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Amylin activates glycogen phosphorylase in the isolated soleus muscle of the rat

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The pancreatic β-cell hormone amylin acts in isolated rat skeletal muscle to decrease insulin-stimulated incorporation of glucose into glycogen. It also increases blood levels of lactate and glucose in fasted rats in vivo. However, it remained uncertain whether amylin exerts direct effects to stimulate muscle glycogenolysis. We now report that amylin caused a dose-dependent increase in activity of muscle glycogen phosphorylase in isolated rat soleus muscle by stimulating phosphorylase *a*. Insulin inhibited amylin-stimulated activation of phosphorylase. Effects of amylin to stimulate muscle glycogenolysis are consistent with observed effects of amylin in vivo and could be a major mechanism whereby amylin modulates carbohydrate metabolism.

Amylin; Muscle; Glycogen; Phosphorylase

1. INTRODUCTION

Amylin is the major protein component of islet amyloid commonly found in the pancreases of patients with non-insulin-dependent diabetes mellitus [1,2]. It is expressed primarily in islet β-cells and secreted from the pancreas following stimulation by nutrients such as glucose or arginine [3]. Several studies have demonstrated that amylin has properties which support the concept that it modulates carbohydrate metabolism [2,4–12].

In vitro studies have shown that amylin can act on skeletal muscle to decrease rates of glucose uptake and incorporation into glycogen [4–9]. Homologous calcitonin gene-related peptide (CGRP) also inhibits net glycogen synthesis in white (extensor digitorum longus) muscles and to a lesser extent in red (soleus) muscles [5,6]. Some studies have proposed that amylin increases glycogenolysis, reduces glycogen content and increases lactate production. However, it has also been proposed that the data might be explained by inhibition of glycogen synthesis [9] and one recent report stated that amylin does not promote muscle glycogen breakdown or enhance lactate production in the presence of insulin [7]. In vivo studies using euglycemic, hyperinsulinemic glucose clamps in rats have shown that amylin decreases insulin-mediated suppression of hepatic glucose output [10,11], and peripheral uptake of glucose [9–11]. Recently, we have shown that amylin acts to increase blood concentrations of lactate and glucose in 18-h fasted rats [12].

Glycogen is the major storage form of carbohydrate in muscle cells. While some of the previous results using isolated muscle might have been explained by stimulation of glycogenolysis, no biochemical mechanism has been identified. In the experiments reported here, we measured the activity of phosphorylase, the rate-limiting enzyme of glycogenolysis, in extracts from rat soleus muscles incubated with different concentrations of rat amylin, and in the presence and absence of 7.1 nM insulin. The phosphorylase assay conditions are designed to minimize the activity of phosphorylase *b* and thus to selectively measure the activity of phosphorylase *a*, the phosphorylated enzyme form that results from the action of phosphorylase kinase on phosphorylase.

2. MATERIALS AND METHODS

2.1. *Materials*

2.1.1. Animals

Harlan Sprague–Dawley rats (200 g males), were housed at 22.7 ± 0.8°C in a 12:12 hour light/dark cycle (experiments being performed during the light cycle) and fed and watered ad libitum (Diet LM-485, Teklad, Madison, WI). Animals were fasted for 4 h before experimentation.

2.1.2. Amylin

Amylin requires the presence within the molecule of both an intact intramolecular Cys^2–Cys^7 disulfide bond and a carboxy-terminal amide group to exert full biological activity to reduce incorporation of ^{14}C-labelled glucose into skeletal muscle glycogen [13].

Bioactivity of commercially available preparations of amylin, as measured by the EC_{50} for inhibition of net insulin-stimulated

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glycogen synthesis in isolated rat soleus muscle [4,5], may vary more than 100-fold (Cooper, G.J.S. et al., unpublished results). Therefore the activity of the rat amylin [14] used in this study (lot no. ZG485, Bachem, Torrance, CA) was first determined using the soleus muscle-based assay; the measured EC_{50} was 6.7 ± 1.3 nM. Recent discrepancies between reported activities of amylin in different biological systems may relate in part to variable purity and biological activity of current synthetic preparations of peptide employed by different experimenters, as well as to the use by some of a non-carboxy-terminally amidated form of the molecule.

Concentrations of amylin in protein-free stock solutions used to apply amylin to muscle preparations were determined using quantitative amino acid analysis as previously described [4].

2.1.3. Chemicals

Soluble insulin, (Humulin R, 100 U/ml) was purchased from Eli Lilly and Co., Indianapolis, IN. The conversion factor between activity units, U, and molar units for insulin used in the present study was 1 μU/ml = 7.1 pM. All other reagents were of analytical grade or better unless otherwise stated.

2.2. *Incubation conditions, measurement of glycogen phosphorylase activity and data analysis*

Isolation and incubation of isolated, stripped rat soleus muscles in the presence of various concentrations of insulin and amylin were performed according to previously described methods [4,5]. Four muscle strips were incubated at each treatment condition.

Control incubations were performed in the absence or presence of insulin (7.1 nM) or amylin (34 nM). Dose-dependent effects of amylin on glycogen phosphorylase *a* activity were studied in the presence of constant insulin (7.1 nM), at increasing concentrations of amylin (0, 0.39, 3.9, 76, 781 nM).

After incubation, muscles were snap-frozen in liquid nitrogen, then stored at −70°C until measurements of glycogen phosphorylase *a* activity were made.

Activity of glycogen phosphorylase *a* in muscle extracts was determined using a previously described modified method [15] omitting AMP in the assay to measure phosphorylase *a*. Enzyme activity is expressed as nmol · glucosyl units transferred/min/mg protein. Concentrations of protein in muscle tissue extracts were measured according to the method of Bradford [16].

All results are presented as mean ± SE. Statistical analysis was performed using the non-paired, two-tailed Student's *t*-test, with significance levels as stated.

3. RESULTS AND DISCUSSION

Treatment of isolated rat skeletal muscle with 34 nM amylin in the absence of insulin increased the activity of muscle glycogen phosphorylase *a* by 2.8-fold, from a basal level of 8.5 ± 0.8 nmol · glucosyl units/min/mg protein to 23.7 ± 4.1 nmol · glucosyl units/min/mg protein ($P < 0.0001$) (Fig. 1).

Amylin produced a dose-dependent increase in the activity of glycogen phosphorylase *a* in the presence of 7.1 nM insulin (Fig. 2). Enzyme activity increased 2.7-fold from a basal level of 6.8 ± 0.7 nmol · glucosyl units/min/mg protein in the absence of amylin to 18.1 ± 2.9 nmol · glucosyl units/min/mg protein at an amylin concentration of 76 nM ($P < 0.0001$).

In the absence of amylin, 7.1 nM insulin did not produce a significant change in glycogen phosphorylase *a* activity (Fig. 1). This is consistent with in vivo dose–response studies in humans where we failed to show an inhibition by insulin of already low glycogen

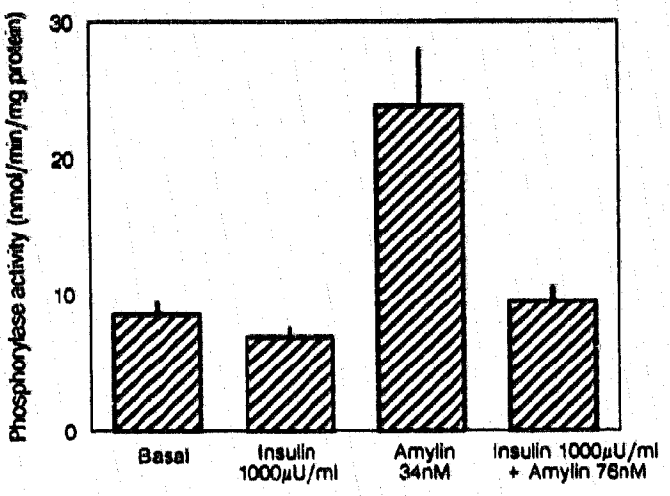

Fig. 1. Phosphorylase activity with amylin and insulin. Glycogen phosphorylase *a* in the isolated soleus muscle after 1 h incubation with insulin alone (7.1 nM), rat amylin alone, (34 nM) or insulin (7.1 nM) plus rat amylin (76 nM). Bars represent mean ± SE, $n = 4$ at each point.

phosphorylase activity [17]. On the other hand, in the presence of amylin, insulin significantly decreased glycogen phosphorylase *a* activity: at 34 nM amylin in the absence of insulin, enzyme activity was 23.7 ± 4.1 nmol · glucosyl units/min/mg protein, while at an insulin concentration of 7.1 nM, phosphorylase *a* activity in the presence of 76 nM amylin fell to 9.4 ± 1.0 nmol · glucosyl units/min/mg protein ($P < 0.02$ compared with activity at 34 nM amylin). We are unaware of any reports demonstrating insulin inhibition of glycogen phosphorylase activity in muscle, although

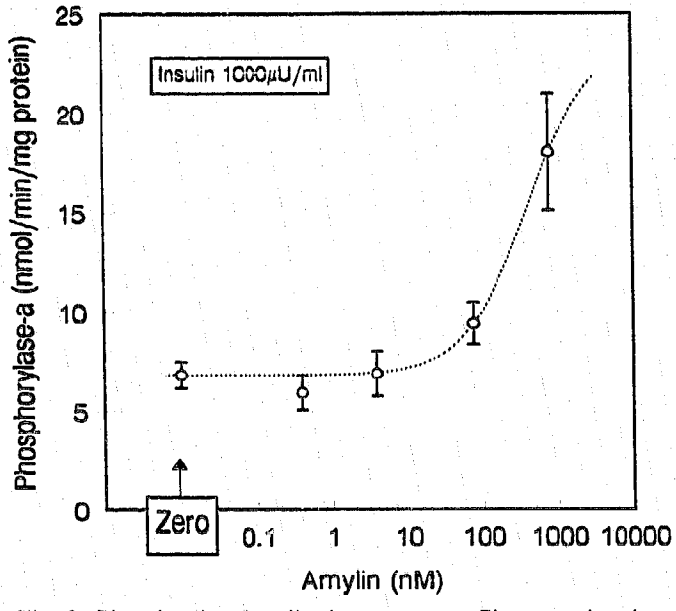

Fig. 2. Phosphorylase/amylin dose-response. Glycogen phosphorylase *a* activity in the isolated soleus muscle following 1 h incubation with increasing concentrations of amylin in the presence of 7.1 nM insulin. Bars represent means ± SE, $n = 4$ at each point.

such an effect had been predicted by the observation that insulin causes dephosphorylation of the enzyme [18]. Significantly, insulin can inhibit the amylin-mediated activation of phosphorylase, while the β-adrenergic [19] activation of the enzyme was not inhibited by insulin at concentrations equivalent to those used in the present study [20].

Glycogen phosphorylase is the rate-limiting enzyme in glycogenolysis [21]. This enzyme catalyzes the sequential phosphorolysis of α-(1->4)-linked glucosyl units from the non-reducing end of glycogen strands to form glucose-1-phosphate. Phosphorylase exists in two interconvertible forms, *a* and *b*. Phosphorylase *b*, the resting form in muscle, is an α_2 dimer of M_r 2 × 97 500 Da. This form is inactive, but is activated by the allosteric effectors AMP and IMP, whereas ATP and ADP act as allosteric inhibitors [21]. Phosphorylase *a* activity, as measured in the present study, is that which is associated with the phosphorylated form of the enzyme and is assayed in the absence of AMP to minimize activity of phosphorylase *b*.

In response to hormonal or neural signals, phosphorylase *b* is converted to phosphorylase *a* by phosphorylation of Ser^{14}, catalyzed by phosphorylase kinase. Phosphorylase *a* is fully active at saturating substrate concentrations, but at low concentrations of P_i its activity is stimulated by AMP. Glucose is an allosteric inhibitor of this enzyme [21].

The ability of amylin to produce dose-dependent increases in the activity of phosphorylase in skeletal muscle in vitro suggests that amylin probably stimulates phosphorylation of the enzyme to effect conversion of the *b* to the *a* form.

In order to activate phosphorylase, amylin presumably acts through an as yet unidentified signalling pathway, in which the first event is binding of amylin to an amylin receptor. Since amylin and CGRP stimulate adenylate cyclase in liver membranes and CGRP increases cAMP levels in skeletal muscle [22–24], one possible mechanism is that the amylin receptors in muscle are linked via Gs to adenylate cyclase, and that activation of phosphorylase is caused by cAMP-dependent protein kinase-mediated activation of phosphorylase kinase. Because amylin-mediated activation was insulin-inhibitable, while adrenalin-mediated activation was not, it is clear that these two hormones cannot induce phosphorylase activation through identical mechanisms.

The effect of amylin to stimulate phosphorylase *a* activity seen in our present study provides a molecular mechanism whereby amylin could exert previously observed biological effects. Stimulation of phosphorylase activity in skeletal muscle, leading to increased rates of glycogenolysis and glycolysis, could account for dose-dependent increases in plasma lactate produced by amylin [Young, A.A. et al. in preparation]. Amylin stimulation of phosphorylase activity could fit with a role for amylin in effecting the previously unexplained increment in phosphorylase *a* activity observed after an oral glucose load in rats [25]. The stimulation of muscle phosphorylase and hence glycogenolysis and lactate production in resting muscle is consistent with the proposed role for amylin as a physiological regulator of muscle glycogen metabolism.

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