Volume 301, number 1, 115–118 FEBS 10954 April 1992


# Unimpaired effect of insulin on glucokinase gene expression in hepatocytes challenged with amylin

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Received 4 March 1992

Amylin appears to interfere with the action of insulin in muscle and possibly in liver. We have attempted to detect a direct antagonism between amylin and insulin in cultured rat hepatocytes. The stimulation of glucokinase gene expression was used as a marker of insulin action. Amylin proved ineffective in suppressing subsequent accumulation of glucokinase mRNA in response to maximal or submaximal doses of insulin. When applied to cells already induced by prior incubation with insulin alone, amylin failed to reverse induction, in contrast to the effectiveness of glucagon under the same conditions. Thus, amylin is not a physiological antagonist of insulin in the control of hepatic glucokinase gene expression.

Amylin; Insulin; Glucokinase; Gene expression; Hepatocyte; Liver

## 1. INTRODUCTION

Amylin, also referred to as islet amyloid polypeptide, is the major protein component of the hyaline deposits found in the islets of Langerhans of a majority of patients with non-insulin-dependent diabetes mellitus [1,2]. This polypeptide is synthesized in precursor form in the $\beta$ cells of the islets. Mature amylin, which is 37 amino acids in length and amidated at its carboxy-terminal end, is secreted from the cells and can either accumulate locally as amyloid fibrils or enter the vascular system and circulate in plasma (for review, see [3]). Circulating amylin is thought to act as a hormone on target tissues. Because of effects that are opposed to those of insulin, amylin may be a contributing factor of the insulin resistance associated with non-insulin-dependent diabetes [4].

The major target of amylin action appears to be skeletal muscle. In the isolated rat soleus muscle preparation, amylin was shown to reduce basal and insulin-stimulated incorporation of glucose into glycogen [5,6]. This effect has recently been traced to a stimulation of phosphorylase a activity, which can be overcome by insulin at submaximal doses of amylin [7]. Consistent with this in vitro effect, the infusion of amylin during a hyperinsulinemic glucose clamp in rats and dogs resulted in a decrease in peripheral glucose utilisation [8,9]. In glucose clamp experiments, amylin was also reported to antagonize the insulin suppression of hepatic glucose production, suggesting that the liver might be an additional target tissue for amylin [8–10]. Concentrations of amylin that are several orders of magnitude higher than measured plasma levels appear to be required to elicit anti-insulin effects, both in intact animals and in isolated tissue preparations [11]. The relevance of these effects to glucose homeostasis in normal or disease states remains therefore uncertain.

The present work was undertaken in an attempt to demonstrate a direct anti-insulin effect of amylin at the level of the liver cell. The insulin response under study was the induction of mRNA coding for the regulatory enzyme of glucose metabolism glucokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1). We [12] and others [13] have shown that acute administration of insulin to streptozotocin-diabetic rats results in a rapid stimulation of glucokinase gene transcription in the liver, leading to a massive accumulation of hepatic glucokinase mRNA. More recently, the same effect of insulin was elicited in rat hepatocytes maintained in primary culture [14,15]. In addition, glucagon and derivatives of cAMP were shown to inhibit the insulin effect in cultured hepatocytes [14]. The glucokinase gene response in cultured hepatocytes is both rapid and of large magnitude, making this system attractive for studying the mechanism of insulin action and for identifying effectors with insulin-like or anti-insulin activities. In the present paper, we make use of this system to address the issue of a direct antagonism between insulin and amylin at the hepatic level.

## 2. MATERIALS AND METHODS

### 2.1. *Cell culture*

Hepatocytes were isolated from 48-h fasted male Wistar rats (approximately 220 g in body weight) by collagenase perfusion of the liver

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and cultured as described previously [14]. Cells were seeded in 55 $cm^2$ plastic dishes at a cell density of $3.5\times10^6$ cells per dish and cultured in RPMI 1640 medium with the following additions. Fetal bovine serum was present at a concentration of 10% (v/v) for a 3-h attachment period and at 5% for the remainder of the experiments. Dexamethasone ($10^{-7}$ M), penicillin (110 U/ml) and streptomycin (110 $\mu$l) were present throughout. In all experiments, the cells were fed fresh medium 20–25 h after seeding and amylin or insulin were added 2 h later.

Rat amylin was a generous gift of Dr. Timothy J. Rink of the Amylin Corporation (San Diego, CA). Two distinct batches of amylin were used in the present experiments with identical results. Both batches were tested for biological activity at the Amylin Corporation and shown to inhibit the insulin-stimulated incorporation of radioactive glucose into glycogen in the rat soleus muscle system [6]. Highly purified human insulin (Actrapid HM from Novo, Copenhagen, Denmark) was used. Highly purified porcine glucagon was a kind gift of Dr. Lise G. Heding of the Novo Research Institute.

2.2. *Northern blot assay of glucokinase mRNA*

At the times selected for RNA isolation, the hepatocytes were rapidly washed 2 times with 4.5 ml of ice-cold phosphate-buffered saline and scraped into 2 ml of guanidinium thiocyanate solution. This solution as well as the subsequent extraction with phenol–chloroform at acid pH were as described by Chomczynski and Sacchi [16]. Following isopropanol precipitation, the RNA pellets were re-dissolved in the guanidinium thiocyanate solution and precipitated again with isopropanol. These steps were repeated once more and the final RNA was dissolved in water, precipitated with ethanol in presence of sodium acetate and finally dissolved in water. Samples of 16 $\mu$g RNA were denatured with glyoxal and dimethylsulfoxide at 65°C, resolved by electrophoresis in 1% (w/v) agarose gels and transferred electrophoretically to uncharged Nylon Membranes (GeneScreen from NEN Research Products, Boston, MA). Pre-hybridization, hybridization and washing of the membranes were performed as described [12]. The probe was the 2.45 kbp glucokinase GK2 cDNA [17], $^{32}$P-labeled by random priming.

## 3. RESULTS

The first aim of this study was to find out whether the addition of amylin to primary cultures of rat hepatocytes would compromise subsequent induction of glucokinase mRNA by insulin. Cells were challenged with increasing doses of amylin 30 min before addition of a fixed dose of insulin. The dose of insulin ($2.6\times10^{-8}$ M) and the time of exposure of the cells to insulin (8 h) were chosen, on the basis of previous work [14] to ensure maximal accumulation of glucokinase mRNA. As may be seen in Fig. 1, the inductive effect of insulin was fully preserved at all amylin concentrations tested. The range of concentrations extended from $10^{-10}$ to $10^{-6}$ M. For comparison, it should be noted that the $EC_{50}$ for the effect of amylin in the isolated soleus muscle was $7\times10^{-9}$ M (T.J. Rink, personal communication).

The above data demonstrate that the maximal effect of insulin on glucokinase gene expression is not diminished by amylin, even at pharmacological concentrations. We next wanted to determine whether the sensitivity of cultured hepatocytes to insulin, i.e. their response to low insulin doses, would be altered in presence of amylin. To this end, dose–response experiments in which the insulin concentration was varied and the amylin concentration was kept constant, were performed. The Northern blot in Fig. 2 (panel A) illustates the response of cells incubated with increasing doses of insulin, alone or in the presence of $10^{-6}$ M amylin. As may be seen, the insulin-dependent accumulation of glucokinase mRNA was similar in absence or presence of amylin. Additional experiments of the same design in which amylin was supplied to cells at a concentration of $10^{-7}$ M, are summarized in Fig. 2 (panel B). The dose–response curves for the insulin effect on glucokinase mRNA were found to be completely superimposable in absence or presence of amylin. Thus, the sensitivity of liver cells to insulin is not impaired in presence of amylin.

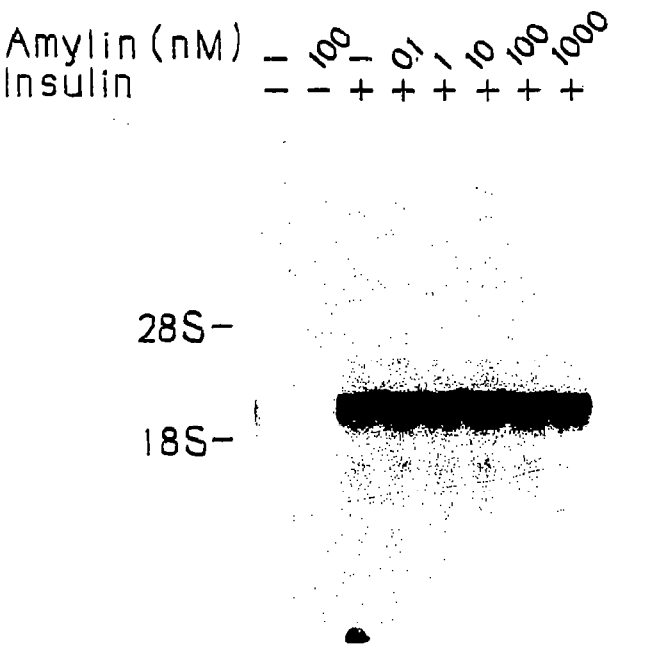


Fig. 1. Effect of a maximal dose of insulin on glucokinase mRNA in presence of various concentrations of amylin. The induction of glucokinase mRNA was studied in cultured rat hepatocytes. Northern blot analysis was performed with total cellular RNA. An autoradiogram of the blot hybridized with a glucokinase cDNA probe is shown.

In the experiments described so far, cells were presented virtually simultaneously with amylin and insulin. We next attempted to determine whether amylin would de-induce glucokinase mRNA in cells cultured initially with insulin alone. In the experiment of Fig. 3, hepatocytes were cultured for 13 h in medium containing $2.6\times10^{-8}$ M insulin, to maximally induce glucokinase mRNA. At the end of this period (time 0 in Fig. 3), amylin was added and culture was continued for the specified time intervals before cell harvest and RNA isolation. For comparative purpose, companion cells were challenged with glucagon instead of amylin. As expected from previous work [14] glucagon caused a rapid drop of glucokinase mRNA such that 20% of the initial level was left after 120 min and less than 4% after

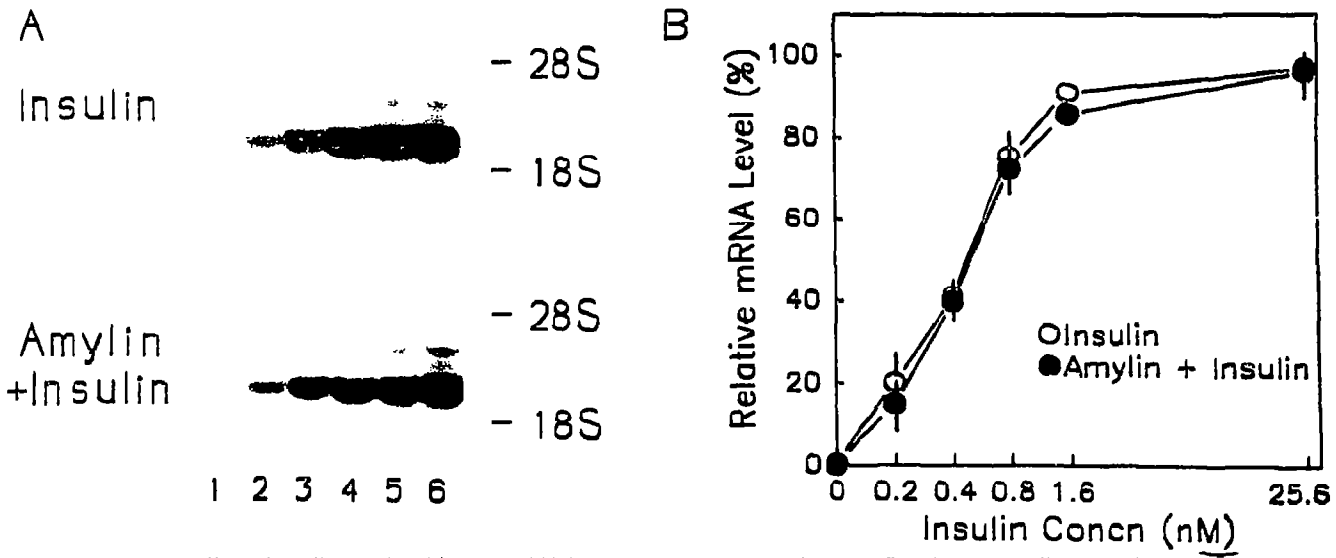


Fig. 2. Dose–response effect of insulin on glucokinase mRNA in presence or absence of amylin. Panel A: autoradiogram of a Northern blot. Where indicated, amylin was present at a fixed concentration of 1 μM. Lane 1, hepatocytes cultured without insulin; lanes 2–6, hepatocytes cultured with 0.2, 0.4, 0.8, 1.6 and 25.6 nM insulin, respectively. Data with or without amylin were obtained with the same batch of hepatocytes. Panel B: quantification of the data from 3 separate experiments by laser densitometer scanning of autoradiograms. Where indicated, amylin was present at a concentration of 100 nM. The means and range of values are given.

240 min. In contrast, the level of mRNA remained unchanged following amylin addition. The amount of message was also stable in cells cultured with insulin and receiving no further addition (not shown).

## 4. DISCUSSION

Amylin does not antagonize the effect of insulin on glucokinase gene expression in the liver cell. As shown previously [14] and further documented here, insulin induces a rapid and massive build-up of glucokinase mRNA in rat hepatocytes maintained in primary culture. Neither the amplitude of the inductive response to supramaximal doses of insulin, nor the cell sensitivity to low doses of insulin, were diminished when amylin was added to the culture medium shortly before insulin. Furthermore, amylin was unable to effect a reduction in the level of the message, when the latter was first raised by culture of the hepatocytes with insulin alone.

The ineffectiveness of amylin is in distinct contrast to the negative control exerted on the glucokinase gene by glucagon and cAMP. We showed in previous work, using a protocol similar to that of Fig. 1, that the insulin-induced accumulation of glucokinase mRNA is progressively inhibited by increasing doses of glucagon, an effect mimicked by 8-chlorophenylthio-cAMP [14]. In addition, as shown in Fig. 3, glucagon rapidly reverses full-blown induction brought on by prior exposure to insulin. The rapid de-induction by glucagon shown here agrees with our earlier kinetic study performed with 8-chlorophenylthio-cAMP [14]. From the contrasting data obtained with glucagon/cAMP on the one hand and amylin on the other, we infer that amylin does not effectively trigger a cAMP signal in the hepatocyte. This conclusion does not support an earlier suggestion of amylin acting via binding to an adenylate cyclase-coupled receptor, the calcitonin gene-related polypeptide receptor, in the plasma membrane of the hepatocyte [18].

During the preparation of this manuscript, Gomez-Foix et al. [19] reported that amylin counteracts the stimulatory effect of insulin on glycogen synthesis in freshly isolated rat hepatocytes. Their data may suggest that amylin could interfere with a branch of the insulin

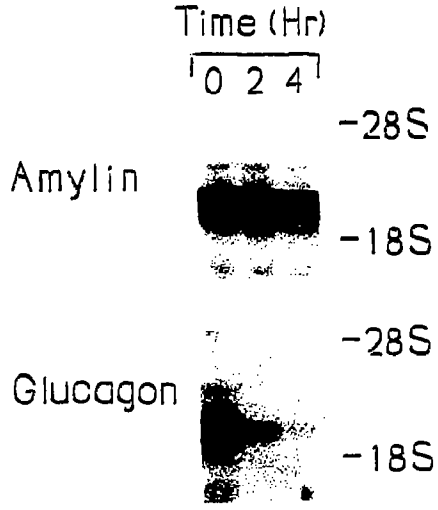


Fig. 3. Reversal of insulin induction by glucagon but not by amylin. Cultured hepatocytes were induced with insulin for 13 h prior to addition of amylin (1 μM) or glucagon (1 nM). Time 0 is the time of these additions. The effects of amylin and glucagon were studied in the same batch of hepatocytes. An autoradiogram of Northern blot is shown.

signal transduction pathway involved in the regulation of hepatic glycogen metabolism, but not in the control of glucokinase gene expression. They are difficult to reconcile, however, with recent metabolic studies using the isolated perfused liver, which failed to reveal an anti-insulin action of amylin [20]. Furthermore, while our work was in progress, Stephens et al. [21] have reported their inability to detect specific binding sites for amylin in the plasma membrane of rat hepatocytes. The lack of receptor for amylin in the liver cell would a fortiori preclude specific effects of the peptide in this cell type. The data presented here are consistent with such a notion.

*Acknowledgements:* We thank Dr. Timothy J. Rink for providing rat amylin. This research was supported by grants from the Swiss National Science Foundation (32.25664.88) and from the Wolfermann-Naegeli Foundation.

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