proteins and Membranes*, p. 85
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Oron, Y., Emerson, J. F., Garrison, J. C. & Lerner, J. (1984) *Cell Calcium* **5**, 143–158
- Payne, M. E., Schworer, C. M. & Soderling, T. R. (1983) *J. Biol. Chem.* **258**, 2376–2382
- Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
- Ray, P. D., Foster, D. O. & Lardy, H. A. (1964) *J. Biol. Chem.* **239**, 3396–3400
- Roach, P. J. & Goldman, M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7170–7172
- Stalmans, W. & Hers, H.-G. (1975) *Eur. J. Biochem.* **54**, 341–350
- Stalmans, W. & Mvumbi, L. (1985) *Biochem. Soc. Trans.* **13**, 890
- Stalmans, W., De Wulf, H. & Hers, H.-G. (1971) *Eur. J. Biochem.* **18**, 582–587
- Stewart, A. A., Ingebritsen, T. S. & Cohen, P. (1983) *Eur. J. Biochem.* **132**, 289–295
- Strickland, W. G., Blackmore, P. F. & Exton, J. H. (1980) *Diabetes* **29**, 617–622
- Strickland, W. G., Imazu, M., Chrisman, T. D. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 5490–5497
- Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979) *J. Biol. Chem.* **254**, 3692–3695
- Takeda, Y., Brewer, H. B., Jr. & Lerner, J. (1975) *J. Biol. Chem.* **250**, 8943–8950
- Tan, A. W. H. (1979) *Biochim. Biophys. Acta* **582**, 543–547
- van de Werve, G. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1323–1329
- Vanstapel, F., Doperé, F. & Stalmans, W. (1980) *Biochem. J.* **192**, 607–612
- Varsányi, M. & Heilmeyer, L. M. G., Jr. (1979) *Biochemistry* **18**, 4869–4875
- Vila, J., Salavert, A., Itarte, E. & Guinovart, J. J. (1982) *Arch. Biochem. Biophys.* **218**, 1–7
- Whitton, P. D. (1981) in *Short-term Regulation of Liver Metabolism* (Hue, L. & van de Werve, G., eds.), pp. 45–62, Elsevier/North-Holland Biomedical Press, Amsterdam
- Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M. & Murachi, T. (1983) *J. Biol. Chem.* **258**, 8883–8889