Amylin influences insulin-stimulated glucose metabolism by two independent mechanisms

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Amylin influences insulin-stimulated glucose metabolism by two independent mechanisms. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E6–E12, 1998.—The effects of amylin on fiber type-specific muscle glucose metabolism under hyperglycemic (10 mmol/l) and hyperinsulinemic (2.1 mmol/l) conditions were investigated using a rat hindlimb perfusion system. Amylin concentration ranged from 1 to 100 nM. Efficacy for inhibition of glucose uptake traced with 2-deoxyglucose by amylin was demonstrated in all three fiber types. The incorporation of 2-deoxy-[3H]glucose tracer decreased from control values by 41% in fast oxidative (FO), 36% in fast glycolytic (FG), and 37% in slow oxidative (SO) muscle with 100 nM amylin. Amylin increased intracellular glucose 6-phosphate (G-6-P), and G-6-P was negatively correlated with 2-deoxyglucose uptake in both FO (r = −0.65; P < 0.01) and FG (r = −0.53; P < 0.01). Muscle glycogen concentration increased under control conditions and decreased in the presence of 100 nM amylin. Lactate arteriovenous efflux across the hindlimb increased significantly above control with 100 nM amylin (5.03 ± 0.81 to 11.28 ± 0.94 µmol·g⁻¹·h⁻¹). Adenosine 3',5'-cyclic monophosphate (cAMP) increased in FO and FG muscle with amylin. Salmon calcitonin-(8—32), an amylin antagonist, ameliorated the effect of amylin on all responses other than 2-deoxyglucose uptake and G-6-P concentration. These results suggest that amylin may work through at least two independent mechanisms, a cAMP-mediated effect on glycogen metabolism and a non-cAMP-mediated inhibition of glycolysis.

skeletal muscle; glucose uptake; glucose 6-phosphate; glycogen metabolism and a non-cAMP-mediated inhibition of glycolysis.

Amylin, a 37-amino acid peptide, is cosecreted with insulin from the pancreatic β-cells (10). It was originally isolated from amyloid plaque formations in type II diabetics and has been shown to inhibit insulin-mediated glucose uptake and disposal in muscle and to raise blood lactate (4, 26). Although the actual role of amylin is unclear, it has been suggested that amylin agonists may be helpful in preventing hyperinsulinemic shock in insulin-dependent diabetes mellitus and that amylin antagonists, such as salmon calcitonin-(8—32) [SCT-(8—32)], may be useful in increasing blood glucose uptake into muscle in the non-insulin-dependent diabetes mellitus (NIDDM) patient (26, 29, 31).

The mechanism of amylin action on skeletal muscle is still not completely understood (26). Amylin appears to inhibit glucose uptake by inhibiting glycogen synthesis and stimulating glycogenolysis possibly through an adenosine 3',5'-cyclic monophosphate (cAMP)-mediated mechanism (5, 16, 18, 23, 33). This may limit glucose uptake by increasing intracellular glucose 6-phosphate (G-6-P) levels, which could cause an inhibition of hexokinase resulting in a decrease in both glucose phosphorylation and net retention of transported glucose. It is unclear whether the inhibition of glycogen synthesis or activation of glycogen breakdown is primarily responsible for amylin action (5, 7, 17, 19, 33). Studies have also conflicted on whether a cAMP-mediated second messenger is responsible for amylin action and whether this mechanism is fiber type specific (5, 16, 23).

The purpose of this study was to test the efficacy and mechanism of amylin action across the basic fiber types of skeletal muscle. In addition, the efficacy of the SCT-(8—32) fragment as an amylin antagonist was tested. Almost all studies on amylin action to date have been conducted in vivo or with isolated muscle preparations. In most of the isolated muscle studies the rat soleus muscle was used. However, the soleus contains 80–90% slow oxidative fibers, and this fiber type accounts for only a small portion of the rat's musculature (1). Therefore, the nonrecirculating hindlimb perfusion technique was used both to examine the effect of amylin across all three basic muscle fiber types simultaneously and to eliminate any counterregulatory effects due to changes in circulating amylin or insulin concentration that could occur in vivo. Emphasis was placed on examining the dose-response mechanism across fiber types by measuring second messengers and metabolic intermediates. Amylin dosages were chosen from those previously shown to be minimally to maximally effective in their ability to inhibit insulin-stimulated glucose disposal in the rat (15, 19). The concentrations of insulin and glucose were selected to explore postprandial conditions of the rat in which amylin could possibly have an important physiological role.

RESEARCH DESIGN AND METHODS

Perfusion and surgical procedure. Forty male Harlan Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), 3–4 wk of age, were purchased, housed three to a cage, and allowed access to food and water ad libitum. The temperature of the animal room was maintained at 23°C, and a 12:12-h light-dark cycle was set. All procedures used in this study were approved by the University of Texas Animal Care and Use Committee.

Rats weighing 168.4 ± 0.5 g were fasted for 4–6 h, which resulted in muscle glycogen concentration averaging 30.91 ± 0.52 µmol/g wet wt, which did not differ among fiber types. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg/100 g body wt). The hindlimbs were surgically isolated, and catheters were placed in the descending aorta and vena cava, as previously described (31). Animals were killed with an intracardiac injection of pentobarbital sodium as the hindlimbs were being washed out with 25 ml Krebs-Henseleit buffer (KHB). Catheters were then
were collected in duplicate and centrifuged at 2,000 g. Deoxyglucose tracers were added, 1-ml venous effluent samples were placed in line with a nonrecirculating perfusion system that included [3H]glucose. At 5, 10, 15, and 20 min after the mannitol and tracer quantities (0.1 µCi/ml) of 2-deoxy-[14C]mannitol and tracer into muscle tissue. 2-Deoxyglucose is a nonmetabolizable glucose analog that has rates of removal from blood into muscle similar to glucose under a physiological range of insulin concentration, but it is not metabolized beyond phosphorylation by hexokinase and provides a good estimate of glucose uptake in muscle tissue (21, 27). Freeze-clamped muscles from the perfusion were sectioned and weighed frozen. A 60- to 100-mg piece of each muscle or muscle section was dissolved in 1 ml 1 N KOH by incubating it for 20 min at 70°C; it was mixed and incubated an additional 10 min at 70°C. An equal volume of 1 N HCl was added to the digested samples and mixed, and aliquots of the neutralized samples were counted for 3H and 14C disintegrations per minute. 2-Deoxyglucose uptake was calculated as a percentage of the tracer concentration in the perfusate (intracellular concentration divided by extracellular concentration × 100). The amount of tracer contained within the extracellular space was determined by the amount of [14C]mannitol retained in the tissue.

Muscle glycogen concentration was determined after its complete enzymatic degradation to glucose with amyloglucosidase (22). An aliquot of the KOH-digested muscle was incubated overnight in 0.3 M sodium acetate buffer, pH 4.8, that contained 5 mg/ml amyloglucosidase (Boehringer Mannheim, Mannheim, Germany). Liberated glucose was then measured using a spectrophotometric Trender reaction (no. 315, Sigma Chemical, St. Louis, MO). Perfusion supernatant samples collected during the tracing period of the perfusions were thawed and mixed well before lactate determination. For muscle tissue lactate, ~100 mg of muscle were powdered under liquid nitrogen and added to three volumes (vol/wt) of 10% perchloric acid (PCA). The sample then was further homogenized in a motorized glass homogenizer and centrifuged at 2,000 g for 15 min. The perfuse and muscle supernatant samples were analyzed for lactate, as described by Hohorst (9). A portion of the muscle supernatant was neutralized with saturated (30%) KHCO3. This neutralized sample was then centrifuged at 2,000 g for 15 min and assayed for G-6-P (20).

A 100-mg portion of the FO and FG quadriceps, excised ~8 min into the equilibrium period of the perfusion, was homogenized in 400 µl 6% PCA at 0°C. The sample was neutralized with saturated 30% KHCO3 before analysis. cAMP was then measured by competitive binding with labeled [3H]cAMP (kaph2, Diagnostic Product, Los Angeles, CA) (8).

Statistical analysis. Individual fiber type responses were analyzed by one-way factorial or repeated-measures analysis of variance (ANOVA). Full multivariate ANOVA was run on all responses to determine overall effects, fiber type specificity, and time differences in response to the different dosages of amylin used. Significance was accepted when P values were <0.05. Individual treatment groups were compared with respective controls using Fisher’s protected least significant difference post hoc test. Correlations were performed between appropriate responses.

RESULTS

Insulin-stimulated 2-deoxyglucose uptake was inhibited by perfusion with amylin in all three basic muscle fiber types, and SCT-(8—32) did not attenuate this inhibition (Fig. 1). The 2-deoxyglucose uptake was negatively correlated with the dose of amylin used, with a maximal uptake inhibition of ~40% in each fiber type. Different fiber types had different absolute uptake responses, but muscle fiber type did not affect the relative response to amylin or amylin plus SCT-(8—32).

Perfusions with amylin significantly affected glycogen metabolism. No differences among fiber types were detected with multivariate ANOVA analysis. Fiber types were therefore pooled for presentation in Table 1. Under control conditions and in the presence of 1 nM amylin, there was an accumulation of muscle glycogen during the perfusion. However, when the hindlimbs were perfused with 10 or 100 nM amylin, there was a reduction in the muscle glycogen concentration. SCT-(8—32) in equal molar concentrations with amylin (100 nM) was able to eliminate all the detectable amylin effect, because glycogen accumulation was essentially the same as control.

G-6-P increased in response to amylin in the FO and FG fibers, whereas SCT-(8—32) was not able to prevent this rise in G-6-P (Fig. 2). G-6-P concentration was correlated positively with amylin dosage and nega-
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Fig. 1. Muscle glucose uptake measured by 2-deoxy-[3H]glucose (DOG) accumulation in three basic fiber types [slow oxidative (SO), fast oxidative (FO), and fast glycolytic (FG) muscle] after exposure to 0, 1, 10, or 100 nM amylin or 100 nM amylin + 100 nM salmon calcitonin [SCT-(8—32)] (ASCT). †All three fiber types demonstrated a significant dose effect (P < 0.05). *Significantly different from its fiber type control.

Fig. 2. Glucose 6-phosphate (G-6-P) concentration of FO and FG muscle excised during equilibration period (A) and at end of perfusion (B). †Dosage effect is significant for all groups (P < 0.05). *Significantly different from control.

Amylin caused an increase in muscle tissue lactate, and SCT-(8—32) was able to attenuate this rise (Fig. 4). There was a significant difference between FO and FG fiber types in lactate response from the equilibration period to the end of perfusion. FO muscle lactate increased with time, whereas FG muscle did not change.

The increase in muscle lactate resulted in an increase in muscle lactate efflux, as represented by increases in venous perfusate lactate levels. SCT-(8—32) prevented this amylin-induced efflux of lactate (Fig. 5).

A rise in cAMP occurred with amylin in both FO and FG fibers (Fig. 6). SCT-(8—32) completely blocked this rise in cAMP. cAMP was elevated in all groups in which glycogenolysis occurred.

DISCUSSION

Although the absolute uptake of 2-deoxyglucose tracer into muscle was different among fiber types, amylin decreased insulin-stimulated uptake of tracer to the same relative extent in all three basic fiber types investigated (Fig. 1). This indicates that skeletal muscle glucose uptake is reduced by amylin, as suggested by earlier observations (7, 24, 33, 34). The different absolute rates of insulin-stimulated glucose uptake across fiber types in both normal and insulin-resistant muscle are well documented (6, 11, 28). The same relative inhibition of 40% across all fiber types demonstrates two important aspects about amylin action on glucose uptake in muscle. First, amylin is effective on all fiber types. Second, amylin efficacy appears to be roughly balanced with insulin efficacy across fiber types.

Table 1. Muscle glycogen changes during hindlimb perfusion

<table>
<thead>
<tr>
<th>Amylin Treatment</th>
<th>All Muscle Fiber Types</th>
<th>SO</th>
<th>FO</th>
<th>FG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>+1.65 ± 0.63 (42)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 nM</td>
<td>+1.24 ± 0.95 (18)</td>
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<td></td>
</tr>
<tr>
<td>10 nM</td>
<td>−2.07 ± 1.05† (18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 nM</td>
<td>−1.60 ± 0.88† (24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 nM + SCT-(8—32)</td>
<td>−1.60 ± 1.18 (18)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of nos. of observations in parentheses and show differences in muscle glycogen concentration between early equilibration period (first 5 min of perfusion) and end of perfusion. SO, FO, and FG, slow oxidative, fast oxidative, and fast glycolytic muscle, respectively. SCT-(8—32), salmon calcitonin fragment. *Significantly different from control (0 nM amylin). †Significantly different from 100 nM amylin plus SCT-(8—32).
An inverse relationship between uptake of tracer and G-6-P concentration occurred with all treatments (Fig. 3). It has been hypothesized that amylin shifts the rate-limiting step of glucose uptake from transport to disposal of glucose into glycogen (24, 33). In our study, G-6-P was found to increase with increasing dosage of amylin and over time, suggesting that amylin could possibly cause an inhibition of glucose uptake by raising the intracellular G-6-P concentration and inhibiting glucose phosphorylation by hexokinase under the conditions tested (Fig. 2). The temporal increase of G-6-P also suggests that this inhibition may not have reached its peak even with 30 min of amylin exposure in the perfused hindlimb. Recently, additional support for a gradual increase in the ability of amylin to inhibit muscle glucose uptake has come from the observation that the rate of glucose disposal (Rd) gradually decreases with prolonged amylin exposure during a hyperinsulinemic euglycemic clamp (13). In addition, it has been demonstrated that amylin in plasma concentration of 300 pM can inhibit the Rd of glucose from the circulation (7). Our observations support these findings and suggest that these decreases in whole body glucose uptake are due in part to decreases in glucose uptake in all three basic muscle fiber types of the rat.

Although the reduction in muscle glucose uptake in the presence of amylin was correlated with the intracellular G-6-P concentration, we cannot exclude the possibility that amylin may have inhibited glucose trans-

Fig. 3. Correlation between muscle G-6-P concentration and insulin-stimulated glucose (DOG) uptake for FO (A) and FG (B) muscle.

Fig. 4. Tissue lactate concentration of FO and FG muscle during equilibration period (A) and at end of perfusion (B). †Dosage effect is significant for FG muscle, and there was an overall dosage response found (P < 0.05). *Significantly different from control.

Fig. 5. Perfusate lactate during hindlimb perfusion. Lactate determination was made on samples of perfusate taken directly from venous line and therefore represents lactate efflux from muscle. †Significant dose and time effect (P < 0.05). Lactate efflux increased significantly with time under all amylin dosages. *Significantly different from control at time point indicated.
It is of interest to note that the intracellular G-6-P levels were elevated to the same extent after amylin exposure with or without the amylin antagonist SCT-(8—32) present, despite inhibition of amylin-activated glycolysis by SCT-(8—32). Because glucose uptake was reduced to the same extent by both the amylin and amylin plus SCT-(8—32) treatments, the G-6-P results suggest that amylin controls intracellular G-6-P concentration by regulating the rate of glycolysis. This conclusion presents a paradox, for amylin alone increased rather than decreased lactate production. This discrepancy may be explained, however, by relating the rate of glycolysis to substrate concentration. That is, all treatment groups containing amylin exhibited an inhibition of glycolysis, as defined by a slower rate of glycolysis for a given G-6-P concentration compared with control. Therefore, amylin may cause a relative inhibition of glycolysis while mobilizing substrate from glycogen. A likely control point for regulation of glycolysis by amylin would be phosphofructokinase, as this enzyme is allosterically regulated by many metabolites.

As previously mentioned, the breakdown in glycogen was associated with an increase in muscle lactate concentration and muscle lactate efflux, which continued to increase with time (Figs. 4 and 5). Young et al. (30) showed that it takes 30 min for blood lactate to peak after a bolus amylin injection. These observations further support our contention that the action of amylin may gradually increase with time. The SCT-(8—32) analog AC-187 has been shown to attenuate lactate efflux from muscle (29), and we have demonstrated that SCT-(8—32) is also effective in eliminating this amylin-induced lactate efflux (Figs. 4 and 5). However, the decreased rate of amylin-induced lactate production with SCT-(8—32) present and G-6-P elevated further supports our contention that the relative rate of glycolysis is slowed by amylin (Figs. 1 and 2).

The increases in glycogenolysis and lactate production in response to amylin were similar to effects that occur with agents that act via cAMP-mediated pathways. In the present study, we found that amylin significantly elevated the cAMP concentration of FO and FG muscle. An intracellular rise in cAMP can cause a prolonged activation of glycogen phosphorylase and inhibition of glycogen synthase through the activation of protein kinase A, but evidence against cAMP as a second messenger for amylin action has been reported (5, 16). In both myocytes and isolated soleus muscles, no increase in intracellular cAMP was found after 15 min of incubation with dosages of amylin that effectively inhibited glucose uptake (16). Furthermore, Deems et al. (5) did not see a rise in cAMP in isolated soleus muscle after 5 min of incubation with 100 nM amylin, but they demonstrated consistent changes in glycogen phosphorylase and glycogen synthase activities. However, Pittner et al. (23) recently reported that 100 nM rat amylin increased the cAMP concentration threefold in isolated soleus muscle strips. The rise in cAMP peaked within 5 min and returned to basal levels between 20 and 40 min of incubation. In response to the rise in cAMP there was a prolonged increase in glyco-
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Amylin may mediate its glycogen effects through a calcitonin gene-related peptide (CGRP) receptor, and SCT-(8–32) may be a CGRP receptor antagonist (12, 25). CGRP is a 37-amino acid peptide that differs only slightly from amylin (4, 25). One of the two types of CGRP receptors is known to be coupled to adenyl cyclase, and amylin can also activate this receptor (25). Evidence exists suggesting that there may be amylin-specific receptors in addition to these CGRP receptors (2, 12, 25). However, until now no evidence existed for multiple postreceptor effects that could be separated by an antagonist. The existence of multiple receptors for amylin and CGRP is based on binding data or sensitivity to antagonism of the same biological effect (2, 12, 25). The demonstration of possibly two distinct pathways of amylin action could help unravel the disparate findings of other researchers (5, 16, 17, 23). However, studies on both binding and functional responses will be needed before it can be determined whether the two functional effects demonstrated are the result of distinct receptors or some anomalous single receptor.

For several metabolic responses, a large dose of amylin was needed before a significant effect could be detected. The magnitude of each response also varied. The largest concentration of amylin used in this study was approximately one order of magnitude greater than concentrations reported in the rat (15) and two orders of magnitude greater than concentrations reported for humans (3). The large discrepancy between effective dosage in vitro and reported in vivo values has cast doubt on any physiological role for amylin. However, several in vivo studies have suggested that amylin is effective at physiological concentrations. Vine et al. (29) demonstrated that an antagonist based on SCT-(8–32) was able to decrease lactate efflux across the rat hindlimb. Frontoni et al. (7) demonstrated that plasma concentrations of 300 pM could change the fraction of plasma glucose that was partitioned to glycolysis and glycogenesis in muscle. Also, Young et al. (33) demonstrated that antibody measurements underestimate in vitro amylin concentration. This observation would suggest that the in vivo measurement of amylin concentration may not be reliable. Regardless of the physiological role of amylin, understanding its mechanism of action is very important, because amylin agonists and antagonists are being developed for use in humans (14, 29).

In conclusion, this is the first study to demonstrate at least two functionally different mechanisms of action for amylin on glucose metabolism. One mechanism is cAMP mediated and acts on glycogen, and the second inhibits or regulates glycolysis, elevates G-6-P, and inhibits glucose uptake independently of cAMP mediation. This could explain why several researchers have not observed cAMP as a mediator of amylin action and could therefore explain some of the discrepancies in the literature (5, 16). We conclude that SCT-(8–32) is an effective antagonist of amylin-activated glycogenolysis and lactate efflux, a view which supports the use of compounds based on this peptide as inhibitors of the Cori cycle for controlling blood glucose in NIDDM (29, 31). However, such compounds may not be effective in attenuating amylin inhibition of insulin-stimulated glucose uptake and would therefore not be expected to reduce peripheral insulin resistance. Thus it appears that amylin may work through several pathways to control glucose metabolism in muscle and that these pathways can be separated with the antagonist SCT-(8–32).

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