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Amylin found in amyloid deposits in human type 2 diabetes mellitus may be a hormone that regulates glycogen metabolism in skeletal muscle

(insulin/insulin action/insulin resistance/diabetes-associated peptide/synthetic peptides)

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ABSTRACT **Diabetes-associated peptide has recently been isolated and characterized from the amyloid of the islets of Langerhans in type 2 (non-insulin-dependent) diabetics, and immunoreactivity with antibodies to the peptide has been demonstrated in islet B cells of both normal and type 2 diabetic subjects. In view of the evidence presented in this paper that this 37-amino acid peptide may be a hormone present in normal individuals, we now propose the name "amylin" to replace "diabetes-associated peptide." Because increased amylin, deposited as amyloid within the islets of Langerhans, is characteristic of type 2 diabetes, the study below was performed to examine the possible effects of amylin on peripheral glucose metabolism. Whole amylin was synthesized by using solid-phase techniques, with formation of the disulfide linkage by oxidation in dilute aqueous solution and recovery of the peptide by lyophilization. The effects of amylin on glucose metabolism were studied in two preparations *in vitro*, isolated rat soleus muscle strips and isolated rat adipocytes. In skeletal muscle exposed to 120 nM amylin for 1 hr, there was a marked decrease in both basal and submaximally insulin-stimulated rates of glycogen synthesis, which resulted in significant reduction in the rates of insulin-stimulated glucose uptake. In muscles treated with amylin there was no response at the concentration of insulin required to stimulate glucose uptake half-maximally in untreated (control) muscles. In marked contrast, amylin had no effect on either basal or insulin-stimulated rates of glucose incorporation into either CO_2 or triacylglycerol in isolated adipocytes. Therefore, amylin may be a factor in the etiology of the insulin resistance in type 2 diabetes mellitus, as both deposition of the peptide in islet amyloid and decreased rates of glucose uptake and glycogen synthesis in skeletal muscle are characteristic of this condition.**

Although it has been known for some time that amyloid of the islets of Langerhans is a feature of the type 2 diabetic pancreas, the monomer has only recently been shown to be the 37-amino acid peptide amylin (1). Specific immunoreactivity with antibodies to amylin is found in islet amyloid and in cells of the islets of Langerhans, where it colocalizes with insulin in islet B cells (2–4). Islet amyloid is a feature of spontaneous diabetes in domestic cats (5) and macaques (6) as well as in humans. The monomer of feline islet amyloid has been partially characterized and shown to be a peptide closely related to human amylin (4). In diabetic macaques, islet amyloid is a prediabetic feature that increases along with the degree of increasing glucose intolerance (7), and the extent of islet amyloid deposition also increases with increasing clinical severity in human type 2 diabetes mellitus (8).

Insulin resistance is a major pathophysiological feature in both obese and nonobese subjects with type 2 diabetes mellitus, and it may be due mainly to a postbinding defect in insulin action (9). Such a defect could be due to an intrinsic property of peripheral cells, or caused by a change in concentration of a humoral factor in plasma, or both. Attempts at demonstrating a humoral factor responsible for insulin resistance have so far yielded conflicting results. Nor has it been possible to demonstrate an intrinsic postbinding defect in insulin action in type 2 diabetes mellitus (10, 11).

The mechanisms of insulin resistance in type 2 diabetes are complex and currently only incompletely understood. Evidence, gleaned mainly from studies on adipose tissue, suggests that in the mildest cases, insulin resistance may largely be accounted for by a decrease in the numbers of insulin receptors on the plasma membranes of peripheral target cells, but as the degree of fasting hyperglycemia increases, a postreceptor defect in insulin action emerges and progressively increases in significance (11). The impaired glucose tolerance accompanying insulin resistance in type 2 diabetes is caused largely by decreased rates of glucose uptake in peripheral tissues, but incomplete glucose-induced suppression of hepatic glucose production is probably also a factor (12). In both obese and nonobese subjects with type 2 diabetes, the insulin dose-response curve is shifted to the right and there is a marked decrease in the maximal rate of glucose disposal and of total-body glucose metabolism in type 2 diabetics compared with nondiabetic subjects (11, 13).

The majority of the glucose in an oral glucose load in humans is utilized in the periphery, where quantitatively the most important tissue is skeletal muscle (14, 15), and it has recently been demonstrated that reduced clearance of glucose into skeletal muscle accounts for the bulk of the decrease in total body glucose uptake in type 2 diabetics (13). The *in vivo* decrease in insulin-mediated glucose disposal in type 2 diabetes is now known to be caused mainly by a marked reduction in nonoxidized glucose storage, primarily in skeletal muscle, rather than by a major shift in glucose or lipid oxidation (16, 17). The degree to which relative insulin deficiency contributes to the overall reduction in whole-body glucose clearance is unclear. Muscle glycogen synthesis has been shown to determine the *in vivo* insulin-mediated glucose disposal rate in humans (18), muscle glycogen synthase activity is well correlated with insulin-mediated glucose storage rates, and the change in muscle glycogen synthase activity corresponds well with the change in muscle glycogen content after an insulin infusion.

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Abbreviation: U, activity unit.
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MATERIALS AND METHODS

Peptide Synthesis and Analysis. The isolation and characterization of amylin were carried out as described previously (1). Amylin was synthesized according to the previously described sequence, using solid-phase techniques (19) on a 430A Applied Biosystems peptide synthesizer with commercially available phenylacetamidomethyl resins and *t*-butoxycarbonyl-protected amino acids and reagents (Applied Biosystems, Foster City, CA). The peptide was cleaved from the resin and the side-chain-protecting groups were simultaneously removed by treatment with anhydrous hydrofluoric acid with anisole as a free radical trap. The side-chain-protecting groups were extracted with diethyl ether. Subsequently, the peptide was dissolved in 15% (vol/vol) acetic acid, filtered from the resin, and lyophilized to yield the crude reduced peptide, which was analyzed by HPLC on a C_8 reverse-phase column (Aquapore RP-300; Brownlee Laboratories, Santa Clara, CA). Sequence and quantitative amino analysis of the synthetic peptide indicated that the material was substantially pure as judged by the sensitive criterion of amino acid sequence analysis. At no point during analysis was a second, contaminating sequence seen. The intramolecular disulfide linkage was formed by oxidation in dilute solution in pH 8 water for 12 hr, after which the peptide was recovered by lyophilization. The percentage of free—SH groups after oxidation was found to equal 7% of the total. The concentration of amylin after dissolution in buffers was measured by quantitative determination of amino acid composition using a Waters Pico Tag amino acid analysis system (Millipore, Harrow, Middlesex, U.K.) (20).

Animals, Chemicals, and Enzymes. Male Wistar rats, 160–180 g, were purchased from Harlan Olac (Bicester, U.K.); they were kept in the Biochemistry Department's animal house for at least 7 days before experimentation and were fed ad lib. Rats were fasted for 12–14 hr prior to each experiment and were killed by cervical dislocation. All chemicals, biochemicals, and enzymes were obtained from sources given previously (21).

Biochemical Procedures and Statistical Analysis. Insulin has major effects on glucose metabolism in skeletal muscle and adipose tissue; it dramatically increases the rates of glucose transport and glycogen synthesis in muscle and glucose transport (measured by CO_2 production) and triacylglycerol formation in adipose tissue. In skeletal muscle preparations, the responses of glucose transport and glycogen synthesis to insulin are indicated by the rate of conversion of glucose to lactate (22–24) and by the rate of incorporation of [U-^{14}C]glucose into glycogen, respectively. The effects of insulin on adipose tissue are indicated by alteration of [U-^{14}C]glucose incorporation into $^{14}CO_2$ and triacylglycerol.

Soleus muscle strips were prepared as previously described (25, 26). The isolated muscles were transferred immediately into silane-treated 25-ml Erlenmeyer flasks containing Krebs–Ringer bicarbonate buffer at 37°C with the following composition (in mM); NaCl, 104; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 6.7; $NaHCO_3$, 22; KCl, 4; $CaCl_2$, 1.1; KH_2PO_4, 1; $MgSO_4$, 1; pyruvate, 5; succinate, 5; L-glutamate, 5; and D-glucose, 5.5. Defatted bovine serum albumin (27) was added to a final concentration of 1.5% (wt/vol) and the pH was adjusted to 7.31 with 1 M NaOH. The medium was gassed with O_2/CO_2 (95:5, vol/vol) during preparation; flasks were gassed with O_2/CO_2 continuously during the incubation. After a 30-min incubation the muscles were transferred into other flasks with identical Krebs–Ringer bicarbonate buffer (without added pyruvate, succinate, or glutamate) containing D-[U-^{14}C]glucose (0.5 μCi/ml; 1 Ci = 37 GBq) and various concentrations of insulin (1–1000 μU/ml) (the conversion between activity units, U, and molar units for insulin is 1 μU/ml ≈ 7.1 pM). Previous studies have demonstrated that similar rates of lactate formation and glycogen synthesis are obtained if muscles are incubated in the absence of insulin or in its presence at 1 μU/ml (21). Furthermore, the maximal response of these processes to insulin is obtained at 1000 μU/ml (21). To determine whether the molecule had biological activity, amylin was used at the single concentration of 120 nM (the experimentally determined maximal soluble concentration of the molecule in a protein-free physiological medium). After 60-min incubation the muscles were quickly removed, blotted, and freeze-clamped in liquid N_2 and [U-^{14}C]glucose incorporation into glycogen was monitored (28). The concentration of lactate in the incubation medium was assayed enzymatically (29).

Adipocytes were prepared and incubated as previously described (30). Adipocytes were incubated in the presence of glucose (1 mM; [U-^{14}C]glucose at 0.25 μCi/ml), insulin (0, 10, or 1000 μU/ml), and amylin (0 or 120 nM). The rate of CO_2 formation was measured by the rate of [U-^{14}C]glucose oxidation to $^{14}CO_2$ (31). The rate of incorporation of [U-^{14}C]-glucose into triacylglycerol was determined according to the following procedures: adipocytes were ruptured by addition of 5 ml of isopropyl alcohol/*n*-heptane/1 M sulfuric acid (4:1:0.1, vol/vol), and the triacylglycerol was extracted by addition of 3 ml of *n*-heptane and 2 ml of water. The aqueous layer was separated by freezing with solid CO_2, the upper heptane layer was transferred to scintillation vials and evaporated to dryness under a stream of compressed air, and the radioactivity incorporated into triacylglycerol was determined after the addition of scintillant.

Results are expressed as mean ± SEM, and statistical significance was determined by Student's *t* test.

RESULTS

The effects of amylin on insulin-stimulated rates of lactate formation and glycogen synthesis in isolated incubated soleus muscles of rats are presented in Table 1. Although there was a trend for amylin to decrease the rate of glycolysis at all insulin concentrations, the effects were never statistically

Table 1. Effects of amylin on insulin-stimulated rates of lactate formation and glycogen synthesis in isolated incubated soleus muscles of rats

Insulin, μU/ml	Rate of lactate formation, μmol/hr per g wet weight		Rate of glycogen synthesis, μmol/hr per g wet weight	
	Control	Amylin (120 nM)	Control	Amylin (120 nM)
1	7.57 ± 0.64 (14)	6.61 ± 0.47 (13)	2.27 ± 0.16 (14)	1.58 ± 0.15 (13)
10	9.95 ± 0.86 (8)	8.41 ± 0.35 (8)	2.59 ± 0.37 (8)	1.79 ± 0.14 (8)
100	11.42 ± 0.63 (14)	10.61 ± 0.41 (13)	5.53 ± 0.28 (14)	4.37 ± 0.23 (13)
1000	13.35 ± 1.00 (8)	12.85 ± 0.45 (8)	5.44 ± 0.72 (8)	5.13 ± 0.39 (8)

All values are mean ± SEM. The number of replicate experiments is included in parentheses. The differences in glycogen synthesis between amylin-treated and control muscles are significant at the $P < 0.05$ level at all concentrations of insulin except 1000 μU/ml. Although a tendency to decreased rates of lactate formation is present in amylin-treated muscles, this does not reach significance at any point.

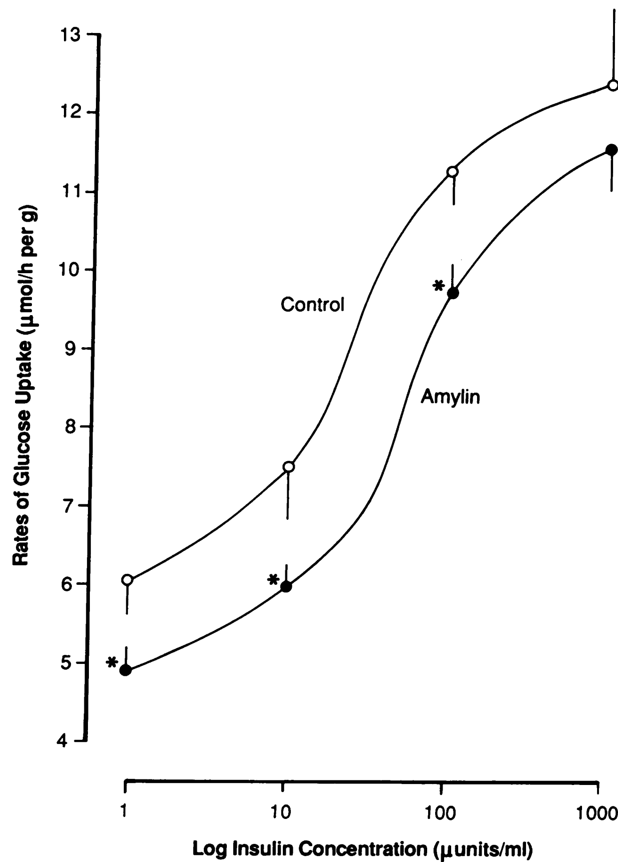

FIG. 1. Effect of amylin on the insulin-stimulated rate of glucose uptake in isolated incubated skeletal muscles of rats. Glucose uptake was calculated by combining the rates of glycolysis and glycogen synthesis.

*Rate of glucose uptake in treated muscles is significantly lower than that in control muscles, $P < 0.05$.

significant. However, amylin caused a marked decrease in both basal and submaximally stimulated rates of glycogen synthesis; in the presence of a supraphysiological concentration of insulin this effect was overcome. The concentration of insulin required to stimulate glucose uptake into soleus muscle half-maximally (i.e., the EC_{50} or sensitivity value) was calculated by combining the rates of glycolysis and glycogen synthesis. The summation of these two rates is an excellent indication of the rate of glucose utilization in soleus muscle, since there is negligible insulin-stimulated flux of glucose through the pentose phosphate pathway (32), to alanine (M.P.-B., unpublished results), or through the Krebs cycle (24, 31). Other studies have shown that the rates of glucose uptake determined in this manner are in excellent agreement with values obtained from studies of uptake and conversion of 2-deoxyglucose to 2-deoxyglucose 6-phosphate (33). Amylin caused a decrease in the rates of glucose uptake at physiologically relevant insulin concentrations (10–100 μU/ml) such that, at the concentration of insulin required to stimulate glucose uptake half-maximally in untreated muscles (i.e., 25 μU/ml), there was no response to insulin in the treated muscles (Fig. 1).

The effects of amylin on the insulin-stimulated rate of glucose metabolism in isolated adipocytes are presented in Table 2. The two major fates for glucose in adipose tissue are the formation of CO_2 and formation of triacylglycerol. However, in contrast to the effects on muscle, amylin had no effect on either basal or insulin-stimulated rates of formation of either CO_2 or triacylglycerol.

DISCUSSION

The 37-amino acid peptide amylin, previously termed diabetes-associated peptide, has been shown to be a major component of the amyloid of the islets of Langerhans in type 2 diabetes. Specific amylin immunoreactivity has been demonstrated in islet amyloid and also in islet B cells from both type 2 diabetics and nondiabetics (2–4). The relationship of islet amyloid itself to the pathogenesis of type 2 diabetes remains unclear, although the specificity of the amyloid for the condition has suggested that it may play a role. In view of the evidence presented in this paper which suggests that amylin may be a hormone with a role in the control of carbohydrate metabolism, and also of the evidence that it is present in the islets of Langerhans of nondiabetics, the name "diabetes-associated peptide" is no longer appropriate, and it has been replaced by the more suitable name "amylin."

The experiments reported here were performed to examine the possible effects of amylin on glucose metabolism in peripheral tissues. The results demonstrate the unexpected fact that synthetic amylin decreases the rates of glucose uptake into skeletal muscle *in vitro*, mainly by decreasing the rate of incorporation of glucose into glycogen—an effect seen in the skeletal muscles of type 2 diabetics *in vivo*. On the other hand, amylin treatment does not significantly alter the rate of glycolysis, as indicated by lactate production, in the same tissue. In muscles treated with amylin there was no response to insulin at the concentration necessary for half-maximal stimulation in non-amylin-treated muscles. The effect of amylin persists even in the presence of low concentrations of insulin. So, for example, amylin produced a 30% reduction in the basal rate of glycogen synthesis in the presence of insulin at 1 μU/ml. In marked contrast, amylin has no significant effect on either the basal or the insulin-stimulated rate of formation of CO_2 or triacylglycerol in isolated adipocytes. The effect of amylin treatment in muscle could be overcome by increasing the concentration of insulin to the supraphysiological 1000 μU/ml.

These results need to be interpreted cautiously in the light of certain factors. First, the experiments reported here were performed with material that was chemically synthesized according to the previously determined sequence of extracted amylin (1), and the reconstitution of the disulfide bridge to give molecules with the natural conformation was incomplete. Since only a portion of synthesized amylin may

Table 2. Effects of amylin on the insulin-stimulated rates of glucose metabolism to CO_2 and triacylglycerol in isolated adipocytes

Insulin, μU/ml	$^{14}CO_2$ production, μmol/hr per g dry weight		Triacylglycerol production, μmol/hr per g dry weight	
	Control	Amylin (120 nM)	Control	Amylin (120 nM)
0	0.44 ± 0.06	0.48 ± 0.05	0.39 ± 0.04	0.44 ± 0.02
10	0.74 ± 0.12	0.86 ± 0.10	0.73 ± 0.10	0.81 ± 0.08
1000	1.18 ± 0.07	1.18 ± 0.07	1.24 ± 0.06	1.20 ± 0.04

All values are mean ± SEM. There are no significant differences between control and amylin-treated groups.

display the natural conformation, it is likely that the specific activity of biologically derived amylin may be greater. Second, these experiments have been performed in rat skeletal muscle *in vitro* with material based on the human sequence. Appropriate caution in generalizing to the diabetic human *in vivo* must therefore be exercised.

It is important to note that the effect of amylin was apparent in muscles that had been treated for only 1 hr. To our knowledge, the nature of these effects on muscle glucose metabolism is unique. Although similar effects on glycogen synthesis are seen in muscle after long-term administration of a glucocorticoid to rats (24), no such effects are seen after acute treatment with this agent. Similarly, although β-adrenergic agonists such as isoprenaline have similar effects on glycogen synthesis (23), they also produce a substantial increase in the rate of glycolysis, as well as marked effects on glucose metabolism in adipocytes. Therefore, it seems that amylin has effects that are relatively specific for the basal and insulin-stimulated rates of glycogen synthesis in muscle. The effect on glycogen synthesis in the absence of insulin stimulation suggests that amylin may exert its action in muscle through a mechanism independent of the insulin receptor, possibly a separate amylin receptor.

The effects of amylin treatment of muscle *in vitro* reported in this paper are consistent with the metabolic state of muscles observed *in vivo* in type 2 diabetes mellitus, in which the pathophysiological state of insulin resistance may be related primarily to a deficiency of glucose uptake into skeletal muscle caused in turn by a decrease in the rate of glycogen synthesis in that tissue. When the reported results are considered in conjunction with the presence of amylin-containing islet amyloid in the majority of type 2 diabetics, then it is a plausible hypothesis that the insulin resistance seen in type 2 diabetes may be caused, in part, by excessive exposure of skeletal muscle to amylin.

The presence of amylin in nondiabetic islets, and the biological activity reported in this paper, suggest that amylin may be a newly recognized hormone, which may be synthesized in the pancreatic islets and may have a role in opposing insulin action in the homeostasis of carbohydrate metabolism. The results also demonstrate that it is possible to chemically synthesize biologically active amylin. The peripheral actions of the peptide appear to be exerted primarily through a reduction in the rate of glucose uptake into skeletal muscle through decreasing the rate of muscle glycogen synthesis.

The hypotheses presented above require much further work to allow for adequate scientific testing. It is necessary to establish the full spectrum of biological activity of the molecule, both *in vitro* and *in vivo*. However, this work will be difficult owing to the extremely restricted supply of material from which to extract the native amylin molecule and the conditions required for this extraction (1). Furthermore, the use of chemically synthesized material must also be interpreted with caution until adequate biological standardization is available.

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