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The effects of amylin on carbohydrate metabolism in skeletal muscle *in vitro* and *in vivo*

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1. The effects of synthetic human amylin on basal and insulin-stimulated (100 and 1000 μunits/ml) rates of lactate formation, glucose oxidation and glycogen synthesis were measured in the isolated rat soleus muscle preparation incubated in the presence of various concentrations of glucose (5, 11 and 22 mM). 2. The rate of glucose utilization was increased by about 2-fold by increasing the glucose concentration from 5 to 22 mM. 3. Synthetic human amylin (10 nM) significantly inhibited (by 46–56 %) glycogen synthesis, irrespective of the concentration of insulin or glucose present in the incubation medium. 4. Amylin (10 nM) did not affect insulin-stimulated rates of 2-deoxy[^{3}H]glucose transport and phosphorylation. 5. Intraperitoneal administration of insulin (100 μg/kg) to rats *in vivo* stimulated the rate of [U-^{14}C]glucose incorporation into glycogen in the diaphragm by about 80-fold. This rate was decreased (by 28 %) by co-administration of amylin (66 μg/kg).

INTRODUCTION

Extensive deposits of pancreatic islet amyloid (fibrillar proteinaceous mass) are found in more than 90 % of people with non-insulin-dependent (type 2) diabetes mellitus (NIDDM) [1], in over 75 % of diabetic Pima Indians [2] and in diabetic cats [3] and monkeys [4]. A 37-amino-acid peptide, called amylin [5], has been completely characterized from human islet amyloid [6]. A similar peptide has been extracted from an insulinoma and called insulinoma/islet amyloid polypeptide [7]. Amylin is expressed in human pancreatic β-cells [8] where it is located in secretory granules [9]; indeed, the content of amylin is high, compared with that of other hormones, in pancreatic islets [10], which secrete amylin *in vitro* in response to glucose [11]. Human islet amyloid is formed from a natural rather than an aberrant molecule, since the predicted amino acid sequence from the amylin gene (see [8] and references therein) and the sequence from the extracted pancreatic peptide [6] are identical. It has been reported that a small content of amylin-like immunoreactivity exists in stomach, duodenum and jejunum [10].

Islet amyloid formation may result from chronic over-production of amylin [12], and since amylin causes marked insulin resistance in skeletal muscle both *in vitro* [13] and *in vivo* [14], a disturbance in amylin homeostasis may play a pathogenic role in NIDDM [12,13]. Skeletal muscle in non-diabetic subjects is known to be the major site (50–70 %) of insulin-stimulated glucose disposal. Most (> 70 %) of an exogenous [1-^{13}C]glucose load is disposed into skeletal muscle glycogen *in vivo* [15]. In NIDDM, insulin-mediated whole-body glucose disposal is dramatically decreased [16]. Studies on Pima Indians strongly indicate that peripheral insulin resistance is a major determinant of the decline in glucose tolerance [17]. Glycogen synthase activity *in situ* or insulin-stimulated rates of glycogen synthesis *in vivo* are dramatically decreased in skeletal muscle from diabetic subjects ([18], and see [12] and references therein). Therefore, an early pathogenic lesion in NIDDM appears to be a decreased response of glycogen synthesis to insulin in skeletal muscle.

The biological activity of a synthetic amylin molecule (a free acid) [5] is about 100-fold less than that of an extracted amylin molecule [13]. However, it has now been established that the post-translational modifications required for a biologically active amylin molecule are *C*-terminal amidation and an intact disulphide bridge between Cys-2 and Cys-7 [8]. In the present study we investigated the effects of a synthetic human amylin molecule (a previous study used amylin extracted from a human pancreas [13]), containing the required post-translational modifications, on rates of basal and insulin-stimulated conversion of glucose to lactate (glycolysis), glycogenolysis and $^{14}CO_2$ formation (glucose oxidation), and on rates of insulin-stimulated hexose transport in isolated incubated soleus muscle. Also, we measured the effect of synthetic amylin on insulin-stimulated rates of glycogen synthesis in rat skeletal muscles *in vivo*. Since high levels of blood glucose and insulin are found in NIDDM, it was considered important to establish if high concentrations of substrate and/or insulin in the incubation medium could decrease the effects of amylin *in vitro*.

MATERIALS AND METHODS

Animals

Male Wistar rats (Harlan–Olac, Bicester, U.K.) were purchased at 5 weeks of age and were kept in the Department's animal quarters until experimentation (body wt. 130–150 g). The animals were housed in controlled conditions (23 ± 1 °C; 12 h light/dark cycle) and received standard laboratory chow and water *ad libitum*, except for the 14 h period before isolation of muscles when food (but not water) was withdrawn. Stripped soleus muscles were routinely prepared between 09:00 and 10:00 h.

Materials

All enzymes, biochemicals and radiochemicals were obtained from sources previously given [19–21]. Amylin was kindly given by Dr. Garth Cooper, Amylin Corporation, San Diego, CA, U.S.A. The concentrations of stock solutions of synthetic amylin were determined with a Waters Pico-Tag analysis system [22].

Isolation and incubation procedures

Strips of soleus muscle were isolated as originally described by Crettaz *et al.* [23] and were tied under tension to stainless-steel

Abbreviations used: NIDDM, non-insulin-dependent (type 2) diabetes mellitus; CGRP, calcitonin-gene-related peptide; EC_{50}, concentration causing 50 % inhibition; EDL, extensor digitorum longus.

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clips. After a 30 min pre-incubation in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 5.5 mM-glucose/4 mM-pyruvate/1.5% defatted BSA, muscles were transferred to flasks that contained identical medium (except that pyruvate was omitted) plus 0.3 μCi of [U-^{14}C]glucose/ml and insulin at concentrations of 0, 100 (physiological) or 1000 (maximal) μunits/ml (the details of the protocol have been previously given [24]). After 60 min of incubation muscles were removed, blotted and frozen in liquid N_2; the concentration of lactate in the incubation medium (spectrophotometric or net) and the rates of incorporation of [^{14}C]glucose into glycogen or conversion to radiochemical lactate were measured [19–21,25]. The rates of oxidation of glucose to $^{14}CO_2$ were measured as described by Leighton *et al.* [26]; measured $^{14}CO_2$ production rates in the present study were not corrected for the decrease in specific radioactivity in pyruvate owing to any glycogenolysis.

The determination of the effects of insulin and amylin on the stimulation of glycogen in the diaphragm *in vivo* was carried out as previously described [27]. This involved intraperitoneal administration of [U-^{14}C]glucose (1.8 μCi) in 0.9% (w/v) sterile saline containing 0.1% (w/v) defatted BSA (1 ml) into fasted male Wistar rats (75 g). After 2 h the rats were killed by cervical dislocation and portions of the diaphragm, intact extensor digitorum longus (EDL) and intact soleus muscle were removed from each animal. The incorporation of [U-^{14}C]glucose into glycogen was measured as described previously [27]. A previous study has established that intraperitoneal injection of insulin has little effect on blood glucose concentration [27]. Glycogen content was measured as previously described [24].

The rates of hexose transport and phosphorylation were determined using 2-deoxy[2,6-^{3}H]glucose (Amersham). A trace amount of 2-deoxy[2,6-^{3}H]glucose (0.5 μCi/ml) was added to the incubation medium, which contained 5.5 mM-glucose [19]. The soleus muscle strips were incubated for 1 h. After incubation, muscles were removed from the medium, blotted and frozen in liquid N_2, and were then powdered and homogenized in 6% (w/v) $HClO_4$. Precipitated protein was removed by centrifugation. The supernatant was neutralized and loaded on to a 2 ml Dowex-2 (formate form) ion-exchange column to separate [^{3}H]deoxyglucose 6-phosphate from [^{3}H]deoxyglucose. The columns were washed with 18 ml of 5 mM-glucose, to elute [^{3}H]deoxyglucose, followed by 18 ml of distilled water. The deoxyglucose 6-phosphate was eluted with 6 ml of 0.3 M-ammonium formate/1 M-formic acid. The radioactivity in the eluate (1 ml) was measured in a liquid scintillation counter.

RESULTS

The dose-related effects of synthetic human amylin on insulin-stimulated rates of glycogen synthesis are given in Table 1. The concentrations of amylin which markedly inhibited the insulin-stimulated (100 μunits/ml) rates of [U-^{14}C]glucose incorporation into glycogen ranged from 6 to 1000 nM. Also, from a plot of the rates of glycogen synthesis versus the various concentrations of amylin, the concentration of amylin which caused half-maximal inhibition (EC_{50} value) of glycogen synthesis was estimated to be 4 nM. Overall, there was no significant effect of amylin on insulin-stimulated rates of spectrophotometric lactate formation (results now shown)

The effect of insulin at different concentrations (0, 100 and 1000 μunits/ml) on rates of net lactate formation (i.e. that from glucose in the medium and from glycogen) and radioisotopic fluxes (i.e. that from [U-^{14}C]glucose in the medium to $^{14}CO_2$, [^{14}C]lactate and [^{14}C]glycogen) were measured in the isolated stripped soleus muscle preparation in the presence of different concentrations of glucose (5.5, 11 and 22 mM) (see Figs. 1–3). The rates of glucose oxidation and glycolytic and glycogenic fluxes were also measured in the presence of 10 nM synthetic amylin.

When the concentration of glucose was increased from 5.5 to 11 or 22 mM (in the absence of amylin), glucose oxidation was stimulated by 132% and 69% respectively (Fig. 1). Overall, amylin had little effect on basal or insulin-stimulated rates of $^{14}CO_2$ formation, except that $^{14}CO_2$ formation was markedly inhibited by amylin in the presence of 11 mM-glucose (and absence of insulin) (see Fig. 1). We cannot explain these results,

Table 1. Dose-related effects of synthetic human amylin on insulin-stimulated rates of glycogen synthesis in isolated incubated soleus muscle preparations from the rat

Values are presented as means ± S.E.M. for at least four separate experiments. Insulin was present in the incubation medium at a concentration of 100 μunits/ml. The statistical significance (Student's t test) of differences between muscle incubated in the presence and absence of amylin is denoted by * $P < 0.05$.

Amylin concn. (nM)	Rates of glycogen synthesis (μmol/h per g wet wt.)
0	4.60 ± 0.18
2	4.16 ± 0.18
6	2.32 ± 0.37*
10	2.27 ± 0.21*
50	1.23 ± 0.18*
100	1.71 ± 0.20*
1000	1.70 ± 0.37*

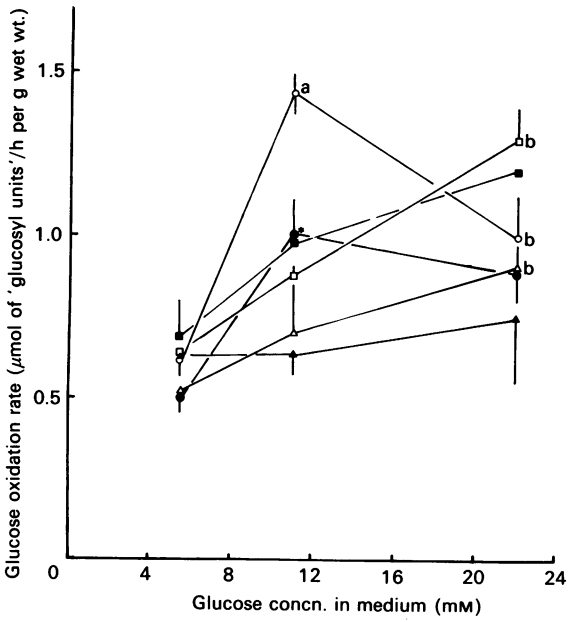

Fig. 1. Effect of glucose on insulin-stimulated rates of glucose oxidation in isolated incubated stripped soleus muscle preparations incubated in the presence of synthetic human amylin

All values are presented as means for four separate experiments. The error bars represent the S.E.M.; in some cases the error bar is omitted for clarity. Insulin was absent (○,●) or present in the incubation medium at a concentration of 100 (△,▲) or 1000 (□,■) μunits/ml. Also, soleus muscle preparations were incubated in the absence (○,△,□) or presence (●,▲,■) of 10 nM-amylin. The statistical significance (Student's t test) of differences between control and amylin-treated preparations is indicated by * $P < 0.05$. The statistical significance of differences between muscles incubated in the presence of either 11 or 22 mM-glucose and 5.5 mM-glucose is indicated by a ($P < 0.05$) and b ($P < 0.05$) respectively.

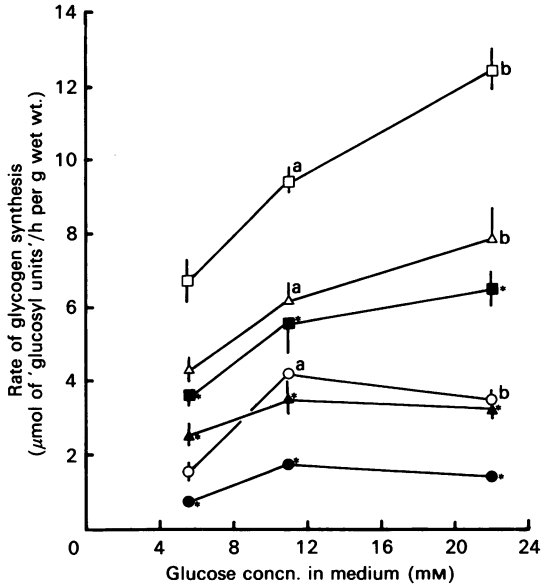

Fig. 2. Effect of glucose on inhibition of insulin-stimulated rates of glycogen synthesis by synthetic human amylin in isolated incubated stripped soleus muscle preparations

All values are presented as means for four separate experiments. The error bars represent the S.E.M.; where the S.E.M. was < 5% of the mean value, no error bar is given. Insulin was absent (○,●) or present in the incubation medium at a concentration of 100 (△,▲) or 1000 (□,■) μunits/ml. Also, soleus muscle preparations were incubated in the absence (○,△,□) or presence (●,▲,■) of 10 nM-amylin. The statistical significance (Student's *t* test) of differences between control and amylin-treated preparations is indicated by $*P < 0.05$. The statistical significance of differences between muscles incubated in the presence of either 11 or 22 mM-glucose and 5.5 mM-glucose are indicated by a ($P < 0.05$) and b ($P < 0.05$) respectively.

Table 2. Effects of insulin and synthetic human amylin on rates of incorporation of [U-^{14}C]glucose into glycogen in diaphragms from fasted rats

Insulin and/or amylin, at the doses indicated (100 μg/kg of insulin is 25 units/kg) were injected intraperitoneally together with [^{14}C]glucose as described in the Materials and methods section. Soleus and EDL muscles were included in this study to gauge the effect of circulating insulin which escaped from the intraperitoneal cavity. All results are presented as means ± S.E.M. for the numbers of separate experiments given in parentheses. The statistical significance of the difference (Student's *t* test) between animals treated with insulin in the presence and absence of amylin is indicated by $*P < 0.05$.

	Rates of [^{14}C]glycogen synthesis (d.p.m./2 h per mg wet wt.		
	Diaphragm	Soleus	EDL
No insulin or amylin	5.1 ± 0.3 (3)	11.2 ± 0.8 (3)	1.6 ± 0.2 (3)
Insulin (100 μg/kg)	407.4 ± 28.9 (5)	46.1 ± 4.2 (5)	5.4 ± 0.9 (5)
Insulin (100 μg/kg) + amylin (66 μg/kg)	292.4 ± 24.3 (6)*	30.9 ± 5.1 (6)*	5.0 ± 1.1 (6)

unless the rate of $^{14}CO_2$ formation in the presence of 11 mM-glucose is an anomalous response.

Amylin markedly inhibited the stimulation of the rate of glycogen synthesis irrespective of the concentration of insulin or glucose present in the incubation medium (Fig. 2). At 5.5, 11 or 22 mM-glucose, the percentage inhibition of the rate of glycogen

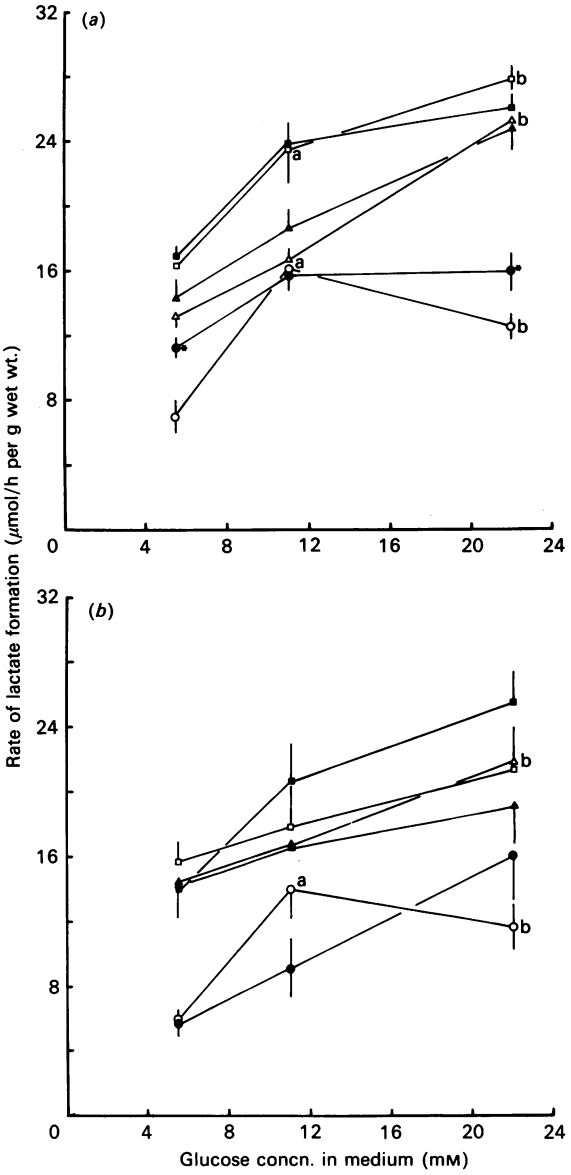

Fig. 3. Effect of glucose on insulin-stimulated rates of (*a*) spectrophotometric and (*b*) radiochemical lactate formation in isolated incubated stripped soleus muscle preparations incubated in the presence of synthetic human amylin

All values are presented as means for four separate experiments. The error bars represent the S.E.M.; in some cases, error bars are omitted for clarity. Insulin was absent (○,●) or present in the incubation medium at a concentration of 100 (△,▲) or 1000 (□,■) μunits/ml. Also, soleus muscle preparations were incubated in the absence (○,△,□) or presence (●,▲,■) of 10 nM-amylin. The statistical significance (Student's *t* test) of differences between control and amylin-treated preparations is indicated by $*P < 0.05$. The statistical significance of differences between muscles incubated in the presence of either 11 or 22 mM-glucose and 5.5 mM-glucose is indicated by a ($P < 0.05$) and b ($P < 0.05$) respectively.

synthesis (includes values obtained with or without insulin) by 10 nM-amylin was 46 ± 3% ($n = 12$), 49 ± 3% (12) and 56 ± 2% (12) respectively. Glucose *per se* elevated the rate of glycogen synthesis by about 2-fold.

Insulin (100 μg/kg; 25 units/kg) stimulated the rate of incorporation of [^{14}C]glucose into glycogen in the diaphragm, soleus and EDL by 80-fold, 4.1-fold and 3.4-fold respectively (Table 2). Amylin (66 μg/kg) significantly inhibited the ability of

Table 3. Effects of synthetic human amylin on insulin-stimulated rates of 2-deoxy[^{3}H]glucose transport and phosphorylation in the stripped soleus muscle preparations of the rat

Values are presented as means ± S.E.M. for the numbers of observations given in parentheses. Amylin was present in the incubation medium at a concentration of 10 nM.

Insulin (μunits/ml)	Rate of 2-deoxy[^{3}H]glucose transport and phosphorylation (d.p.m. of 2-deoxy-D-[^{3}H]glucose 6-phosphate/h per mg wet wt.)	
	Control	+Amylin (10 nM)
10	215 ± 27 (8)	214 ± 18 (7)
100	285 ± 31 (8)	282 ± 23 (7)
1000	343 ± 39 (8)	298 ± 29 (8)

insulin to stimulate glycogen synthesis in the diaphragm (28%) and soleus (33%) but not in the EDL.

Amylin significantly increased the rate of spectrophotometric (net) lactate formation in soleus muscle incubated in the presence of 5.5 or 22 mM-glucose, but only in the absence of insulin (Fig. 3*a*). Similar increases in the rates of radiochemical lactate formation were not observed at these glucose concentrations (Fig. 3*b*). Glycogen content (μmol/g) in the isolated soleus muscle preparation *in vitro* (incubated in the absence of insulin) was decreased by synthetic human amylin [control, 31.7 ± 2.7 (5); amylin (10 nM), 27.6 ± 1.7 (5); amylin (100 nM), 22.7 ± 1.3 (5)]. However, the decrease was only significant for 100 nM-amylin. Generally, there was good agreement between the insulin-stimulated rates of spectrophotometric and radiochemical lactate formation in the presence of amylin, indicating that amylin did not stimulate glycogenolysis to a measurable extent in the isolated incubated muscle preparation. Glucose, at concentrations of 11 or 22 mM, significantly increased the rate of lactate formation in the absence of amylin or insulin.

The rate of glucose transport and phosphorylation was determined by monitoring the rate of accumulation of 2-deoxy[^{3}H]glucose 6-phosphate from 2-deoxy[^{3}H]glucose added to the incubation medium (Table 3). Insulin stimulated 2-deoxyglucose transport and phosphorylation, but this stimulation was not affected by amylin.

DISCUSSION

In the present study we have examined the interaction between insulin, glucose and the novel pancreatic hormone amylin [5,6,8,12,13] on glucose metabolism in the isolated incubated soleus muscle preparation. Amylin has a high degree of primary amino acid sequence identity with the neuropeptide calcitonin-gene-related peptide (CGRP) [6]. Both rat CGRP-1 [13,28,29] and native human pancreatic amylin [13] are potent inhibitors of insulin-mediated glycogen synthesis in isolated incubated skeletal muscle preparations. In the present study we found that synthetic human amylin was also a potent inhibitor of insulin-stimulated glycogen synthesis (see Table 1).

Effects of glucose

When the glucose concentration in the incubation medium was increased from 5.5 to 22 mM, the rate of glucose utilization was increased by about 2-fold. There is evidence to suggest that high glucose concentrations *per se* can diminish the rate of glucose utilization [30–32] and cause insulin resistance in skeletal muscle [33]. However, these latter effects are exhibited after skeletal muscle has been exposed to high levels of glucose for at least 3 h. In marked contrast, it has been demonstrated that short-term (4 days) hyperglycaemia increases the sensitivity of glucose utilization of peripheral tissues to insulin *in vivo* [34]. The present study showed that there was increased glucose utilization in soleus muscle in the presence of 22 mM-glucose and without any change in insulin sensitivity after a shorter incubation period (1 h). We hypothesize that the alteration in insulin sensitivity observed in skeletal muscle incubated over a longer period is linked to the likely increase in the intracellular level of glucose and/or one of its metabolites during the early stages of the incubation.

Le Marchand-Brustel & Freychet [35] observed that glucose alone had no effect on the activity of glycogen synthase in isolated mouse soleus muscle after a period of incubation. The stimulation of the rate of glycogen synthesis by glucose (22 mM) demonstrated in the present report (Fig. 2) is at variance with this observation. The difference may be explained by increases in the content of allosteric activators of glycogen synthase in soleus muscle *in vitro* (e.g. glucose 6-phosphate). Glucose 6-phosphate would probably increase the activity of glycogen synthase in the incubated muscle preparation, but this effect would be absent in the enzyme assay employed by Le Marchand-Brustel & Freychet [35]. Also, glycogen synthase activity is increased in the presence of a maximal concentration of insulin and 20 mM-glucose [35]. Our results are in agreement with this finding [see Fig. 2 for a comparison of the effects of 22 mM-glucose with those of 11 mM-glucose in the presence of physiological (100 μunits/ml) and maximal (1000 μunits/ml) concentrations of insulin].

Effects of amylin on glycolysis and glucose transport

Amylin, in the absence of insulin, increased the rate of net lactate formation (i.e. from glucose in medium and from muscle glycogen) but not the rate of radiochemical lactate formation (i.e. from [^{14}C]glucose in the medium). Similar observations have been made with high concentrations of rat CGRP-1 [13,28,29]. This strongly indicates that, in the absence of insulin, 10 nM-amylin stimulated glycogenolysis (this is indicated by the differences between rates of net and radiochemical lactate formation; see [36]). Indeed, glycogen content in incubated soleus muscle preparations (see the Results section) was decreased by amylin. In the presence of insulin in the incubation medium 10 nM-amylin had little effect on glycogenolysis. Interestingly, CGRP-1 stimulates glycogenolysis in skeletal muscle composed of fast-twitch fibres (soleus is largely populated with slow-contracting fibres) [28]. However, about 100-fold less CGRP-1 is required to stimulate glycogenolysis in fast-twitch fibres than in slow-contracting fibres [28]. The effect of amylin on fast-twitch fibres has not been examined.

Previous studies have demonstrated that the rate of lactate formation from [^{14}C]glucose is a reasonable measure of the rate of glucose transport into skeletal muscle [19,25,36,37]. Since 10 nM-amylin did not affect the rates of radiochemical lactate formation, this would suggest that amylin exerts little effect on glucose transport. To test this idea we measured the effects of 10 nM-amylin on insulin-stimulated rates of 2-deoxy[2,6-^{3}H]glucose transport and phosphorylation. During a 1 h incubation period there was no measurable effect of amylin on these processes in isolated incubated soleus muscle. Amylin (10 nM) did not alter the basal rates of 2-deoxyglucose uptake and phosphorylation when soleus muscles were incubated in the absence of insulin (results not shown). Also, the rates of 2-deoxyglucose uptake and phosphorylation (and net lactate formation [19]) were the same when muscles were incubated in the absence or the presence of a basal insulin concentration (10 μunits/ml).

Effects of amylin on glycogen synthesis

A previous study has shown that human amylin purified from a diabetic pancreas is a potent inhibitor of insulin-stimulated glycogen synthesis in incubated rat soleus muscle preparations [13]. Similar responses were found in the present study with synthetic human amylin, but additional findings were that amylin inhibited both glycogen synthesis (in the absence of insulin) *in vitro* and insulin-stimulated rates of glycogen synthesis in skeletal muscle *in vivo*. A maximal dose (66 μg/ml) of amylin was used for the study *in vivo*, and this significantly inhibited rates of glycogen synthesis in diaphragm and soleus (but not EDL) muscles. However, soleus and EDL muscles, which responded poorly to both peptides, were included in this study to gauge the effect of circulating insulin which might have been absorbed into the bloodstream. CGRP-1 is a potent inhibitor of insulin-stimulated rates of glycogen synthesis in isolated incubated EDL muscle preparations [28].

An important observation was that the increased extracellular glucose concentration did not significantly influence the inhibition of glycogen synthesis by amylin. This point is particularly relevant to the putative involvement of amylin in the pathogenesis of NIDDM [12,13]. In this disorder, higher than normal levels of blood glucose and insulin are frequently observed. It is interesting to note that, in order to obtain a similar physiological rate of glycogen synthesis (i.e. in the presence of 100 μunits of insulin/ml and 5.5 mM-glucose) with 10 nM-amylin present, the required concentrations of glucose and insulin were 11 or 22 mM and 1000 μunits/ml respectively (see Fig. 2). Also, if the results from the present study can be extrapolated to the situation *in vivo*, then it is suggested that the hyperglycaemia found in NIDDM cannot overcome the likely effects of amylin in skeletal muscle *in vivo*.

The effects of amylin reported in the present study are quantitatively similar to those of CGRP-1 in similar isolated incubated muscle preparations [13,28]. There is evidence for localization of CGRP in both sensory nerves [38] and the motor axon terminal [39] in skeletal muscle. High-affinity binding sites for CGRP are present in sarcolemmal membranes prepared from rat [40] and human [41] skeletal muscle, rat soleus muscle [42] and L6 myocytes [43]. However, little is known about amylin receptors. Therefore, characterization of the amylin and CGRP receptor(s) in skeletal muscle will help to elucidate the mechanism of both peptides in modulation of insulin responses.

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