Amylin impairment of insulin effects on glycogen synthesis and phosphoenolpyruvate carboxykinase gene expression in rat primary cultured hepatocytes

Susanna BAQUÉ, Joan J. GUINOVART and Anna M. GÓMEZ-FOIX*

Departament de Bioquímica i Fisiologia, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain

The ability of amylin to impair hepatic insulin action is controversial. We have found that the effect of amylin in primary cultured hepatocytes is strongly dependent on the culture conditions. Only in hepatocytes preincubated in the presence of fetal serum did amylin, at concentrations ranging from 1 to 100 nM, reduce insulin-stimulated glycogen synthesis rate and glycogen accumulation without showing direct effects. Neither basal glycogen synthase nor glycogen phosphorylase activity was modified by amylin treatment. Nevertheless, amylin (100 nM)

blocked the activation of glycogen synthase by insulin. Amylin also proved capable of opposing the reduction in the expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene induced by insulin, whereas the basal mRNA level of PEPCK was unaffected by amylin treatment. Thus, these results show that, in cultured rat hepatocytes, amylin is indeed able to interfere with insulin regulation of glycogenesis and PEPCK gene expression, favouring the hypothesis that amylin may modulate liver sensitivity to insulin.

INTRODUCTION

Amylin, or islet amyloid polypeptide, is a pancreatic hormone co-secreted with insulin by the β -cells, which accumulates in amyloid deposits in the islets of type 2 (non-insulin-dependent) diabetic patients [1]. A possible role of amylin in the induction of the muscle and liver insulin insensitivity associated with non-insulin-dependent Diabetes mellitus has been revealed *in vivo* [2–6] and *in vitro* [7–10]. Therefore, although it is widely accepted that muscle is a target tissue for amylin action, the controversy regarding hepatic sensitivity to amylin remains.

In conscious rats, several studies have shown that amylin infusion antagonizes the ability of insulin to suppress hepatic glucose output [4–6] and decreases liver glycogen content [2]. More contradictory results have been obtained in hepatic systems *in vitro*. A lack of effect of amylin on insulin-stimulated glucose metabolism has been described in perfused isolated livers [11,12]. With respect to isolated or cultured cells, conflicting observations have been reported. In isolated hepatocytes, we previously showed anti-insulin effects of amylin on glycogen metabolism [9], whereas Stephens et al. [13] did not observe any action of amylin on glucose metabolism. Amylin also failed to modify insulin-challenged glucokinase gene expression in cultured hepatocytes [14]. In contrast, in HepG2 cells, Ciaraldi et al. [10] reported that amylin reduced glucose oxidation and enhanced both glycogenolysis and gluconeogenesis.

The present work aimed to study the effects of amylin in the liver cell using cultured hepatocytes. We have found that eliciting of amylin action is highly dependent on the culture conditions. We show that amylin is capable of counteracting hepatocyte insulin effects on glycogen synthesis and phosphoenolpyruvate carboxykinase (PEPCK) gene expression only in hepatocytes cultured in the presence of fetal serum, suggesting a permissive

role of fetal serum. Our results are consistent with hyper-insulinaemic clamp studies, showing that amylin may induce hepatic insulin resistance.

EXPERIMENTAL

Hepatocyte culture and incubation

Hepatocytes were prepared from 24 h starved male Wistar rats as described [15]. Cells were finally resuspended in Dulbecco's Modified Eagle's Medium (DMEM) without glucose (Biochrom KG), supplemented with 10 mM glucose, 100 nM insulin, 100 nM dexamethasone and 10% (v/v) fetal bovine serum (FBS) (Whittaker), and plated onto collagen-coated tissue-culture dishes (Corning). Unattached cells were removed, after 4 h, by replacing the medium with DMEM containing 5 mM glucose and 10% fetal bovine serum (unless otherwise stated), and the incubation was continued for a further 12 h. Before hormone additions, cells were washed with PBS and preincubated with serum-free DMEM containing 5 mM glucose and 0.2% bovine serum albumin (Sigma). After hormone treatments, cell monolayers were rinsed with PBS and frozen in liquid N₂ until analysis, except for RNA isolation. Insulin was obtained from Boehringer Mannheim and amidated amylin was from Peninsula.

Glycogen determination

Glycogen synthesis rate was measured in hepatocytes incubated in DMEM supplemented with 10 mM D-[U-¹⁴C]glucose (0.06 μ Ci/ μ mol) (Sigma). After hormone treatment, cell monolayers were scraped into 5% (w/v) trichloroacetic acid and

Abbreviations used: cAMP, cyclic AMP; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PEPCK, phosphoenolpyruvate carboxykinase.

* To whom correspondence should be addressed.

centrifuged (5000 *g*, 15 min). Supernatants were spotted onto Whatman 31 ET papers and glycogen was precipitated by immersing the papers in ice-cold 66% (v/v) ethanol. Papers were subjected to two additional washes, dried and counted for radioactivity.

To determine glycogen content, cells were scraped from the plates into 30% (w/v) KOH. The homogenates were boiled for 15 min and centrifuged (5000 *g*, 15 min). The glycogen present in the supernatants was precipitated onto Whatman 31 ET papers as described above. Papers were then incubated for 90 min at 37 °C in 0.2 M sodium acetate buffer, pH 4.8, supplemented with 25 units/ml α -glucosidase (1 unit = 1.0 mg of glucose from starch in 3 min at 55 °C). Glucose released from glycogen was measured enzymically with the aid of a Cobas-Bio autoanalyzer with a GlucoQuant (Boehringer Mannheim) kit. Protein concentration was measured as described by Bradford [16], using the Bio-Rad protein assay reagent.

Enzyme activities

For determining synthase and phosphorylase activities, cell monolayers were homogenized in a 10 mM Tris/HCl buffer, pH 7.0, containing 150 mM KF, 15 mM EDTA, 15 mM β -mercaptoethanol, 10 μ g/ml leupeptin, 1 mM benzamidin and 1 mM phenylmethanesulphonyl fluoride. Homogenates were centrifuged at 10000 *g* for 15 min, and supernatants were tested for enzyme activity. The glycogen synthase activity ratio was measured by a radioactive technique in the absence or presence of 10 mM glucose 6-phosphate, as described [17]. Glycogen phosphorylase *a* was assayed as described [18], in the presence of 1 mM caffeine.

Northern blot analysis

Total RNA was isolated from hepatocytes by a single-step guanidium thiocyanate-phenol-chloroform method [19]. Total RNA (25 μ g) was separated by electrophoresis through 1.5% (w/v) agarose gels and transferred to nitrocellulose filters. Filters were hybridized with an antisense 32 P-labelled riboprobe, prepared with a Riboprobe System (Promega) from a 1.1 kb *Pst*I fragment of the rat liver PEPCK cDNA. After hybridization at 65 °C in the presence of 50% (v/v) formamide, membranes were washed to a final stringency of $0.1 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% (w/v) SDS at 65 °C and subjected to autoradiography.

RESULTS

Effects on glycogen

Anti-insulin effects of amylin on cultured hepatocytes were found to be highly sensitive to culture conditions. When we attempted to reproduce in primary cultured cells, incubated for 12 h in serum-free medium (5 mM glucose-DMEM), our previous observations on glycogen metabolism in isolated hepatocytes, no effect of amylin on insulin action was observed. Moreover, no action of amylin was observed in hepatocytes exposed to 100 nM dexamethasone for 12 h before the experiment began. Hormone effects were also tested in the presence of a range of different concentrations of glucose and amino acids. In all of these conditions, even though insulin was able to exert its actions, no effect of amylin was detected.

Cells were next cultured in the presence of 10% fetal serum and preincubated in the absence of serum, before hormone additions, in order to eliminate the interference of serum components with hormone action. Insulin and amylin effects on glycogen synthesis rate were studied after preincubation for

Figure 1 Time-dependent effect of serum-deprivation on the ability of amylin to impair insulin-stimulated glycogen synthesis

Hepatocytes were preincubated for 12 h in 10% FBS-containing medium and then incubated for different times in a serum-free medium. Afterwards, cells were incubated for 2 h in DMEM containing 10 mM [14 C]glucose and 0.2% (w/v) BSA, in the absence of effectors (○) or in the presence of 100 nM amylin (●), 10 nM insulin (□) or 10 nM insulin plus 100 nM amylin (■). Glycogen synthesis is expressed as a percentage of the rate measured in control cells (37.8 ± 1.4 nmol of [14 C]glucose incorporated/h per mg of protein; means $\pm$ S.E.M., $n = 4$). * $P < 0.005$, ** $P < 0.001$ versus insulin-treated cells.

Table 1 Amylin counteraction of insulin-stimulated glycogen synthesis rate in hepatocytes incubated in the continuous presence of 1% fetal serum

Hepatocytes were incubated for 12 h in the presence of 10% FBS and afterwards preincubated for the times indicated in a 1% FBS-containing medium. Next, cells were incubated for 2 h in DMEM containing 10 mM [14 C]glucose and 0.2% (w/v) BSA, in the absence of effectors (control) or in the presence of 100 nM amylin, 10 nM insulin or both hormones together (Ins + Amy). Glycogen synthesis is expressed as a percentage of the rate measured in control cells (53.2 ± 4.1 nmol [14 C]glucose incorporated/h per mg of protein; mean $\pm$ S.E.M. of 2 independent experiments each performed in triplicate). * $P < 0.0001$ versus insulin-treated cells.

Time (h)	Glycogen synthesis rate (%)			
	Control	Insulin	Amylin	Ins + Amy
1.0	100	149 $\pm$ 4	102 $\pm$ 3	98 $\pm$ 4*
3.0	100	176 $\pm$ 7	100 $\pm$ 3	102 $\pm$ 3*
5.0	100	181 $\pm$ 7	102 $\pm$ 4	106 $\pm$ 4*

different times in serum-deprived medium. As shown in Figure 1, insulin caused a 2-fold stimulation of [14 C]glucose incorporation into glycogen. Amylin (100 nM) showed a maximal 40% counteraction of insulin effect after 1 h preincubation without serum. Longer preincubations in the absence of serum reduced amylin inhibition of insulin, and no effect of amylin, assayed at concentrations ranging from 1 pM to 1 μ M, was observed when preincubations were extended for more than 3 h. In another approach, cells were incubated with 10% fetal serum and then preincubated for different times in the presence of 1% fetal serum, which was maintained during hormone treatments (Table 1). Under these conditions, 100 nM amylin was able to inhibit insulin-stimulated glycogen synthesis activity for the duration of the study (5 h preincubation). In addition, when fetal bovine serum was replaced by horse serum, no insulin counteraction by amylin on glycogen synthesis rate was detected (results not

Figure 2 Dose-response of amylin on insulin-stimulated glycogen synthesis

Cells, preincubated for 12 h in the presence of 10% FBS, were incubated for 1 h without serum, and then further incubated for 2 h in DMEM containing 10 mM [U- 14 C]glucose, 0.2% BSA with different concentrations of amylin, in the absence (□) or the presence (■) of 10 nM insulin. Glycogen synthesis is expressed as a percentage of the rate measured in control cells. Data represent means $\pm$ S.E.M., $n \geq 5$. * $P < 0.05$, ** $P < 0.0003$, *** $P < 0.0001$ versus insulin-treated cells.

Table 2 Counteraction by amylin of insulin effect on glycogen accumulation and glycogen synthase activity

Hepatocytes, preincubated for 12 h in the presence of 10% FBS, were incubated for 1 h in the absence of serum, and then exposed for 30 min (to measure glycogen synthase activity) or 1 h (to measure glycogen content), to no effectors (control cells), 10 nM insulin, 10 nM insulin plus 100 nM amylin, or 100 nM amylin. G6P, glucose 6-phosphate. Data represent means $\pm$ S.E.M., $n = 4$. * $P < 0.01$, ** $P < 0.0001$ versus insulin-treated cells.

	Glycogen (μ g of glucose/mg of protein)	Glycogen synthase (—G6P/ + G6P activity ratio)
Control	59.8 $\pm$ 1.7	0.22 $\pm$ 0.02
Insulin	104.0 $\pm$ 6.8	0.34 $\pm$ 0.02
Insulin + amylin	84.8 $\pm$ 3.6*	0.26 $\pm$ 0.01**
Amylin	59.8 $\pm$ 2.5	0.23 $\pm$ 0.05

shown). However, the optimal conditions to observe insulin and amylin effects in the absence of any other effectors present in serum proved to be the culture of hepatocytes in 10% fetal serum-DMEM and the preincubation in the absence of serum for 1 h before hormone treatments. Thus, all the following experiments were performed under these conditions.

The dose-dependence of the amylin inhibition effect was studied (Figure 2). Amylin, at concentrations above 1 nM, significantly reduced insulin (10 nM)-stimulated [14 C]glucose incorporation into glycogen, and the highest inhibition was reached with 100 nM amylin. At 0.1 nM or lower doses, amylin failed to show any effect when added together with insulin. Basal glycogen synthesis rate was unchanged by amylin at all concentrations tested.

Similar insulin counteraction by amylin was observed when hepatocyte glycogen content was measured. As illustrated in Table 2, amylin (100 nM) did not modify basal levels of glycogen but reduced the accumulation of the polysaccharide induced by insulin by 40%.

Figure 3 Time-dependence of amylin effect on insulin-reduced PEPCK mRNA levels

Hepatocytes were preincubated for 12 h in the presence of 10% FBS, subsequently incubated for 1 h in a serum-deprived medium, and then treated with 10 nM insulin (I, □), 100 nM amylin (A, ●), 10 nM insulin plus 100 nM amylin (IA, ■) or left untreated (C, ○). At indicated times, cells were harvested and PEPCK mRNA levels were measured. (a) Representative autoradiographs of Northern blots. (b) Quantification of data by densitometric analysis. Data are expressed as a percentage of the density measured in control cells (mean $\pm$ S.E.M., $n \geq 3$). * $P < 0.002$ versus insulin-treated cells.

Effects on glycogen synthase and phosphorylase

The possibility that amylin opposition to insulin action was exerted at the level of glycogen synthase activity was next examined. Glycogen synthase activity ratio was increased 50% above control by incubation with 10 nM insulin (Table 2). In the presence of 100 nM amylin, insulin activation of glycogen synthase was partially counteracted (70% inhibition). Amylin by itself did not modify the activity ratio of the enzyme.

The ability of amylin to alter glycogen phosphorylase activity was also studied. Phosphorylase *a* basal activity (44 $\pm$ 2 m-units/mg of protein) was unaffected by amylin from 10 to 60 min of incubation and at concentrations ranging from 0.1 to 100 nM (results not shown). As expected, glycogen phosphorylase was not modified by insulin treatment.

Effects on PEPCK gene expression

Amylin has been shown to counteract insulin inhibition of hepatic glucose output in conscious rats [4–6]. Because PEPCK is a key point in the regulation of gluconeogenesis by insulin, we tested whether amylin was able to interfere with insulin regulation of PEPCK gene expression. As expected, in primary cultured hepatocytes, insulin reduced PEPCK mRNA level (Figure 3), whereas 100 nM amylin did not cause significant changes. The decrease in the mRNA level of PEPCK obtained after 1–1.5 h incubation with insulin was blocked by the simultaneous presence of 100 nM amylin. This effect of amylin appeared to be time-dependent, as no counteraction was observed after 2 h of treatment. The time dependence of the amylin action on serum deprivation was next investigated. To this end, hepatocytes were preincubated for different periods in the absence of serum, and further incubated with amylin, insulin or both hormones together

Figure 4 Time-dependent effect of serum-deprivation on the ability of amylin to impair insulin-reduced PEPCK gene expression

Hepatocytes were preincubated for 12 h in DMEM containing 10% FBS, and subsequently incubated, for different times, in a serum-free medium. Cells were then incubated for 1 h in the absence of effectors (○) or in the presence of 100 nM amylin (●), 10 nM insulin (□) or 10 nM insulin plus 100 nM amylin (■). PEPCK mRNA was quantified by scanning the autoradiograms of Northern blot analysis. Data are expressed as a percentage of the density measured in control cells (mean $\pm$ S.E.M., $n \geq 2$). * $P < 0.02$, ** $P < 0.002$ versus insulin-treated cells.

for 1 h. As shown in Figure 4, insulin counteraction by amylin was again dependent on the presence of serum, as it was only observed within 2 h following deprivation of serum. Thus, these results corroborate a permissive role of fetal serum in hepatic amylin action.

DISCUSSION

The results presented in this paper show that in order to be able to observe amylin counteraction of insulin effects in cultured hepatocytes, the conditions of culture are critical. Of the different incubation media assayed, only in the presence of fetal serum were the effects of amylin elicited. Interestingly, fetal serum seems to be specifically required for amylin to exert its anti-insulin actions, because when fetal serum was replaced by horse serum, no inhibition by amylin was observed. Moreover, when cells were preincubated in fetal serum-deprived medium for 3 h or more before hormone addition, no effect of amylin was detected in insulin-regulated glycogen synthesis or PEPCK gene expression, not even at 1 μ M amylin. Thus, a permissive effect of fetal serum on amylin action is suggested, which disappears with time when cells are deprived of the fetal serum. In contrast, when cells were incubated in the continuous presence of 1% fetal serum, amylin inhibition of insulin-stimulated glycogen synthesis rate was observed for the duration of the study. A possible explanation of this serum dependence is that the presence of serum permits the expression of (a) cell component(s) that would be required for amylin action. Accordingly, it is well known that culturing of hepatocytes under serum and hormone-free conditions causes the progressive and rapid decline of some hepatic functions, such as a decrease in the expression of the genes of key proteins in glucose metabolism such as PEPCK [20], glucokinase [21] or pyruvate kinase [22]. In primary cultured hepatocytes, serum or hormones need to be added to the incubation media to prevent this decline and to permit the study of the regulation of the expression of such of proteins in response to different effectors. It can be postulated that the presence of serum is also

required for maintenance of the expression of the protein(s) mediating amylin interference of insulin action, which at present is(are) unknown. Alternatively, it might be considered that (a) factor(s) present in fetal serum may be acting synergistically or potentiating the amylin effect, or that amylin may be boosting the action of a serum component.

Several studies performed on conscious rats have demonstrated that liver is sensitive to amylin. Infusion of amylin during hyperinsulinaemic-clamp studies decreases liver glycogen content [2] and antagonizes insulin-suppression of hepatic glucose output [4–6]. Here we show that amylin is able to decrease insulin-stimulated glycogen accumulation and glycogen synthase activation in hepatocytes. In addition, we found that amylin opposes insulin reduction of the mRNA level of PEPCK, a key enzyme in the regulation of the gluconeogenic pathway. Therefore, this observation provides an explanation for the reported effects of amylin on hepatic glucose production *in vivo*. Because hepatocytes in primary culture are a well controlled and easily reproducible system, they can offer a good model for studying amylin action, which proved to be difficult to assess in other systems *in vitro*, where many discrepancies have been reported. Indeed, cultured hepatocytes have been shown to be the most useful model for studying glucose metabolism control in response to different hormones [20–22].

In hepatocytes cultured in the optimal conditions previously stated, amylin impaired insulin action on glucose metabolism at doses of 1–100 nM. At the highest concentration tested (100 nM), amylin partially counteracted insulin stimulation of glycogen accumulation and glycogen synthase activity, whereas it blocked the insulin effect on PEPCK expression almost completely. The doses shown to be effective in cultured hepatocytes are in the same range as the doses known to be active in other systems of liver or muscle cells *in vitro*. In our conditions, the increase in amylin concentration was always positively correlated to greater inhibition of insulin effects. No biphasic dose response was observed, as previously reported in HepG2 cells [10]. Although the time-dependent effects of amylin on PEPCK expression were studied (Figure 4), no clear-cut conclusion about the duration of the effect of amylin could be reached, as the interpretation of these results is complicated by the fact that serum-deprivation *per se* is altering amylin action, as discussed above.

No effect of amylin by itself on glycogen metabolizing enzymes or basal glycogen levels was observed at any hormone concentration tested. These results differ from those reported in the HepG2 cells [10], where amylin directly stimulated glycogenolysis and gluconeogenesis, although it did not affect glucose incorporation into glycogen. This discrepancy might be related to the dedifferentiated state of HepG2 cells. The lack of effect of amylin on glycogen phosphorylase activity indicates that amylin does not act through a cyclic AMP (cAMP)-mediated mechanism, as phosphorylase is very sensitively regulated by changes in the concentration of the cyclic nucleotide. Indeed, to our knowledge, no change of cAMP levels by amylin has been demonstrated in hepatocytes. Only in liver systems in which other type(s) of cell were present has amylin been shown to increase cAMP production [11,23]. Similarly, the effects of amylin on glucose metabolism in muscle cells are believed to be mediated through a cAMP-independent mechanism [24].

In conclusion, our studies demonstrate that hepatocytes in primary culture, when preincubated in the presence of fetal serum, are able to respond to amylin. In these cells, amylin antagonizes the effects of insulin on glycogen synthesis and PEPCK gene expression. Our data strengthen the hypothesis that the liver is a target for amylin action. The observation that amylin modulates the effects of insulin on two key steps of

hepatic glucose metabolism may support a possible role of amylin in the induction of insulin resistance.

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