Control of Glycogen Synthase and Phosphorylase by Amylin in Rat Skeletal Muscle

HORMONAL EFFECTS ON THE PHOSPHORYLATION OF PHOSPHORYLASE AND ON THE DISTRIBUTION OF PHOSPHATE IN THE SYNTHASE SUBUNIT*

(Received for publication, December 30, 1993)

John C. Lawrence, Jr. and Ji-nan Zhang

From the Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

The effects of amylin and insulin on the phosphorylation of glycogen synthase and phosphorylase were investigated using rat diaphragms incubated with $^{32}$P, Muscles were preincubated (200 units/ml of amylin (200 ng/ml) for 30 min before extracts were prepared. The $^{32}$P contents of the enzymes were determined after immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Amylin increased both the activity ratio $(-\text{AMP/}+\text{AMP})$ and the $^{32}$P content of phosphorylase by approximately 2-fold. Insulin alone was without significant effect on phosphorylase, but insulin blocked the effect of amylin on increasing the phosphorylation of phosphorylase. Insulin increased the glycogen synthase activity ratio (low glucose-6-P/high glucose-6-P) by approximately 80%. Amylin decreased this ratio from 0.14 to 0.08 and increased the phosphorylation of synthase by approximately 40%. To investigate changes in phosphorylation of different sites in the synthase, the enzyme was subjected to exhaustive proteolysis with trypsin, and $^{32}$P-labeled fragments were separated by reverse phase high performance liquid chromatography. Insulin decreased the $^{32}$P contents of sites 3(a + b + c) and 2(a + b), which appears to account for the increase in synthase activity. Amylin increased phosphorylation of sites 1a, 1b, and 3a + b + c, but not sites 2(a + b). With insulin plus amylin, phosphorylation of none of the sites was significantly changed. The results indicate that the effects of amylin on glycogen synthase must involve more than activation of cAMP-dependent protein kinase, as this kinase phosphorylates site 2 and does not phosphorylate sites 3(a + b + c).

Glycogen metabolism in skeletal muscle plays a major role in the control of glucose homeostasis by insulin, the most important regulator of blood glucose levels. In humans, skeletal muscle accounts for the bulk of insulin-stimulated glucose uptake (1–3), and most of the glucose that enters muscle fibers in response to insulin is converted to glycogen (4). Opposing insulin are several counter-regulatory hormones, such as epinephrine and glucagon, which stimulate glycogenolysis in skeletal muscle and liver, respectively (5). Islet amyloid polypeptide or amylin, a 37-amino acid peptide having approximately 50% identity with calcitonin gene-related peptides, is another potential counter-regulator of glycogen metabolism in skeletal muscle (6, 7). Amylin is synthesized and stored in the pancreatic b cells, and it appears to be released with insulin in response to glucose (8). Amylin has been shown to inhibit glycogen synthesis (9–14) and stimulate glycogenolysis (15) in skeletal muscle, both in vitro and in vivo. Glycogen synthase and phosphorylase, the enzymes immediately responsible for the synthesis and degradation of glycogen, are controlled by phosphorylation (16–19). Insulin stimulates dephosphorylation of both glycogen synthase (20–22) and phosphorylase (23) in skeletal muscle, thereby promoting enzymatic changes which favor glycogen accumulation. Epinephrine increases the phosphorylation of both enzymes (20, 23–25) leading to glycogen degradation. Amylin has been reported to decrease the activity ratio $(-\text{glucose-6-P/}+\text{glucose-6-P})$ of glycogen synthase (26) and to increase the phosphorylase activity ratio $(-\text{AMP/}+\text{AMP})$ (13, 26), effects that are indicative of increased phosphorylation of the enzymes. However, effects of amylin on the phosphorylation of glycogen synthase and phosphorylase have not been assessed directly.

The apparent complexities in the control of the covalent modification of glycogen synthase and phosphorylase differ greatly. Phosphorylase is activated when phosphorylated by a single enzyme, phosphorylase kinase, which introduces phosphate into a site located near the NH$_2$ terminus of the enzyme (16, 27). In contrast, glycogen synthase is inactivated by multiple protein kinases that phosphorylate 1 or more of 10 Ser/Thr phosphorylation sites, all of which are found within the first 10 amino acid residues from the NH$_2$ terminus and in a stretch of 71 amino acid residues near the COOH terminus (17, 28). Site 2 (Ser$^7$) and Ser$^{10}$ are located in the NH$_2$-terminal region; sites 1a, 1b, 3a, 3b, 3c, 4, and 5 are in the COOH-terminal region. Although there is overlap in their specificities, the different protein kinases that act on glycogen synthase exhibit defined preferences for certain sites (17). Thus, determining which sites are phosphorylated in response to a hormone is useful in identifying the kinases involved. Experimental in the present report were performed to investigate the effects of amylin on the phosphorylation of glycogen synthase and phosphorylase. Rat diaphragms were incubated with $^{32}$P, to label the enzymes, then treated with insulin and/or

1 The NH$_2$-terminal region of rat skeletal muscle glycogen synthase has been sequenced and shown to contain Ser$^7$ and Ser$^{10}$ (50). However, the amino acid sequence of the entire protein has not been determined. Consequently, it is not clear that the residue numbers assigned to the different COOH-terminal sites in rabbit glycogen synthase are applicable to the rat enzyme. For this reason we will continue to use the original site designations (51). To be consistent, we will refer to Ser$^{10}$ as site 2b. Trypsin does not cleave between sites 3a, 3b, and 3c, sites 4 and 5, or site 2 and Ser$^{10}$. The sites will be referred to collectively as site 3(a + b + c), site 4 + 5, and site 2a + b, respectively.

* This research was supported in part by National Institutes of Health Grants DK28312 and AR41180. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Amylin. The effects of the hormones on glycogen synthase and phosphorylase were assessed by measuring their $^{32}$P contents after immunoprecipitation. To determine the amounts of $^{32}$P in phosphorylase were assessed by measuring their $^{32}$P contents by reverse phase HPLC.3

**EXPERIMENTAL PROCEDURES**

**Incubation of Tissue and Preparation of Extracts—**Muscles were incubated with $^{32}$P essentially as described previously (20). Briefly, diaphragms from 80-120-g male rats (Sprague-Dawley) were trimmed of external fat, connective tissues, and perirenal fat. The diaphragms were then transferred to a beaker containing low P, medium (6 ml/muscle) composed of low P, (0.1 mM) DMEM. All media and buffers used in incubating tissues were directly gassed by bubbling with a mixture of 95% O$_2$ and 5% CO$_2$ (20). Muscles were incubated in medium containing 0.5 mM $\text{Na}^+$/$\text{Mg}^+$-P, for 4 h at $37^\circ$C, then transferred to low P, buffer (119 mM NaCl, 4.6 mM KCl, 2.4 mM CaCl$_2$, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$, 5 mM glucose, and 0.1 mM K$_2$HPO$_4$, pH 7.4) for hormonal treatments. Incubations were terminated by freezing the muscles in liquid nitrogen. The tissue was powdered by using a porcelain mortar and pestle chilled with liquid nitrogen. The powdered muscle was homogenized (approximately 0.1 g of tissue/mL of buffer) composed of 100 mM NaF, 8 mM EDTA, 2 mM EGTA, and 50 mM Tris-HCl, pH 7.8) in a glass homogenization tube with a Teflon pestle driven at 1,000 rpm. The homogenates were centrifuged at 10,000 x g for 30 min. The supernatants were retained for analyses.

**Immunoprecipitation and Measurements of Glycogen Synthase and Phosphorylase Activities—**The protein content of each sample was measured by using biocinchonic acid (30) and adjusted to an equal concentration by using homogenization buffer. Glycogen synthase activity was measured using the method of Guinovart et al. (31), in which the activity ratio represents activity measured at low (0.2 mM) glucose-6-P divided by activity observed with high (10 mM) glucose-6-P. Phosphorylase activity was measured using the method of Gilboe et al. (32). The $^{32}$P-labeled proteins were immunoprecipitated from extracts as described previously (23).

**Glycogen Synthase Phosphorylation Site Analyses—**Samples were subjected to SDS-PAGE (33), and autoradiograms were prepared to identify $^{32}$P-labeled glycogen synthesis and phosphorylase. The $^{32}$P contents of the proteins were measured by Cerenkov counting of gel slices. Synthase was eluted from the slices by using an ISCO sample concentrator (model 1750, 3-watt setting for 5 min). The pellet was rinsed with acetone. Samples were incubated at 30 °C for 18 h in 100 ml of solution containing 100 µM Tris-HCl, pH 8.2, 1 mg/mg trypsin (1-t-losyalidro-2-phenylethylcholomethyl ketone-treated, Worthington), and 1% hydrogenated Triton X-100 (35). Samples were subjected to reverse phase HPLC as previously described (36). $^{32}$P was measured in 1-ml fractions by scintillation counting.

**Statistical Analyses—**To allow comparisons among experiments, values for $^{32}$P-labeled enzymes or fragments were normalized to correct for radioactive decay, for the differing amounts of muscle protein used in immunoprecipitations. and for the specific radioactivity of the $^{32}$P used in incubations. The statistical significance of differences were evaluated by one-way analysis of variance and Dunnett's method for comparing multiple treatments to a single control. The method of Tukey (38) was used when comparisons were made between all treatment groups.

**Other Materials—**[U-14C]Glucose-1-P was obtained from DuPont NEN. UDP-[U-14C]glucose was synthesized from [U-14C]glucose (ICN Radiochemicals) as described by Thomas et al. (39). Rat amylin (Lot AR084-52.1) was supplied by Dr. Garth Cooper, Amylin Corp. Porcine insulin (crystalline) was a gift from Eli Lilly Co.

**RESULTS**

**Inactivation and Phosphorylation of Glycogen Synthase in Response to Amylin—**The effects of insulin and amylin on glycogen synthase activity were assessed by measuring changes in the low/high glucose-6-P activity ratio (31) after incubating diaphragms with the hormones. The activity ratio in control muscles was 0.14 (Fig. 1). Consistent with its well-established effect of activating synthase, insulin increased the ratio by approximately 80%. In contrast, amylin decreased the ratio by approximately 40%. Moreover, amylin blocked the effect of insulin on activating glycogen synthase. Neither insulin nor amylin affected the total (+10 µM glucose-6-P) activity of glycogen synthase, indicating that the amount of enzyme was not changed within the time frame of these experiments.

Stable hormonal effects on glycogen synthase activity are indicative of changes in the phosphorylation state of the enzyme (40). To investigate the role of phosphorylation in the actions of amylin and insulin, rat diaphragm muscles were incubated with $^{32}$P, then treated with the hormones. Extracts were prepared and glycogen synthase was immunoprecipitated and subjected to SDS-PAGE. After autoradiography, the $^{32}$P-labeled enzyme appeared as a species of apparent $M_r = 90,000$ in samples from all treatment groups (Fig. 2). Amylin increased $^{32}$P-labeled synthase by approximately 40% (Fig. 3). The effect of insulin on decreasing the overall $^{32}$P content of synthase was not statistically significant, although insulin blocked the increase in $^{32}$P produced by amylin.

**Phosphorylation Site Analyses of Glycogen Synthase—**To resolve sites phosphorylated in response to amylin, immunoprecipitated glycogen synthase was subjected to exhaustive proteolysis with trypsin, and $^{32}$P-labeled fragments were resolved by reverse phase HPLC (Fig. 4). By using this strategy, it is possible to resolve fragments containing sites 1a, 1b, 2a + b, 4 + 5, and 3(a + b + c).5 The elution profiles of $^{32}$P-labeled trypsin fragments of synthase from control and hormonally treated diaphragms, and the presumed location of the different phosphorylation sites, are shown in Fig. 4. With the exception of the peak located between fractions 4 and 6, peaks of $^{32}$P-labeled fragments were observed in the same positions as those containing known phosphorylation sites.

The large peak in fractions 4–6 was not previously noted when comparable experiments were performed using synthase immunoprecipitated from $^{32}$P-labeled epithrochlearis muscles. It seems clear that the $^{32}$P was derived from glycogen synthase, because the immunoprecipitated enzyme was purified by SDS-
Amylin-stimulated Phosphorylation

Figure 2. Electrophoretic analysis of glycogen synthase immunoprecipitated from \(^{32}P\)-labeled diaphragms. Diaphragms were incubated as described in the legend to Fig. 1, except that the media were supplemented with 0.5 mCi/ml \(^{32}P\). Glycogen synthase was immunoprecipitated from extracts (250 \(\mu\)g of protein) of control and hormone-treated muscles. The immunoprecipitates (GS Ab IP) and samples (25 \(\mu\)g of protein) of the extracts (EXTRACT) were subjected to SDS-PAGE using a 7.5% resolving gel. A picture of an autoradiogram of the dried gel is presented. The positions of the following standards are indicated to the right: MYO, myosin; PHOS, phosphorylase a; BSA, bovine serum albumin; OVAL, ovalbumin; CA, carbonic anhydrase; and BPB, bromphenol blue.

Figure 3. Increased phosphorylation of glycogen synthase in response to amylin. Glycogen synthase was immunoprecipitated from \(^{32}P\)-labeled muscles and subjected to SDS-PAGE as described in the legend to Fig. 2. The \(^{32}P\)-labeled enzymes were sliced from the gel, and the amounts of \(^{32}P\) were determined. The results presented are mean values of 5 experiments. *, \(p < 0.05\) versus control.

PAGE prior to trypsin treatment. Moreover, SDS-PAGE and subsequent electrophoresis and ethanol precipitation would have been expected to remove \(^{32}P\) that was not covalently bound to synthase. When subjected to SDS-PAGE on a 15% polyacrylamide gel, the \(^{32}P\)-labeled material from fractions 4–6 ran just behind the dye front. Thus, it was separated from \(^{32}P\), which had a higher electrophoretic mobility. The least hydrophobic of the peptides containing known phosphorylation sites is the site 1a peptide, which would not be expected to bind to the column if phosphorylated in two sites. Approximately half of the residues in this small peptide are Ser and Thr (17), although to our knowledge no other residue except 1a has been shown to be phosphorylated. As we are unsure of the identity of the \(^{32}P\)-labeled species in fractions 4–6, this peak has been designated site ?. Site ? may represent multiple \(^{32}P\)-labeled species, as fractions 4–6 contain material that was not appreciably retained by the column.

In synthase immunoprecipitated from control muscles, the amounts of \(^{32}P\) in the different sites, when expressed as percentages of the total \(^{32}P\) recovered in all the peak fractions,

4 J. Zhang and J. C. Lawrence, Jr., unpublished observations.

Figure 4. Phosphorylation site analysis of \(^{32}P\)-labeled glycogen synthase. Glycogen synthase was immunoprecipitated from extracts of \(^{32}P\)-labeled diaphragms, then subjected to SDS-PAGE. The \(^{32}P\)-labeled enzyme was eluted from gel slices, precipitated with ethanol, and incubated at 30 °C for 18 h with 1 mg/ml trypsin. Samples were then applied to a reverse phase (C-18) column, and \(^{32}P\) fragments were eluted at 1 ml/min by using an increasing gradient of acetonitrile. The amounts of \(^{32}P\) in 1-ml fractions were determined by scintillation counting. The results represent average values from 5 experiments. The values in fraction 5, which are off-scale, were as follows (counts/mi): CON, 559; INS, 627; AMY, 889; and INS + AMY, 574. To identify the fragments that contained different phosphorylation sites, purified rat skeletal muscle glycogen synthase was phosphorylated in vitro with \(^{32}P\)/ATP and different kinases having defined specificities as described previously (36). The presumed site assignments are indicated above the peaks and were based on the position of fragments generated by the following protein kinases: CAMP-dependent protein kinase, sites 1a, 1b, and 2a + b; phosphorylase kinase, sites 2a + b; casein kinase II, sites 2a + b; and Fas/GSK-3, sites 3a + b + c. were as follows (means ± S.E.): site 1, 26.5 ± 8.4; site 1a, 5.6 ± 0.2; sites 4 + 5, 4.7 ± 0.5; sites 3a + b + c, 28.5 ± 1.0; site 1b, 19.3 ± 1.7; and sites 2a + b, 15.4 ± 0.7. Thus, site 3a + b + c contained the highest proportion of the total \(^{32}P\), followed closely by site 1a.

Amylin increased the phosphorylation of several sites (Fig. 4). The largest percentage change occurred in site 1a, which was increased by approximately 60% (Fig. 5). Amylin increased the \(^{32}P\) content of sites 3a + b + c and 1b by approximately 35% and 45%, respectively; but, it did not significantly change the phosphorylation of sites 2a + b or 5. Insulin significantly decreased the phosphorylation of sites 1a, 3a + b + c, 1b, and 2a + b. In absolute terms, the largest decrease in \(^{32}P\) occurred in sites 3a + b + c, which contained more \(^{32}P\) than any of the other peak fractions. However, the largest percentage changes were observed in phosphorylation of sites 1a and 2a + b, which were decreased by approximately 50% (Fig. 5). Incubating muscle with the combination of insulin plus amylin did not significantly change the \(^{32}P\) content of any site.

Phosphorylation of Phosphorylase in Response to Amylin—Incubating diahpgrams with amylin increased the phosphorylase activity ratio (-AMP/+AMP) by approximately 2.5-fold, without affecting total (+AMP) phosphorylase activity (Fig. 6). Insulin did not cause a statistically significant decrease in phosphorylase activity ratio in either the presence or absence of amylin, although average activity ratios in the presence of insulin were lower. To examine changes in the phosphorylation state of phosphorylase more directly, the enzyme was immunoprecipitated from the same extracts used for analyses of \(^{32}P\)-labeled glycogen synthase. After SDS-PAGE and autoradiography, \(^{32}P\)-labeled phosphorylase from control and hormonally treated muscles appeared as a single species having exactly the same mobility as the rabbit muscle phosphorylase standard (Fig. 7). To determine changes in phosphorylation, the amount of \(^{32}P\)-labeled phosphorylase was measured after slicing the gels (Fig. 8). Amylin increased the \(^{32}P\) content of phosphorylase.
Amylin-stimulated Phosphorylation

FIG. 5. Effects of amylin and insulin on the phosphorylation of different sites in glycogen synthase. Glycogen synthase was immunoprecipitated from muscle extracts, subjected to SDS-PAGE, eluted from gel slices, and incubated with trypsin. The 32P fragments were resolved by reverse-phase HPLC as described in the legend to Fig. 4. The amounts of 32P in the presumed sites were determined by summing the 32P contents of the peak fractions as follows: site ?, fractions 4–8; site 1a, fractions 17–28; sites (4 + 5), fractions 35–38; sites 3(a + b + c), fractions 48–59; site 1b, fractions 60–70; and sites 2(a + b), fractions 83–90. The results represent mean values ± S.E. from 5 experiments. The S.E. for site ? from the amylin group was 332 cpm, *p < 0.05 versus control.

FIG. 6. Effects of amylin on phosphorylase activity. Muscles were incubated in low P, DMEM for 4 h at 37 °C, then transferred to low P buffer and incubated for 30 min with no additions, 200 nM amylin, 200 nM insulin, or the combination of insulin plus amylin. Extracts were prepared and phosphorylase activities were measured. Total activities, measured in the presence of 2 mM AMP, were as follows (in nanomoles/min/mg of extract protein): control, 510 ± 34; insulin, 481 ± 40; amylin, 575 ± 40; and insulin plus amylin, 615 ± 38. The results presented are activity ratios, determined by dividing the activity measured without AMP by the activity measured with AMP. Mean values ± S.E. from 7 experiments are presented. **, p < 0.01 versus control.

by approximately 2-fold. The increase produced by amylin was abolished by insulin.

DISCUSSION

The present results provide the first direct demonstration of increased phosphorylation of phosphorylase and glycogen synthase in response to amylin. In addition, new information concerning the sites in the glycogen synthase subunit that are phosphorylated in response to amylin and insulin has been presented. These findings provide an explanation for the stable changes in synthase and phosphorylase activities produced by insulin and amylin. Moreover, the findings have important implications with respect to the mechanisms of action of the hormone.

Consistent with previous findings in soleus muscles (13, 26), amylin increased the phosphorylase activity ratio in the present experiments. Phosphorylase is activated when a single site, Ser14, is phosphorylated (16, 27). In the present experiments, amylin increased the 32P content of phosphorylase by approximately 2-fold, which was similar to the increase in the phosphorylase activity ratio produced by the hormone. As all available evidence indicates that Ser14 is the only phosphorylation site in the enzyme, it is likely that the increase in 32P occurred in this site.

The present findings are consistent with a previous study in which amylin was found to decrease the glycogen synthase activity ratio (26). Phosphorylation site analyses indicated that amylin increased the phosphorylation of sites 1a, 3(a + b + c), and 1b (Figs. 4 and 5). While decreases in the synthase activity ratios are indicative of increased phosphorylation of the enzyme, neither the extent nor the sites of phosphorylation could have been predicted from the changes in activity ratio. Most studies of the effect of phosphorylation have been conducted by introducing phosphate into fully dephosphorylated synthase. Using this strategy, the sites have been shown to differ markedly with respect to their influence on the kinetic properties of the enzyme. For example, phosphorylation of sites 5 or 1b has no discernible effect on synthase activity (17). Phosphorylation of the site 3 region in general (17), and site 3a in particular (41), has the greatest effect on decreasing the synthase activity ratio. Thus, it seems likely that the effect of amylin on increasing phosphorylation of one or more sites in the 3(a + b + c) region...
accounts for the effects of the hormone on the synthase activity ratio.

Phosphorylase is thought to be controlled by a relatively small number of enzymes: phosphorylase kinase and the protein phosphatases, PPlγ and calcineurin. Thus, the effect of amylin on phosphorylase presumably results from activation of the kinase and/or from inactivation of at least one of the protein phosphatases. Amylin has been shown to increase cAMP in L6 myotubes (12). Although this effect appears to be mediated by receptors for calcitonin gene-related peptides (12), a sufficient rise in cAMP would lead to sequential activation of cAMP-dependent protein kinase, phosphorylase kinase, and phosphorylase (16).

There is evidence that amylin does not exert its effects via increased cAMP in skeletal muscle. For example, amylin has been reported to activate phosphorylase and inactivate glycogen synthase (26) and to oppose insulin-stimulated glycogen synthesis (12), without changing cAMP. Our findings with glycogen synthase do not eliminate a role of cAMP-dependent protein kinase in amylin action, but they do provide two strong arguments that the effect of amylin cannot be solely accounted for by an increase in cAMP-dependent protein kinase. Firstly, amylin did not significantly increase the phosphorylation of site 2 (Fig. 5), which is one of the sites phosphorylated by cAMP-dependent protein kinase. This distinguishes the action of amylin from that of epinephrine, which increases phosphorylation of sites 2a and 2b (25, 42). It might also be noted that several kinases, including phosphorylase kinase, calmodulin-dependent protein kinase, and protein kinase C phosphorylate site 2 in vitro (17). The failure of amylin to increase phosphorylation in site 2 suggests either that these enzymes are not involved in amylin action or that amylin affects other factors which interfere with phosphorylation of site 2. Secondly, amylin increased the phosphorylation of sites 3(a + b + c) (Fig. 5). It is unlikely that these sites are phosphorylated directly by cAMP-dependent protein kinase, as none conform to the consensus sequence for a cAMP-dependent protein kinase phosphorylation site (43). The activation of cAMP-dependent protein kinase could indirectly involve GSK-3, which appears to be the kinase that phosphorylates sites 3a, 3b, and 3c. Although the preferred sites of cAMP-dependent protein kinase are sites 1a, 2, and 1b, site 4 is slowly phosphorylated (44). By phosphorylating site 4, cAMP-dependent protein kinase generates a phosphoserine recognition site, which is needed to initiate phosphorylation by GSK-3 (28). Activation of cAMP-dependent protein kinase could also lead to phosphorylation of sites 3(a + b + c) by inhibiting the activity of PPlγ, the glycogen-bound form of the Type 1 protein phosphatase (45). Phosphorylation of inhibitor-1 and the glycogen binding subunit of the PPlγ by cAMP-dependent protein kinase decrease the activity of the phosphatase (46). As the substrate specificity of PPlγ is relatively broad (46), decreasing PPlγ activity would be expected to result in a general increase in synthase phosphorylation, including sites not phosphorylated by cAMP-dependent protein kinase.

As previously observed in rat epithelial cells (36), insulin prompted the dephosphorylation not only of sites 3(a + b + c), but also of sites 2(a + b). These findings are consistent with the hypothesis that insulin activates PPlγ (47), as this phosphatase is able to dephosphorylate these sites at a reasonable rate (17). Because phosphorylation of the site 3 region has the greatest effect on the synthase activity ratio in vitro, it has been concluded that the activation of synthase is due to dephosphorylation of sites 3(a + b + c) (21, 40). However, this interpretation may not be entirely correct. Roach et al. (48) have recently investigated the role of phosphorylation by expressing glycogen synthase mutants having Ser to Ala substitutions at different phosphorylation sites. When expressed in COS cells, wild-type synthase had an activity ratio of only 0.015, indicating that it was very highly phosphorylated. Mutation of any single Ser had little effect on the ratio observed. The ratio was significantly increased only by expressing synthase having a double mutation that included site 3a and one of the NH₂-terminal sites (Ser6 or Ser58) (48). Thus, the insulin-stimulated dephosphorylation of site 2(a + b) observed in our experiments may have an important role in the control of synthase activity.

The effect of neither insulin nor amylin on the phosphorylation of glycogen synthase prevailed when muscles were incubated with both hormones (Figs. 1, 3, and 6). This mutual antagonism was not strictly a case of cancellation of equal but opposite actions, as amylin alone did not increase phosphorylation of site 2(a + b), but blocked the decrease in this site produced by insulin (Fig. 6). The concentration (200 nm) of insulin used in the present experiments was sufficient to fully saturate insulin receptors (49). Thus, the present findings are consistent with previous results indicating that amylin produces insurmountable antagonism of insulin action on glycogen synthesis in skeletal muscle (14). More complete definition of the signal transduction pathways involved will be needed to understand the noncompetitive antagonism between insulin and amylin.

REFERENCES

Amylin-stimulated Phosphorylation