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Protein Phosphorylation and the Control of Glycogen Metabolism in Skeletal Muscle [and Discussion]

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Protein phosphorylation and the control of glycogen metabolism in skeletal muscle

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Glycogen metabolism in mammalian skeletal muscle is controlled by a regulatory network in which six protein kinases, four protein phosphatases and several thermostable regulatory proteins determine the activation state of glycogen phosphorylase and glycogen synthase, the rate-limiting enzymes of this process. Thirteen phosphorylation sites are involved, twelve of which have been isolated and sequenced and shown to be phosphorylated *in vivo*. The effects of adrenalin and insulin on the state of phosphorylation of each site have been determined.

The neural control of glycogen metabolism is mediated by calcium ions and involves phosphorylase kinase, and a specific calmodulin-dependent glycogen synthase kinase. The β-adrenergic control of the system is mediated by cyclic AMP, and involves the phosphorylation of phosphorylase kinase, glycogen synthase and inhibitor 1 by cyclic-AMP-dependent protein kinase. Inhibitor 1 is a specific inhibitor of protein phosphatase 1, the major phosphatase involved in the control of glycogen metabolism. The stimulation of glycogen synthesis by insulin results from the dephosphorylation of glycogen synthase at sites $(3a+3b+3c)$, which are introduced by the enzyme glycogen synthase kinase 3.

The structure, regulation and substrate specificities of the protein phosphatases involved in glycogen metabolism are reviewed. Protein phosphatase 1 can exist in an inactive form termed the Mg-ATP-dependent protein phosphatase, which consists of a complex between the catalytic subunit and a thermostable protein termed inhibitor 2. Activation of this complex is catalysed by glycogen synthase kinase 3. It involves the phosphorylation of inhibitor 2 and its dissociation from the catalytic subunit. Protein phosphatase 2A can be resolved into three forms by ion exchange chromatography. These species contain the same catalytic subunit and other subunits that may have a regulatory function. Protein phosphatase 2B is a Ca^{2+}-dependent enzyme composed of two subunits, A and B. Its activity is increased tenfold by calmodulin, which interacts with the A-subunit. The B-subunit is a Ca^{2+}-binding protein that is homologous with calmodulin. Its N-terminus contains the unusual myristyl blocking group, only found previously in the catalytic subunit of cyclic-AMP-dependent protein kinase. Protein phosphatase 2C is a Mg^{2+}-dependent enzyme that accounts for a very small proportion of the glycogen synthase phosphatase activity in skeletal muscle. It is likely to be involved in the regulation of other metabolic processes *in vivo* such as cholesterol synthesis.

Recent evidence suggests that many of the proteins involved in the control of glycogen metabolism have much wider roles, and that they participate in the neural and hormonal regulation of a variety of intracellular processes.

1. Introduction

It is now almost 50 years since glycogen phosphorylase was shown to exist in two forms termed *b* and *a* (Cori *et al.* 1938). The *b* form was completely dependent on adenylic acid (AMP) for activity, whereas the *a* form was almost fully active in the absence of AMP. It was reasoned that the *a* form must contain tightly bound AMP, and that its conversion to the *b* form,

catalysed by a PR (prosthetic group removing) enzyme must involve the liberation of this nucleotide (Cori & Green 1945). However, AMP could never be detected as a product of the reaction, and it was not until the mid-1950s that the interconversion of phosphorylase *b* and *a* was shown to involve a phosphorylation–dephosphorylation process (Krebs & Fischer 1955) catalysed by phosphorylase kinase and a protein phosphatase still occasionally referred to as PR (phosphate releasing!) enzyme. The conversion of phosphorylase *b* to *a* results from the formation of a single phosphoserine residue near the N-terminus of the polypeptide chain (Titani *et al.* 1975, 1979).

The activity of phosphorylase kinase was also found to be controlled by phosphorylation–dephosphorylation (Krebs *et al.* 1959), a discovery that eventually led to the identification of cyclic-AMP-dependent protein kinase (Walsh *et al.* 1968). The third enzyme reported to be regulated in this manner was glycogen synthase, which is converted from a high-activity dephosphorylated form to phosphorylated forms that are dependent on glucose 6-phosphate for activity (Friedman & Larner 1963). Studies of the control of glycogen metabolism have therefore been of unique importance to our understanding of the role of protein phosphorylation in enzyme regulation, and this system continues to act as the model with which others are compared. This article will summarize current knowledge of the neural and hormonal control of glycogen metabolism in skeletal muscle, with emphasis on recent developments.

2. Neural control of glycogen metabolism via calcium ions

When muscle is stimulated electrically, calcium ions released from the sarcoplasmic reticulum not only initiate muscle contraction but also activate phosphorylase kinase (Heilmeyer *et al.* 1970; Brostrom *et al.* 1971). As a result, the phosphorylation state of glycogen phosphorylase is increased (Danforth & Helmreich 1969; Helmreich & Cori 1966; Danforth & Lyon 1964), and glycogenolysis accelerated to provide the ATP needed to sustain muscle contraction. The steady-state level of phosphorylation increases, and the time taken to reach the steady-state decreases, with increasing frequency of electrical stimulation. At frequencies that cause the muscle to go into tetanus, a phosphorylation level of *ca.* 70 % is reached within 1 s (Danforth & Lyon 1964). Thus the rate of glycogenolysis is linked to the strength and duration of contraction.

There appear to be two methods by which calcium ions activate phosphorylase kinase. The purified enzyme has the subunit structure ($\alpha\beta\gamma\delta$), where the δ subunit is identical to the Ca^{2+}-binding protein calmodulin (Cohen *et al.* 1978; Grand *et al.* 1981). However, phosphorylase kinase also interacts with a second molecule of calmodulin, termed the δ' subunit, which strongly activates the dephosphorylated form of the enzyme (Shenolikar *et al.* 1979; Cohen 1980*a*). The δ' subunit only binds to phosphorylase kinase in the presence of Ca^{2+}, where it interacts with the α and β subunits. In contrast, the integral molecule of calmodulin (the δ subunit) remains complexed with the catalytic γ subunit even in the absence of Ca^{2+} (Shenolikar *et al.* 1979; Picton *et al.* 1980). Calmodulin is closely related in structure and Ca^{2+}-binding properties to troponin-C (Watterson *et al.* 1980), the protein on the thin filaments of muscle that confers Ca^{2+}-sensitivity on the contractile apparatus. Troponin-C, the troponin complex, and even artificial thin filaments of muscle, can substitute for the δ' subunit, and several lines of evidence suggest that troponin-C, rather than the δ' subunit, may be the important activator *in vivo* (Cohen 1980*a*; Cohen *et al.* 1980). Thus while the binding of Ca^{2+} to the δ subunit (calmodulin) is essential for the activation of phosphorylase kinase, as much as 20–30-fold higher

activation could be achieved through the interaction with troponin-C (Cohen 1980*a*). Activation of glycogenolysis and muscle contraction by the same Ca^{2+}-binding protein (troponin-C) would represent an attractive mechanism for coupling these two processes (figure 1), and perhaps this is why phosphorylase kinase is so large (M_r = 1300000 (Cohen 1973)), because if these ideas are correct it would have to span the distance between the protein–glycogen complex and thin filaments. Knowledge of its precise intracellular location in contracting muscle will be required to test this hypothesis.

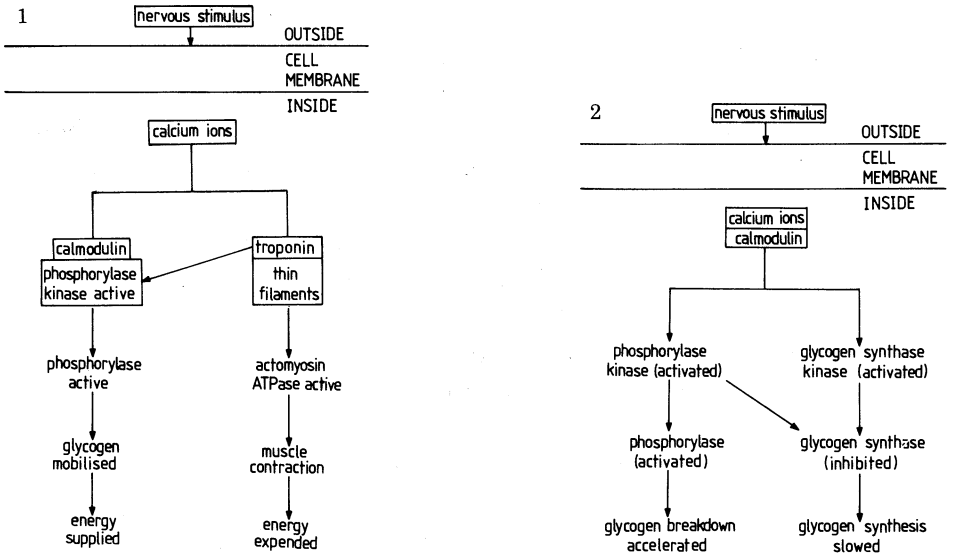

FIGURE 1. Role of calcium ions, calmodulin and troponin-C in synchronizing glycogenolysis and muscle contraction.

FIGURE 2. Role of calcium ions and calmodulin in the regulation of glycogenolysis and glycogen synthesis.

There is increasing evidence that calcium ions not only activate glycogenolysis but also inhibit the synthesis of glycogen (figure 2). Glycogen synthase can be phosphorylated by two Ca^{2+}-dependent protein kinases that decrease its activity *in vitro*. One of these is phosphorylase kinase (Roach *et al.* 1978), and the other is a Ca^{2+}-calmodulin-dependent glycogen synthase kinase that does not act on glycogen phosphorylase (Woodgett *et al.* 1982). Both protein kinases phosphorylate the same residue (serine 7) on glycogen synthase (§3). At physiological concentrations of glycogen phosphorylase (7 mg ml^{-1}, 70 μM) and glycogen synthase (0.25 mg ml^{-1}, 3 μM), phosphorylase kinase phosphorylates the former enzyme preferentially (Woodgett *et al.* 1982). This suggests that serine 7 of glycogen synthase may initially be phosphorylated by the Ca^{2+}-calmodulin-dependent glycogen synthase kinase during contraction, phosphorylase kinase only acting on this site subsequently, after the phosphorylation of glycogen phosphorylase has been accomplished. However, it should be emphasized that the studies needed to test this hypothesis have not yet been done. In particular, it still needs to be demonstrated that serine 7 becomes phosphorylated *in vivo* during muscle contraction.

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3. Adrenergic control of glycogen metabolism via cyclic AMP

Adrenergic stimulation of glycogenolysis represents a mechanism for mobilizing glycogen in resting muscle during periods of stress, in anticipation of an increased energy demand. The interaction of adrenalin with its β-receptor on the outer surface of the plasma membrane triggers the activation of adenylate cyclase located on the outer surface of the plasma membrane (Robison *et al.* 1971). This results in an increase in the intracellular concentration of cyclic AMP, which transmits the hormonal signal by activating cyclic-AMP-dependent protein kinase (Walsh *et al.* 1968). This protein kinase appears to stimulate glycogenolysis by phosphorylating three proteins, namely phosphorylase kinase, inhibitor 1 and glycogen synthase.

Phosphorylation of phosphorylase kinase by cyclic-AMP-dependent protein kinase is accompanied by the phosphorylation of one major serine residue on the α subunit and one on the β subunit, at physiological concentrations of Mg^{2+} (Cohen 1973; Yeaman *et al.* 1975). Phosphorylation of the β subunit correlates with changes in the activity of the enzyme (Cohen & Antoniw 1973; Cohen 1980*b*), although both serines become phosphorylated *in vivo* in response to adrenalin (Yeaman & Cohen 1975). Phosphorylation increases the activity of phosphorylase kinase 15–20-fold at saturating concentrations of Ca^{2+} and decreases the $A_{0.5}$ for Ca^{2+} from *ca.* 20 to *ca.* 1 μM, under the standard assay conditions at 8 mM Mg^{2+} (Cohen 1980*a*). These results suggest that phosphorylation of the α and β subunits not only increases the catalytic activity of the γ subunit, but allows activation to occur when fewer calcium ions are bound to calmodulin. Calmodulin binds four calcium ions per mole at micromolar concentrations (Klee *et al.* 1980), the affinity of the two Ca^{2+}-binding sites in the N-terminal portion being ten times those in the C-terminal portion (Kilhoffer *et al.* 1980). It seems likely that phosphorylation allows activation to occur when only the high-affinity sites are saturated with Ca^{2+}, whereas all four sites must be filled to activate the dephosphorylated form of the enzyme. The activity of the phosphorylated form at 0.1 μM Ca^{2+} is comparable with that of the dephosphorylated form at 10 μM Ca^{2+} (Cohen 1980*a*). This observation, coupled with the activation of inhibitor 1 (see below), may explain why the phosphorylation state of glycogen phosphorylase can reach a high level in resting muscle during adrenergic stimulation.

Protein phosphatase 1 is the major enzyme responsible for the dephosphorylation of glycogen phosphorylase, phosphorylase kinase (β subunit) and glycogen synthase in skeletal muscle (§5). It is inhibited *in vitro* by nanomolar concentrations of a thermostable protein, termed inhibitor 1, whose activity is only expressed after phosphorylation by cyclic-AMP-dependent protein kinase (Huang & Glinsmann 1976; Nimmo & Cohen 1978). Inhibitor 1 has a molecular mass of 18 640 Da and the elucidation of its primary structure (Aitken *et al.* 1982*a*, *b*) has identified the site of phosphorylation as a threonine, 35 residues from the N-terminus. Its molar concentration *in vivo* (*ca.* 1.8 μM) is higher than that of protein phosphatase 1, which is below 1 μM (Foulkes & Cohen 1980). The extent of phosphorylation of inhibitor 1 *in vivo* is increased by adrenalin (Foulkes & Cohen 1979; Khatra *et al.* 1980; Foulkes *et al.* 1982). These results suggest that the inhibition of protein phosphatase 1 by inhibitor 1 may represent an important mechanism for increasing the phosphorylation states of glycogen phosphorylase, phosphorylase kinase and glycogen synthase during adrenergic stimulation (figure 3). This idea is supported by recent studies of the effects of adrenalin on the phosphorylation of glycogen synthase.

Glycogen synthase is an excellent substrate for both cyclic-AMP-dependent protein kinase (Schlender *et al.* 1969; Soderling *et al.* 1970) and phosphorylase kinase (Roach *et al.* 1978; Embi

et al. 1979) *in vitro*. Cyclic-AMP-dependent protein kinase phosphorylates the enzyme at sites 1*a*, 1*b* and 2, while phosphorylase kinase is specific for site 2 (figure 4). These observations suggested that the inactivation of glycogen synthase by adrenalin *in vivo* (Parker *et al.* 1982)

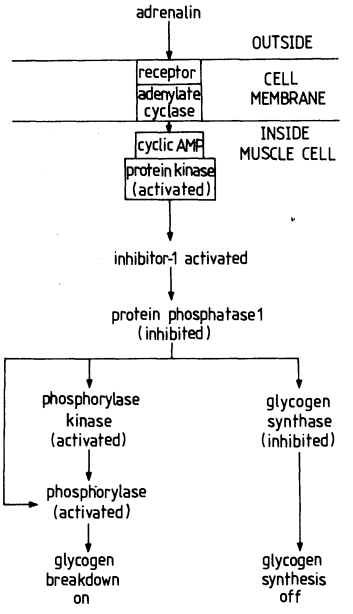

FIGURE 3. Role of inhibitor 1 in the β-adrenergic control of glycogen metabolism in mammalian skeletal muscle.

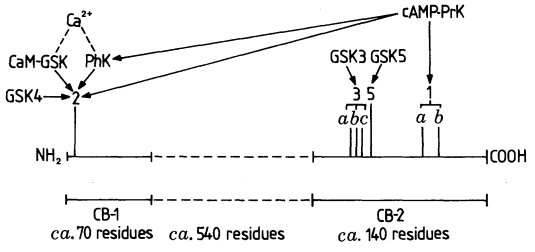

FIGURE 4. Organization of the phosphorylation sites in rabbit skeletal muscle glycogen synthase. Site 2 is 7 residues from the N-terminus (Embi *et al.* 1981) and sites 3*a*, 3*b*, 3*c*, 5, 1*a* and 1*b* are 30, 34, 38, 46, 87 and 100 residues from the N-terminus of a large CNBr-peptide (CB-2) at the C-terminal end of the molecule (Picton *et al.* 1982*a*). Sites 3*a*, 3*b* and 3*c* are located on the same tryptic peptide. Abbreviations: cAMP-PrK, cyclic-AMP-dependent protein kinase; PhK, phosphorylase kinase; GSK, glycogen synthase kinase; CaM, calmodulin.

would either result directly from phosphorylation catalysed by cyclic-AMP-dependent protein kinase, or indirectly through the activation of phosphorylase kinase by cyclic-AMP-dependent protein kinase. Surprisingly, however, neither of these mechanisms appears to account for the action of adrenalin.

Glycogen synthase is now known to be phosphorylated on seven serine residues by at least six protein kinases (figure 4), and all seven sites are phosphorylated *in vivo*. In normally fed animals that have been injected with the β-adrenergic antagonist propranolol there are *ca.* 3 mol phosphate bound covalently to each subunit, but this value rises to *ca.* 5 mol per subunit after the administration of maximally effective doses of adrenalin. Although the phosphorylation of site 2 is increased by *ca.* 0.6 mol mol^{-1} subunit and sites 1*a* and 1*b* each by *ca.* 0.25 mol mol^{-1}

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subunit, half of the increased phosphorylation occurs at sites ($3a+3b+3c$), which are introduced by an enzyme termed glycogen synthase kinase 3 (Parker *et al.* 1982). Since the phosphorylation of sites ($3a+3b+3c$) decreases the activity of glycogen synthase to a greater extent

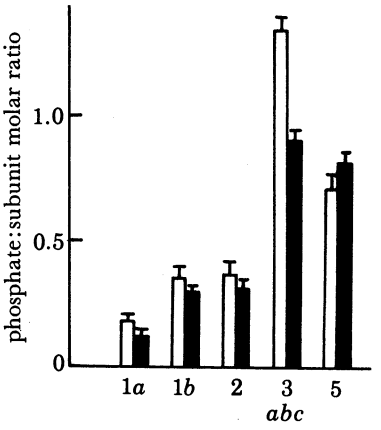

FIGURE 5. Effect of insulin on the phosphorylation of the seven phosphoserine residues of rabbit skeletal muscle glycogen synthase (figure 4). The open bars show the phosphate contents of the various sites in animals injected with propranolol, and the filled bars the phosphate contents after injection of propranolol plus insulin. Further experimental details are given in the legend to table 1, and the data are taken from Parker *et al.* (1983).

TABLE 1. EFFECT OF INSULIN ON THE KINETIC PROPERTIES AND PHOSPHATE CONTENT OF SKELETAL MUSCLE GLYCOGEN SYNTHASE

(Female New Zealand white rabbits were starved for 24 h. Propranolol (2 mg kg^{-1}) and insulin (16.5 μg kg^{-1}) were administered intravenously 15 min before killing, and a further injection of insulin (16.5 μg kg^{-1}) was given 7.5 min before killing. The animals were killed with a lethal dose of 6% sodium pentobarbitone (4.0 ml) and the muscle from the hind limbs and back homogenized in the presence of EDTA and NaF to freeze the state of phosphorylation. After centrifugation for 45 min at 6000 *g* the supernatant was decanted, and samples were passed through Sephadex 25 and assayed for glycogen synthase activity. The activity ratio ∓ glucose 6-phosphate and K_a for glucose 6-phosphate were measured. Glycogen synthase was then purified to homogeneity and the alkali-labile phosphate bound covalently to the enzyme determined. The values are given ± s.e.m. and the number of determinations are given in parentheses. (Taken from Parker *et al.* (1983).)

treatment	activity ratio ∓ glucose-6P	K_a, glucose-6P / mM	phosphate content / mol mol^{-1} subunit
L-propranolol	0.18 ± 0.02 (11)	1.2 ± 0.1 (11)	2.74 ± 0.09 (14)
L-propranolol + insulin	0.35 ± 0.02 (12)	0.6 ± 0.05 (12)	2.33 ± 0.09 (14)

than other sites, the increased phosphorylation at these three residues makes a major contribution to the inhibition of glycogen synthase by adrenalin. The activity of glycogen synthase kinase 3 is unaffected by cyclic AMP (Embi *et al.* 1980), and nor is there yet any evidence that it is a substrate for cyclic-AMP-dependent protein kinase (B. A. Hemmings & P. Cohen, unpublished). It therefore seems likely that the increased phosphorylation at sites ($3a+3b+3c$), and perhaps site 2 as well, results from the inhibition of protein phosphatase 1. One mechanism by which this could occur is through the phosphorylation of inhibitor 1 (figure 3). However, a further possibility is that increased phosphorylation of glycogen phosphorylase decreases the rate of dephosphorylation of glycogen synthase, owing to competition for protein phosphatase 1. In view of the high concentration of glycogen phosphorylase (*ca.* 70 μM) relative to glycogen synthase (*ca.* 2.5 μM) in skeletal muscle, this alternative explanation cannot yet be discounted.

Site 5 is almost fully phosphorylated *in vivo* under all conditions so far examined (Parker

et al. 1982, 1983), yet this phosphorylation does not affect the kinetic properties of glycogen synthase *in vitro* (Cohen *et al.* 1982; Picton *et al.* 1982*b*). Recently it has been demonstrated that this modification is a prerequisite for phosphorylation by glycogen synthase kinase 3 (Picton *et al.* 1982*b*). Thus glycogen synthase kinase 5 would appear to be an example of a protein kinase whose function is to form the recognition site for another protein kinase.

4. Stimulation of glycogen synthesis by insulin

Larner and coworkers were the first to show that insulin stimulated glycogen synthesis through an activation of the enzyme glycogen synthase (Villar-Palasi & Larner 1960). Insulin increased the activity of glycogen synthase in the absence, but not in the presence, of glucose 6-phosphate, suggesting that dephosphorylation of the enzyme had taken place (§1). This in turn implied that insulin had either decreased the activity of a glycogen synthase kinase or increased the activity of a glycogen synthase phosphatase. The effect occurred without any detectable alteration in the concentration of cyclic AMP (Goldberg *et al.* 1967), and was not secondary to the effects of insulin on glucose transport (Villar-Palasi & Larner 1961).

Recently the activation of glycogen synthase by insulin has been shown to result from a specific dephosphorylation of sites (3*a* + 3*b* + 3*c*) (table 1 and figure 5). Because the residues phosphorylated by cyclic-AMP-dependent protein kinase (sites 1*a*, 1*b* and 2) are unaffected by insulin, the results exclude the possibility that insulin acts by decreasing cyclic AMP concentration or by inhibiting cyclic-AMP-dependent protein kinase through any other mechanism. Insulin must therefore either decrease the activity of glycogen synthase kinase 3 or increase the activity of a protein phosphatase that acts on sites (3*a* + 3*b* + 3*c*) *in vivo*. Two enzymes, termed protein phosphatase 1 and protein phosphatase 2A, are capable of dephosphorylating these sites, protein phosphatase-1 being the dominant activity (§5). However, neither enzyme is specific for sites (3*a* + 3*b* + 3*c*), and site 2 is dephosphorylated with almost equal rapidity *in vitro* (Parker *et al.* 1983). Thus current evidence suggests that activation of glycogen synthase by insulin should involve a specific inhibition of glycogen synthase kinase 3 (but see §5).

Glycogen synthase kinase 3 has been purified *ca.* 50000-fold to a state approaching homogeneity (Hemmings *et al.* 1981) and its intracellular concentration is *ca.* 2×10^{-8} M, or *ca.* 50 times higher than the normal circulating levels of insulin. One possibility is that the insulin receptor interaction triggers the formation of a 'second messenger' that inhibits glycogen synthase kinase 3. However, an intriguing alternative possibility is that the insulin receptor is a protein kinase that only becomes activated when it interacts with insulin. The precedent for this idea is epidermal growth factor. The binding of this hormone to its receptor causes the latter protein to be phosphorylated by a protein kinase (Cohen *et al.* 1981) that appears to be the receptor itself (Buhrow *et al.* 1982). Similar results have now been reported for the insulin receptor (Kasuga *et al.* 1982). In both cases, phosphorylation of the receptor occurs on tyrosine residues. This is most unusual, since phosphotyrosine represents only 0.03 % of the phosphorylated amino acids in normal cells, phosphoserine and phosphothreonine accounting for 99.97 % (Sefton *et al.* 1980). These findings raise the possibility that hormones like epidermal growth factor, insulin, and platelet-derived growth factor (Ek & Heldin 1982; Nishimura *et al.* 1982) do not act through the formation of intracellular 'second messengers'. The idea would be that the receptor is a transmembrane protein, the binding site for insulin being located on the outer

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surface, and the protein kinase activity on the inner surface, of the plasma membrane. Interaction of the hormone with its receptor would then trigger a conformational change in the latter protein, leading to activation of its protein kinase activity, and the phosphorylation of intracellular proteins. An important possibility that needs to be examined is whether glycogen synthase kinase 3 can be phosphorylated (on a tyrosine residue) and inactivated by the insulin receptor.

TABLE 2. CONTRIBUTION OF TYPE 1 AND TYPE 2 PROTEIN PHOSPHATASES TO THE TOTAL PROTEIN PHOSPHATASE ACTIVITY IN RABBIT SKELETAL MUSCLE ACTING ON THE PHOSPHORYLATED PROTEINS OF GLYCOGEN METABOLISM AT PHYSIOLOGICAL pH (7.0) AND Mg^{2+} CONCENTRATION (1.0 mM)

(Assays for protein phosphatases 1, 2A and 2C were done in the presence of 1.0 mM EGTA and 1.0 mM $MgCl_2$ ('resting muscle'). Protein phosphatase 2B was measured in the presence of 1.0 mM EGTA and 0.85 mM $CaCl_2$ ('contracting muscle', 3 μM free Ca^{2+}). Other conditions are described in Ingebritsen *et al.* (1983*b*).)

		relative contribution (%)			
substrate	condition	PrP1	PrP2A	PrP2B	PrP2C
phosphorylase	'resting muscle'	88	12	0	0
glycogen synthase (site 1*a*)	'resting muscle'	62	36	0	< 1
glycogen synthase (site 2)	'resting muscle'	74	24	0	2
glycogen synthase (sites 3*a* + 3*b* + 3*c*)	'resting muscle'	75	24	0	< 1
phosphorylase kinase (β subunit)	'resting muscle'	94	6	0	0
phosphorylase kinase (α subunit)	'resting muscle'	17	79	0	4
	'contracting muscle'	6	29	63	2
inhibitor 1	'resting muscle'	0†	100	0	0
	'contracting muscle'	0	35	65	0

† Protein phosphatase 1 can catalyse the dephosphorylation of inhibitor 1 *in vitro* because inhibitor 1 does not inhibit its own dephosphorylation (Nimmo & Cohen 1978*b*). However, this dephosphorylation reaction is unusual because in contrast to the dephosphorylation of other substrates it has an absolute requirement for Mn^{2+} (Ingebritsen *et al.* 1983*b*). Because high Mn^{2+} concentrations do not exist *in vivo*, protein phosphatase 1 may not act as an inhibitor-1 phosphatase in muscle (but see discussion in Ingebritsen *et al.* 1983*b*).

5. STRUCTURE AND REGULATION OF THE PROTEIN PHOSPHATASES INVOLVED IN THE CONTROL OF GLYCOGEN METABOLISM

There are four protein phosphatases in skeletal muscle, and other tissues, that are capable of dephosphorylating the phosphoproteins involved in the control of glycogen metabolism. These four enzymes can be conveniently grouped into two classes, type 1 and types 2, depending on whether they dephosphorylate the β subunit of phosphorylase kinase and are inhibited by the two thermostable proteins termed inhibitor 1 and inhibitor 2 (protein phosphatase 1), or whether they dephosphorylate the α subunit of phosphorylase kinase preferentially and are insensitive to the inhibitor proteins (type 2 protein phosphatases) (Ingebritsen & Cohen 1983*a*, *b*; Ingebritsen *et al.* 1983*a*, *b*). The development of methods for quantitating these four enzymes in tissue extracts has allowed their relative contributions to the total phosphatase activity acting on each phosphorylation site to be assessed (table 2).

(*a*) *Protein phosphatase* 1

This is the major phosphatase acting on each of the phosphorylation sites that alter the kinetic properties of the enzymes of glycogen metabolism (the β subunit of phosphorylase kinase, sites 1*a*, 2 and (3*a* + 3*b* + 3*c*) of glycogen synthase and glycogen phosphorylase). Its

important role in this process is further emphasized by the finding that *ca.* 50 % of the protein phosphatase 1 in skeletal muscle sediments with the protein–glycogen particles (Ingebritsen & Cohen 1983*b*), the functional complex on which glycogenolysis and glycogen synthesis are thought to take place *in vivo* (Meyer *et al.* 1970). In contrast, protein phosphatases 2A, 2B and 2C are exclusively cytosolic enzymes (Ingebritsen *et al.* 1983*b*), at least under the extraction conditions so far employed.

The regulation of protein phosphatase 1 by the thermostable protein inhibitor 1 was described in §3 (see figure 3), providing a way of controlling protein phosphatase 1 activity during adrenergic stimulation. The *in vivo* phosphorylation state of inhibitor 1 can be decreased by insulin, which antagonizes the effects of low (0.5 nM) concentrations of the β adrenergic agonist isoproterenol. This appears to be due to the ability of insulin to suppress the small rise in cyclic AMP concentration caused by these concentrations of isoproterenol (Foulkes *et al.* 1982). However, insulin is unable to alter cyclic AMP or decrease the phosphorylation state of inhibitor 1 in the presence of high (10 nM) concentrations of isoproterenol, where the level of phosphorylation reaches 70 %, or in the presence of propranolol, where it is less than 10 % (Foulkes *et al.* 1982). Therefore, although the ability of insulin to decrease the phosphorylation of inhibitor 1 should be physiologically important, it does not account for the activation of glycogen synthase by this hormone, which is observed even in the presence of propranolol (table 1).

An enzyme has been isolated, termed the Mg-ATP-dependent protein phosphatase, whose activity is only expressed after preincubation with Mg-ATP and another protein factor (Goris *et al.* 1979, 1980) that has been identified as glycogen synthase kinase 3 (Hemmings *et al.* 1981). Once it has been activated, the properties of this phosphatase are remarkably similar to those of protein phosphatase 1, suggesting that the two enzymes possess the same catalytic subunit (Stewart *et al.* 1981). This idea has recently been established by the finding that a Mg-ATP-dependent protein phosphatase can be reconstituted from the M_r = 33000 subunit of protein phosphatase 1 and the thermostable protein inhibitor 2 (M_r = 30500), and consists of a 1:1 complex between these two proteins. Activation by glycogen synthase kinase 3 and Mg-ATP results from the phosphorylation of inhibitor 2 on a threonine residue and is accompanied by its dissociation from protein phosphatase 1 (Hemmings *et al.* 1982*a*, *b*). Thus the relation between the Mg-ATP-dependent protein phosphatase and protein phosphatase 1 appears to be closely analogous to that which exists between cyclic-AMP-dependent protein kinase and its catalytic subunit.

Because the activity of glycogen synthase kinase 3 appears to be under the control of insulin, the phosphorylation of inhibitor 2 and activation of the Mg-ATP dependent protein phosphatase may also be under hormonal control. However, the role of this interconversion system *in vivo* is not yet clear, because the two actions of glycogen synthase kinase 3, namely to phosphorylate glycogen synthase and to convert the Mg-ATP-dependent protein phosphatase to protein phosphatase 1, seem to be antagonistic.

(*b*) *Protein phosphatase 2A*

This enzyme accounts for an appreciable percentage of the glycogen synthase phosphatase activity in skeletal muscle (table 2) and for a small proportion of the phosphorylase phosphatase and phosphorylase kinase (β subunit) phosphatase activity. In the absence of Ca^{2+} (i.e. resting muscle), protein phosphatase 2A is the major phosphorylase kinase (α subunit) phosphatase. However, the role of phosphorylation of the α subunit in the regulation of phosphorylase kinase

activity is unknown (see §2). Protein phosphatase 2A may also be the only enzyme capable of dephosphorylating inhibitor 1 in resting muscle if protein phosphatase 1 is unable to carry out this reaction *in vivo* (see legend to table 2).

Protein phosphatase 2A is resolved by chromatography on DEAE-cellulose into three forms termed $2A_0$, $2A_1$ and $2A_2$ (Ingebritsen *et al.* 1983*a*). The apparent relative molecular masses of these species, determined by gel filtration, are 210000, 210000 and 150000 respectively. Each form contains the same M_r = 38000 catalytic subunit, which can be dissociated from the higher M_r complexes by freezing and thawing in the presence of 250 mM 2-mercaptoethanol or by treatment with 80 % ethanol at room temperature. Protein phosphatases $2A_1$ and $2A_2$ have been purified to homogeneity from tissues other than skeletal muscle (Pato *et al.* 1983; Pato & Adelstein 1980; Tamura & Tsuiki 1980; Crouch & Safer 1980; Tamura *et al.* 1980; Werth *et al.* 1982). Protein phosphatase $2A_1$ contains two additional subunits, M_r = 60000 and 55000, in addition to the catalytic subunit, whereas protein phosphatase $2A_1$ only contains the M_r = 60000 component complexed with the catalytic subunit. There is evidence that protein phosphatase $2A_2$ may be derived from $2A_1$ during ion-exchange chromatography (Ingebritsen *et al.* 1983*a*). Dissociation of the catalytic subunit results in a severalfold activation of protein phosphatase $2A_1$ and a much smaller activation of protein phosphatase $2A_2$ (Ingebritsen *et al.* 1983*a*, *b*). It seems likely that the M_r = 60000 and 55000 components are regulatory subunits, but whether they interact with allosteric effectors or are subject to phosphorylation–dephosphorylation is unknown. Protein phosphatase $2A_0$ is completely inactive, and its activity is only expressed after dissociation to the catalytic subunit. This species has not yet been obtained in pure form and hence its subunit composition is unknown.

(*c*) *Protein phosphatase 2B*

The activity of this enzyme is completely dependent on calcium ions ($A_{0.5} \approx 1.0$ μM), suggesting that it is only active during muscle contraction. Under optimal conditions it is the major phosphorylase kinase (α subunit) phosphatase and inhibitor 1 phosphatase in skeletal muscle (table 2), but whether either of these proteins represent physiological substrates of this enzyme is unknown (discussed in Stewart *et al.* (1983) and Ingebritsen & Cohen (1983*a*)). Protein phosphatase 2B does not dephosphorylate glycogen phosphorylase, glycogen synthase or the β subunit of phosphorylase kinase.

Protein phosphatase 2B is composed of two subunits, A ($M_r \approx 60000$) and B ($M_r \approx 18000$) in a molar ratio 1:1 (Stewart *et al.* 1982, 1983). The B subunit is a calcium-binding protein, which, like calmodulin, binds four calcium ions per mole with affinities in the micromolar range (Klee *et al.* 1979). The determination of the primary structure of the B subunit shows that it has 35 % sequence identity with calmodulin and 29 % with troponin-C, with most of the identities occurring in the region of the four calcium-binding sites (Aitken *et al.* 1983). The activity of protein phosphatase 2B is increased *ca.* tenfold by calmodulin (Stewart *et al.* 1982), which binds to the A subunit. Thus protein phosphatase 2B resembles phosphorylase kinase (see §2) in being regulated by two different, but structurally related, calcium-binding proteins.

The N-terminus of the B subunit is blocked, and the blocking group has recently been identified as the C_{14} saturated fatty acid myristic acid, which is linked by an amide bond to the N-terminal glycine residue (Aitken *et al.* 1982*a*, *b*). This is of considerable interest because the only other protein known to possess such a blocking group is the catalytic subunit of cyclic-AMP-dependent protein kinase (Carr *et al.* 1982). Whether N-terminal myristyl groups are

confined to protein kinases and phosphatases remains to be determined. However, if this were true it would imply that the myristyl group plays a role in the recognition of protein substrates. Protein phosphatase 2B and the catalytic subunit of cyclic-AMP-dependent protein kinase both start with the N-terminal sequence myristyl-Gly-Asn-Xaa-Ala, suggesting that the enzyme that links myristyl-CoA (presumably) to the protein may recognize this sequence.

(*d*) *Protein phosphatase 2C*

This enzyme was first identified in liver and cardiac muscle as a glycogen synthase phosphatase that was unable to dephosphorylate glycogen phosphorylase (Kikuchi *et al.* 1977; Binstock & Li 1979). It is a Mg^{2+}-dependent enzyme ($A_{0.5} \approx 1.0$ mM) and consists of a single polypeptide chain $M_r = 43000$ (Pato & Adelstein 1980; Hiraga *et al.* 1981). It dephosphorylates site 2 of glycogen synthase more rapidly than site 1*a* or sites (3*a* + 3*b* + 3*c*) (Ingebritsen & Cohen 1982*b*). However, it accounts for a negligible percentage of the measurable glycogen synthase phosphatase in skeletal muscle extracts (table 2), and it is probably involved in the regulation of other metabolic pathways *in vivo*, such as cholesterol synthesis (see Ingebritsen *et al.* (1983*b*) and §6).

6. Concluding remarks

A striking feature of recent investigations into the control of glycogen metabolism has been the discovery that many of the regulatory proteins possess multiple functions. In fact, many of these proteins may have even wider roles. Cyclic-AMP-dependent protein kinase and calmodulin regulate many cellular processes, while protein phosphatases 1, 2A and 2C appear to be the only phosphatases involved in the control of glycolysis–gluconeogenesis, fatty acid synthesis, cholesterol synthesis and protein synthesis as well as glycogen metabolism (reviewed in Cohen (1982), Ingebritsen & Cohen (1983*a*) and Cohen *et al.* (1983)). The participation of protein phosphatase 1 in different metabolic events implies that inhibitor 1, inhibitor 2 and glycogen synthase kinase 3 may also have much more general functions. Glycogen synthase kinase 5 is also known to phosphorylate a number of proteins *in vivo* (Picton *et al.* 1982*b*; Hemmings *et al.* 1982*a*, *b*). Thus evidence is emerging that the major pathways of intermediary metabolism are controlled by common protein kinases, protein phosphatases and small 'modulator' proteins that regulate their activities, creating an integrated network of regulatory pathways by which diverse cellular processes are coordinated by neural and hormonal stimuli. A major goal of future research must be to establish the structure of this regulatory network.

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Discussion

T. Goldstone (*Biochemistry Department, University College, London, U.K.*). It appears that calcium, acting via protein phosphatase 2B on inhibitor 1, stimulates glycogen resynthesis while promoting glycogen breakdown by its effects on phosphorylase kinase. Has Professor Cohen looked at the time course of the effects of calcium ions on inhibitor 1, and in the light of this does he have any comments on the proposed role of calcium ions in insulin action?

P. Cohen. Protein phosphatase 2B is potentially the most powerful protein phosphatase acting on the α subunit of phosphorylase kinase (table 2). However, phosphorylation of the α subunit does not appear to alter the activity of phosphorylase kinase significantly *in vitro* (Cohen 1980*b*). Therefore protein phosphatase 2B does not appear to be involved in the regulation of glycogenolysis because it is also unable to dephosphorylate glycogen phosphorylase.

Protein phosphatase 2B is also potentially the most powerful inhibitor 1 phosphatase in skeletal muscle (table 2). The dephosphorylation of inhibitor 1 by this Ca^{2+}-dependent enzyme could therefore provide a mechanism for activating protein phosphatase 1 during muscle contraction. Because protein phosphatase 1 is the major enzyme in skeletal muscle acting on glycogen phosphorylase, glycogen synthase and the β subunit of phosphorylase kinase, its activation should stimulate the rate at which glycogen is resynthesized when contraction ceases. Thus the rate of glycogen synthesis in resting muscle would be linked to the strength and duration of the previous contraction and the extent of glycogen depletion. However, this hypothesis is only very speculative at present because studies of the effects of muscle contraction on the phosphorylation state of inhibitor 1 *in vivo* have not yet been performed.

There is no evidence that calcium ions play any role in the action of insulin on glycogen synthesis. The activation of glycogen synthase by insulin results from decreased phosphorylation of sites ($3a+3b+3c$) (figure 5). These sites are phosphorylated and dephosphorylated by enzymes whose activities are unaffected by calcium ions (glycogen synthase kinase 3 and protein phosphatases 1 and 2A). The only serine residue phosphorylated by calcium-dependent protein kinases (site 2) is unaffected by insulin. A normal activation of glycogen synthase by insulin is observed in ICR/IAn mice that lack one of the Ca^{2+}-dependent glycogen synthase kinases, namely phosphorylase kinase (Le Marchand Brustel, Y., *et al.* (1979*a*) *FEBS Lett.* **105**, 235–238).

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