Vol. 114, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1983 Pages 148-154

COORDINATED FEEDBACK REGULATION OF MUSCLE GLYCOGEN METABOLISM: INHIBITION OF PURIFIED PHOSPHORYLASE PHOSPHATASE BY GLYCOGEN

Ronald L. Mellgren and Michael Coulson

Department of Pharmacology and Therapeutics
Medical College of Ohio
Toledo, Ohio 43699

Received May 25, 1983

An assay method was devised for accurately measuring the activity of phosphorylase phosphatase in the presence of glycogen. Both oyster glycogen and rabbit liver glycogen inhibited the activity of purified phosphorylase phosphatase catalytic subunit in a concentration-dependent manner. In the presence of 120 mM KCl, micromolar AMP increased the sensitivity of the phosphatase to glycogen inhibition. The present studies suggest that glycogen feedback inhibition may be a coordinated mechanism producing a decrease in phosphorylase phosphatase activity as well as glycogen synthase phosphatase activity.

The feedback inhibition of muscle glycogen synthase activity by glycogen has been recognized for many years (1,2). Increased concentrations of glycogen inhibit synthase phosphatase activity, resulting in a decrease in the rate of conversion of glycogen synthase to the active I form, and a decreased rate of glycogen biosynthesis. Similar studies of the effect of glycogen on impure muscle phosphorylase phosphatase activities have produced equivocal results (reviewed in 3). In general, either activation of phosphorylase phosphatase by glycogen (4), or no effect (2,5) at physiological glycogen concentrations has been observed. Stalmans et al. (6) observed a very minor degree of inhibition of crude mouse liver phosphorylase phosphatase by 48 mg/ml glycogen. Lower glycogen concentrations appeared to activate the phosphatase (6). We have reassessed the possible role of glycogen in feedback regulation of rabbit skeletal muscle phosphorylase phosphatase activity utilizing a highly purified rabbit muscle phosphorylase phosphatase catalytic subunit.

MATERIALS AND METHODS

Materials. Oyster glycogen and rabbit liver glycogen were obtained from Sigma Chem. Co., and stock solutions were further purified by treatment

0006-291X/83 $1.50

with a deionizing resin (7). Bio-Gel A-1.5 m was obtained from Bio-Rad. DEAE-Sepharose, Phenyl-Sepharose, and Sephadex G-200 Superfine were products of Pharmacia Fine Chemicals obtained through Sigma. ^{32}P-Labeled rabbit muscle phosphorylase a was prepared from crystallized phosphorylase b as previous described (8). Grade IV 5'-nucleotidase from Crotalus atrox venom was obtained from Sigma. Rabbit muscle phosphorylase phosphatase catalytic subunit was purified to near-homogeneity from the partially purified holoenzyme by a procedure which will be described in detail elsewhere. Briefly, the purification steps were: chromatography of muscle extract on DEAE-cellulose followed by successive column chromatography on Bio-Gel A-1.5 m,Phenyl-Sepharose, and Sephadex G-200 Superfine. The Phenyl-Sepharose step brought about dissociation of the holoenzyme complex. The purified catalytic subunit used in these studies had a Stokes radius of 2.3 nm on Sephadex G-200 gel filtration, and a molecular weight of 31,000 determined by SDS-polyacrylamide gel electrophoresis. It had a specific activity of 11,000 units/mg protein utilizing the phosphatase assay and unitage in (8), and it had relatively little activity on phosphorylase kinase, phosphoprotamine or phosphorylated lysine-rich histone. Thus, it had properties similar to the previously described catalytic subunit of rabbit muscle phosphorylase phosphatase (9). All other materials used were of reagent grade or equivalent quality.

Methods. Phosphorylase phosphatase was assayed at 37^{o}C. All components of the assay mixture except the phosphatase were preincubated at 25^{o}C for 2 hours, and at 37^{o}C for 30 min. The incubation mixture contained in a volume of 50 µl, 25 µg [^{32}P] phosphorylase a, 2-3.5 milliunits of phosphatase, and various concentrations of glycogen, in 50 mM Tris-acetate, 1 mM dithiothreitol, pH 7.5. After 10 min. of reaction, the incubation was terminated by the addition of 50 µl of 30% w/v trichloroacetic acid and the test tubes placed on ice. After 10 min. 50 µl of 95% ethanol was added to each tube to precipitate any glycogen: phosphorylase complex present. After a further 20 min. on ice, the samples were centrifuged and 50 µl aliquots were withdrawn for liquid scintillation counting of ^{32}P released by phosphatase activity. In all experiments involving KCl or AMP, these constituents were present in controls as well as glycogen-containing assays. Thus, the data presented in this paper represent specific effects of glycogen.

RESULTS

Modified assay of phosphorylase phosphatase in the presence of glycogen. In early studies, the phosphorylase phosphatase assay was terminated by the addition of trichloroacetic acid to 10% w/v. After 10 min. at 4^{o}C the precipitated [^{32}P] phosphorylase was sedimented and samples of the supernatant were counted in a liquid scintillation counter (8). The results from these studies were variable; glycogen appearing in various experiments to activate, inhibit, or have no significant effect on phosphorylase phosphatase activity. In a control experiment, it was observed that the addition of 20 mg/ml rabbit liver glycogen to 0.2 mg/ml [^{32}P] phosphorylase a prevented nearly half of the phosphorylase from being precipitated by 10% trichloroacetic acid (10 min. incubation at room temperature). In the absence of glycogen, essentially all of the

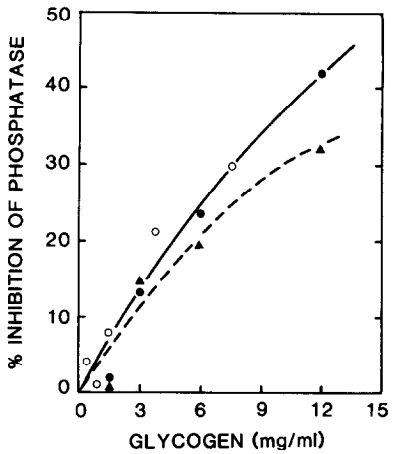


Figure 1. Inhibition of purified phosphorylase phosphatase by shellfish glycogen and rabbit liver glycogen. Phosphorylase phosphatase activity was measured as described in the Experimental section in the presence of various concentrations of shellfish (●) or rabbit liver (○) glycogen. In one experiment with shellfish glycogen 4.8 μM AMP was included in all assay mixtures (▲---▲). The phosphatase concentration in each experiment was 40 milliunits/ml. This concentration of phosphatase produced 30.0% dephosphorylation of phosphorylase in 10 min. in the absence of AMP and glycogen, and 16.2% dephosphorylation in the presence of 4.8 μM AMP and no glycogen.

phosphorylase was precipitated under the same conditions. Thus, the variable results in the phosphatase assays may have been due to this effect of glycogen. By terminating the phosphatase incubation with 15% trichloroacetic acid and then precipitating glycogen with the further addition of ethanol to 32% v/v, there was complete precipitation of [^{32}P]phosphorylase in the presence of glycogen. Including ethanol in the assay scheme did not influence reagent blank values or the measurement of phosphatase activity in the absence of glycogen.

Inhibition of phosphorylase phosphatase by glycogen.

Dephosphorylation of phosphorylase a by the purified rabbit muscle phosphorylase phosphatase was inhibited in a concentration-dependent manner by glycogen from oyster or rabbit liver (Fig. 1). The inhibition occurred at concentrations of glycogen normally present in muscle (5-10 mg/g muscle - ref. 10). Although the glycogen used in these studies was treated to remove ionic contaminants, we considered the possibility that traces of the potent phosphatase inhibitor AMP ($I_{0.5}$ = 5.0 μM under the assay conditions used

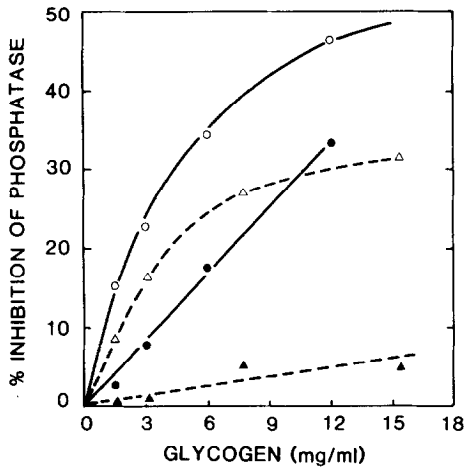


Figure 2. The effects of KCl and AMP on glycogen inhibition of phosphorylase phosphatase. The inhibition of phosphatase by shellfish glycogen was studied in buffers containing 120 mM KCl (●) or 120 mM KCl and 4.8 μM AMP (○). In a separate experiment, rabbit liver glycogen was used: ▲ ,plus 120 mM KCl; △ , plus 120 mM KCl and 4.8 μM AMP. The phosphatase concentration in both experiments was 70 milliunits/ml. At this concentration of phosphatase and in the absence of glycogen and AMP, phosphorylase a was 14.9% dephosphorylated in the 10 min. assay. In the presence of 4.8 μM AMP and absence of glycogen, the phosphorylase a was 10.7% dephosphorylated.

in Fig. 1) might produce the observed inhibition. However, the inhibitory effect could not be abolished by treatment of oyster glycogen with activated charcoal (ca. 1 mg/mg glycogen) or by a 60 min. incubation with 50 μg/ml snake venom 5'-nucleotidase at 30°C. Under these conditions, the nucleotidase could completely hydrolyze a 1 mM solution of AMP within 15-30 minutes.

Effect of KCl and AMP on glycogen inhibition. Since the ionic strength of the standard phosphatase assay mixture used in the above studies was lower than would occur in the cell, the effect of 120 mM KCl on glycogen inhibiton was studied. This concentration of KCl decreased the phosphatase activity measured in the absence of glycogen by 3.5-fold. KCl greatly decreased the inhibition by rabbit liver glycogen, and slightly decreased the inhibitory effect of oyster glycogen (Fig. 2). However, the addition of 4.8 μM AMP increased the inhibitory effect of both glycogen samples. The increase in inhibition by glycogen was dependent on AMP concentration (Table I), and the effect was most pronounced at low concentrations of glycogen

Table I. The effect of AMP concentration on inhibition of phosphorylase phosphatase by shellfish glycogen in the presence of 120 mM KCl.

Glycogen (mg/ml)	AMP[a] (μM)	%Inhibition[a] by Glycogen
3.2	0	9.7
3.2	2.4	13.1
3.2	4.8	22.7
8.0	0	34.4
8.0	2.4	36.7
8.0	4.8	37.4
8.0	9.6	45.1

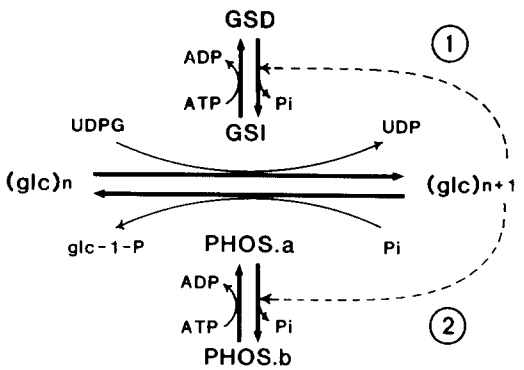
[a]When present, AMP was added to control (no glycogen) as well as glycogen-containing assays. Thus, the % inhibition in the third column represents specific inhibition by glycogen.

(Fig. 2, Table I). In the absence of KCl, AMP had little effect on glycogen inhibition of phosphorylase phosphatase (Fig. 1).

DISCUSSION

Glycogen, AMP, glucose, glucose-6-phosphate and various other metabolites, have been shown to influence the activities of various protein kinase and phosphatase activities involved in glycogen metabolism by binding to their substrates, glycogen synthase and phosphorylase (3,11,12). Previous studies have suggested that glycogen either activates phosphorylase phosphatase (4), or does not influence its activity (2,5). Recent literature reviews have stated that glycogen is an activator of muscle phosphorylase phosphatase (11,12). The results presented here suggest that under physiologic conditions glycogen may inhibit phosphorylase phosphatase. There are several possible reasons for these different results.

1. Glycogen inhibition is evident only in certain conditions. For instance, in the presence of 120 mM KCl, 1.6-16 mg/ml rabbit liver glycogen did not significantly inhibit phosphatase unless AMP was present (Fig. 2).
2. Although AMP is known to increase the affinity of rabbit muscle phoshorylase a for glycogen (13), no studies to our knowledge have investigated the possibility that AMP may sensitize phosphorylase



Scheme 1.

phosphatase to glycogen effects. 3. Since impure phosphatase preparations were used in previous studies showing glycogen activation, it is conceivable that these preparations contained a phosphatase activity which was stimulated by glycogen, or a factor may have been present which allowed glycogen stimulation of the phosphatase.

The discovery that glycogen can inhibit the purified catalytic subunit of phosphorylase phosphatase under near physiologic buffer conditions suggests a dual role for the polysaccharide in the feedback inhibition of phosphatase activity. Dashed arrow 1 in Scheme 1 shows the previously described effect of glycogen in decreasing the conversion of glycogen synthase (GS) to the active I form by inhibiting synthase phosphatase (1,2). The present studies indicate that glycogen may also increase the steady state concentration of phosphorylase a by decreasing the rate of its dephosphorylation and inactivation by phosphorylase phosphatase (dashed arrow 2). The combined effect would lead to a decrease in the steady state glycogen concentration by an initial decrease in the rate of its biosynthesis and an increase in its breakdown. A further factor which may contribute to this feedback mechanism is the previously described activation of rabbit muscle phosphorylase kinase by glycogen (14). The concentration-dependent effect of AMP on glycogen inhibition would tend to increase the sensitivity of phosphorylase phosphatase to glycogen inhibition during anaerobic glycosis, thus allowing more efficient glycogen

utilization. Evaluation of the importance of this possible mechanism of regulation requires more extensive studies involving other effectors of phosphorylase phosphatase activity including glucose-6-phosphate and ATP. A combined effect of ATP and glycogen in inhibition of leukocyte synthase phosphatase has been described (15).

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Keith Schlender for his comments on the manuscript and for supplying some of the materials used in this study. This work was supported by NIH grant AM28277 and by a grant from the American Heart Association.

REFERENCES

1. Danforth, W.H. (1965) J. Biol. Chem. 240, 588-593.
2. Villar-Palasi, C. (1969) Ann. N.Y. Acad. Sci., 166, 719-730.
3. Curnow, R.T., and Larner, J. (1979) Biochemical Actions of Hormones, 6, 77-119.
4. Martensen, T.M., Brotherton, J.E., and Graves, D.J. (1973) J. Biol. Chem. 248, 8329-8336.
5. Khandelwal, R.L. (1977) Biochim. Biophys. Acta. 248, 379-390.
6. Stalmans, W. DeWulf, H., and Hers, H.-G. (1971) Eur. J. Biochem. 18, 582-587.
7. Thomas, J.A., Schlender, K.K. and Larner, J. (1968) Anal. Biochem. 25, 486-499.
8. Killilea, S.D., Aylward, J.H., Mellgren, R.L., and Lee, E.Y.C. (1978) Arch. Biochem. Biophys. 191, 638-646.
9. Lee, E.Y.C., Silberman, S.R., Ganapathi, M.K., Petrovic, S., and Paris, H. (1980) Adv. Cyclic Nucleotide Res. 13, 95-131.
10. White, A., Handler, P., and Smith, E.L. (1973) Principles of Biochemistry, Fifth Edition, p. 479, McGraw-Hill, New York.
11. Li, H.-C. (1982) Curr. Top. Cell Regul. 21, 129-174.
12. Krebs, E.G., and Beavo, J.A. (1979) Ann. Rev. Biochem. 48, 923-959.
13. Helmreich, E., Michaelides, M.C., and Cori, C.F. (1967) Biochemistry 6, 3695-3710.
14. Carlson,G.M., Bechtel, P.J., and Graves, D.J. (1979) Adv. Enz. Regul. 50, 41-116.
15. Wang, P., and Bantle, G. (1974) Biochem. Biophys. Res. Commun. 57, 148-153.