Anti-insulin effects of amylin and calcitonin-gene-related peptide on hepatic glycogen metabolism

Anna M. GÓMEZ-FOIX,* Joan E. RODRIGUEZ-GIL† and Joan J. GUINOVART*‡

*Department of Biochemistry and Physiology, School of Chemistry, University of Barcelona, Martí i Franquès 1, 08028-Barcelona, Spain, and †Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Autonomous University of Barcelona, 08193-Bellaterra (Barcelona), Spain

To evaluate the effects of amylin and calcitonin-gene-related peptide (CGRP) as anti-insulin agents in hepatic tissue, we have studied whether these two agents counteracted the action of insulin on glycogen metabolism in isolated rat hepatocytes. In this system insulin stimulates [¹⁴C]glucose incorporation into glycogen and activates glycogen synthase. Incubation of the cells with insulin in the presence of amylin or CGRP markedly blocked the insulin stimulation of these two parameters, whereas amylin or CGRP acting alone did not induce any effect. We also examined the ability of amylin and CGRP to modify the anti-glucagon effects of insulin. In the presence of 100 nM-amylin or -CGRP, 10 nM-insulin was almost unable to counteract the inactivation of glycogen synthase and the activation of phosphorylase induced by glucagon. In contrast, neither amylin nor CGRP modified the effect of glucagon on these two enzymes. Our results indicate that amylin and CGRP are able to impair the action of insulin on hepatic glycogen metabolism.

INTRODUCTION

The liver is a crucial site for insulin action in the regulation of normal glucose homeostasis. Insulin stimulates the rate of glycogen synthesis and increases the glycogen content of hepatocytes [1,2]. Direct activating effects of insulin on hepatocyte glycogen synthase, the enzyme responsible for glycogen synthesis, have been observed [2–5]. However, under our conditions insulin does not modify basal unstimulated glycogen phosphorylase activity in isolated rat hepatocytes [6].

In type 2 non-insulin-dependent diabetes mellitus (NIDDM), there is an impairment in glucose metabolism that has been partially attributed to the insensitivity of liver, skeletal muscle and adipose tissue to insulin action. Pancreatic islets of human NIDDM patients characteristically contain an interstitial amyloid deposition. The major component of these deposits, named islet amyloid polypeptide (IAPP) or amylin, has been isolated and sequenced, showing sequence similarity to the neuropeptide calcitonin-gene-related peptide (CGRP) [7,8]. CGRP results from an alternative specific splicing of the transcript of the calcitonin gene [9]. Amylin appears to be a tissue-specific constituent of β -cells [10] and shows co-localization with insulin in the secretory granules [11].

Several observations support the hypothesis that these peptides may be implicated in the pathogenesis of type II diabetes. In isolated pancreas, amylin [12] and CGRP [13] have been shown to inhibit insulin secretion. Moreover, studies have demonstrated that these peptides may partially account for the peripheral insulin resistance observed in type II diabetes. In rat skeletal muscle, amylin and CGRP are able to inhibit the insulin-stimulated rate of glycogen synthesis [14–16]. In myocytes CGRP also inhibits the insulin-stimulated hexose uptake [17,18]. On the contrary, amylin [16] or CGRP [17] did not modify insulin action in isolated adipocytes. When administered to whole animals *in vivo*, CGRP and amylin have been shown to antagonize insulin stimulation of glucose disposal [17]. In addition, Yamaguchi *et al.* [19] observed the induction of hyperglycaemia after intravenous infusion of CGRP to the rats.

The aim of this work was to study whether the anti-insulin effects of amylin and CGRP extended to the liver. Here we show that amylin and CGRP are able to counteract the effects of insulin on glycogen metabolism in isolated rat hepatocytes.

MATERIALS AND METHODS

Materials

Rat amylin was from Peninsula Laboratories. CGRP, insulin, glucagon and D-[U-¹⁴C]glucose were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

Preparation and incubation of hepatocytes

Isolated parenchymal liver cells were prepared from 24 h-starved male Sprague–Dawley rats (200–250 g) as previously described [6]. Cells were resuspended in Krebs bicarbonate buffer (pH 7.4) equilibrated with O₂/CO₂ (19:1) deprived of glucose unless otherwise stated [6].

To assess the rate of glycogen synthesis, hepatocytes were incubated for 1 h in Krebs bicarbonate buffer in the presence of 10 mM-D-[U-¹⁴C]glucose (0.06 μ Ci/ μ mol), and then 1.0 ml samples of the cell suspension were centrifuged (3000 g, 20 s). Cell pellets were added with 0.5 ml of 30% (w/v) KOH and heated at 100 °C for 15 min; glycogen was precipitated with ethanol and measured by the method described in [20].

Enzymic assays

To determine glycogen synthase and phosphorylase activities, hepatocytes were centrifuged (3000 g, 20 s) and cell pellets were immediately homogenized as previously described [21]. Glycogen synthase activity ratio was measured by the low glucose 6-phosphate (0.25 mM)/high glucose 6-phosphate (10 mM) method as in [22]. Glycogen phosphorylase activity was measured in cell extracts by the method of Gilboe *et al.* [23], but caffeine was included in the assay as in [24], except that the final caffeine concentration was 1 mM. Protein was determined by the biuret method [25].

Abbreviations used: CGRP, calcitonin-gene-related peptide; NIDDM, non-insulin-dependent (type 2) diabetes mellitus.

‡ To whom all correspondence should be addressed.

RESULTS

Effects of amylin and CGRP on insulin-stimulated glycogen synthesis

In rat hepatocytes incubated with 10 mM-D-[U-¹⁴C]glucose, 10 nM-insulin caused a 1.6-fold increase in the incorporation of ¹⁴C into glycogen (Table 1), whereas 100 nM-CGRP or -amylin acting alone did not produce any significant effect. When cells were incubated with 10 nM-insulin in the presence of 100 nM-amylin or 100 nM-CGRP, the effect of insulin was almost completely abolished. Thus both effectors clearly counteracted the insulin-stimulated glycogen synthesis.

Table 1. Counteraction by CGRP and amylin of the insulin-induced stimulation of glycogen synthesis and glycogen synthase activity ratio

Hepatocytes were incubated without effectors or with 10 nM-insulin, 100 nM-CGRP, 100 nM-amylin or combinations of these agents as indicated. Length of incubation was 60 min to determine glycogen synthesis, or 10 min to determine glycogen synthase activity ratio. Results of glycogen synthesis are the means $\pm$ S.E.M. of four different experiments, and are expressed as a percentage of the rate measured in control cells (7.2 ± 0.4 nmol of [¹⁴C]glucose incorporated/h per mg of protein). Results of glycogen synthase activity ratio are means $\pm$ S.E.M. of eight independent experiments. The statistical significances of differences calculated by Student's paired test were: ^a $P \leq 0.01$ versus basal; ^b $P \leq 0.05$ versus basal; ^c $P \leq 0.01$ versus insulin; ^d $P \leq 0.05$ versus insulin.

	Glycogen synthesis (% of control)	Glycogen synthase activity ratio
Control	100	0.40 ± 0.01
Insulin	160 ± 5^a	0.43 ± 0.01^b
Insulin + CGRP	98 ± 4^c	0.41 ± 0.01^d
CGRP	106 ± 6	0.39 ± 0.02
Insulin + amylin	110 ± 7^c	0.41 ± 0.01^d
Amylin	109 ± 11	0.39 ± 0.02

Fig. 1. Dose-response of CGRP and amylin on insulin stimulation of glycogen synthesis

After 20 min preincubation with 10 mM-glucose, hepatocytes were further incubated for 60 min with [U-¹⁴C]glucose in the presence of 10 nM-insulin and increasing concentrations of CGRP ($\blacktriangle$) or amylin ($\blacksquare$). Glycogen synthesis is expressed as a percentage of the rate measured in control cells ($\circ$) (7.2 ± 0.4 nmol of [¹⁴C]glucose incorporated/h per mg of protein). Results are means $\pm$ S.E.M. of three experiments performed with different cell preparations.

Concentration-dependence of the effects of amylin and CGRP to counteract the stimulation of glycogen synthesis by insulin

Isolated hepatocytes were incubated with 10 nM-insulin in the presence of increasing concentrations of either amylin or CGRP. As shown in Fig. 1, amylin and CGRP counteracted the effects of insulin in a dose-dependent manner. Half-maximal counteraction of the effect of 10 nM-insulin was observed with concentrations of CGRP or amylin of about 1 nM. Both CGRP and amylin were practically ineffective at 0.1 nM.

Effect of amylin and CGRP on the insulin-mediated activation of glycogen synthase

The action of CGRP and amylin to counteract insulin-stimulated glycogen synthesis was correlated with their effects on glycogen synthase. In hepatocytes treated with 10 nM-insulin for 15 min, a consistent activation of glycogen synthase activity ratio above its basal level was observed (Table 1). When hepatocytes were incubated with 10 nM-insulin in the presence of 100 nM-CGRP or 100 nM-amylin, the insulin-mediated activation of glycogen synthase was reversed. No statistically significant differences between effects of insulin plus amylin or CGRP versus amylin or CGRP alone were observed. Neither CGRP nor amylin significantly modified basal synthase activity ratio up to 15 min of incubation (results not shown).

Interaction between amylin or CGRP and glucagon

Since insulin and glucagon usually have opposite effects on liver glycogen metabolism, we next examined whether CGRP or amylin, in addition to counteracting insulin effects, was able to affect glucagon action. When hepatocytes were preincubated for 10 min with 100 nM-CGRP or -amylin and then further incubated for 5 min with 1 nM-glucagon, no interaction between these effectors as regards glycogen synthase and phosphorylase activities was observed (Table 2). Amylin or CGRP did not

Table 2. Effects of CGRP and amylin on glucagon action and on insulin-counteracted glucagon action on glycogen synthase and phosphorylase activities

Hepatocytes were preincubated for 10 min in the absence of effectors (control cells), or in the presence of 10 nM-insulin, 10 nM-insulin plus 100 nM-CGRP, 100 nM-CGRP, 10 nM-insulin plus 100 nM-amylin or 100 nM-amylin. Then cells were incubated for 5 min more with no further additions or with 1 nM-glucagon, as indicated. Results are means $\pm$ S.E.M. of four experiments (glycogen phosphorylase activity) or of eight independent experiments (glycogen synthase activity ratio). The statistical significance of differences calculated by Student's paired test were: ^a $P \leq 0.01$ versus basal; ^b $P \leq 0.05$ versus glucagon; ^c $P \leq 0.01$ versus glucagon; ^d $P \leq 0.05$ versus insulin + glucagon; ^e $P \leq 0.01$ versus insulin + glucagon.

	Glycogen synthase activity ratio	Glycogen phosphorylase <i>a</i> (m-units/mg of protein)
Control	0.40 ± 0.01	40 ± 11
Glucagon	0.29 ± 0.02^a	104 ± 15^a
Glucagon + insulin	0.34 ± 0.01^b	78 ± 10^c
Glucagon + insulin + CGRP	0.31 ± 0.01^d	107 ± 13^e
Glucagon + CGRP	0.29 ± 0.02	104 ± 16
CGRP	0.39 ± 0.02	41 ± 18
Glucagon + insulin + amylin	0.31 ± 0.01^d	104 ± 13^e
Glucagon + amylin	0.29 ± 0.02	103 ± 19
Amylin	0.39 ± 0.02	39 ± 10

counteract or potentiate the effects of glucagon on these two enzymes.

Counteraction by CGRP or amylin of the ability of insulin to oppose glucagon actions

One of the most important effects of insulin in the liver is to counteract the actions of glucagon [6,26]. Therefore we assayed whether amylin or CGRP was able to block the anti-glucagon effects of insulin. Cells were preincubated for 10 min with 10 nM-insulin in the absence or presence of 100 nM-CGRP or 100 nM-amylin; then 1 nM-glucagon was added and the incubation was continued for 5 min more. Amylin or CGRP significantly blocked the effects of insulin, since in the presence of CGRP or amylin insulin was practically unable to counteract the effects of glucagon on glycogen synthase and phosphorylase activities (Table 2). Neither CGRP nor amylin modified basal phosphorylase activity.

DISCUSSION

Glucose storage as liver glycogen, which plays an important role in mammalian glucose homeostasis, is stimulated by insulin [1,2]. Here we report that incubation of isolated hepatocytes with either CGRP or amylin results in a dose-dependent counteraction of the insulin-stimulated rate of glycogen synthesis. With a maximal concentration of insulin (10 nM), 1 nM-amylin and 1 nM-CGRP significantly decreased the rate of glycogen synthesis, and 100 nM of either completely blocked the effects of insulin. Thus both compounds appear to be equally powerful anti-insulin agents.

Recently, it has been suggested that amylin and CGRP may be partially responsible for the pathogenesis of NIDDM. In NIDDM, insulin-insensitivity in peripheral and hepatic tissues has been suggested to be the major abnormality [27,28]. It is not understood whether this defect results from relative insulin deficiency and chronic hyperglycaemia, or is caused by a change in the concentration of a hypothetical humoral factor. It has been speculated that CGRP and amylin may belong to this class of humoral factors [15,17,29].

Our results support the hypothesis that amylin and CGRP may account for the decrease in hepatic insulin sensitivity, since both effectors markedly antagonize in rat hepatocytes the stimulation of glycogen synthesis by insulin. Our results are in keeping with the data reported in skeletal muscle *in vitro* [15], in which CGRP and amylin also oppose the insulin-induced increase in glycogen synthesis rate. The concentrations of CGRP or amylin shown to be effective in muscle are similar to those that we have determined in hepatocytes. In marked contrast, neither CGRP nor amylin has been shown to counteract insulin action on glucose conversion into CO₂ or triacylglycerol in isolated rat adipocytes [16,17].

Moreover, we show that the anti-insulin effects of CGRP and amylin on hepatocyte glycogen synthesis rate can be correlated with changes in glycogen synthase activity ratio. As we have previously demonstrated [5], insulin activates basal glycogen synthase in isolated rat hepatocytes. Here we observe that both CGRP and amylin are able to antagonize the insulin-induced activation of glycogen synthase.

We have also examined the effects of amylin and CGRP on the ability of insulin to counteract effects of glucagon. Our data indicate that, in cells treated with glucagon and insulin, the addition of CGRP or amylin concomitantly with insulin partially restores the ability of glucagon to inactivate glycogen synthase and to activate glycogen phosphorylase. Furthermore, one of the most interesting conclusions of this paper is that, although

CGRP or amylin is able to counteract all assayed actions of insulin, even its capacity to oppose glucagon, amylin or CGRP by themselves do not potentiate the effects of glucagon on glycogen-metabolizing enzymes. This is a striking point, since it may well be assumed that an anti-insulin agent may favour the action of glucagon.

CGRP is thought to activate adenylate cyclase in skeletal muscle [30] and rat liver membranes [31]. For this reason, it has been postulated that cyclic AMP may mediate certain biological actions of CGRP in these tissues. However, in isolated hepatocytes we could not observe any direct effect of 100 nM-CGRP on glycogen synthase or glycogen phosphorylase activity, enzymes that respond in a very sensitive way to all agents that increase cyclic AMP. A similar conclusion about the non-involvement of cyclic AMP in the action of CGRP was reached by Leighton & Cooper [15] in studies performed in stripped soleus muscle.

In conclusion, our studies indicate that CGRP and amylin may produce insensitivity of liver to the action of insulin. Our results are in keeping with the recent observations *in vivo* by Molina *et al.* [17], which demonstrated that intravenous infusion of CGRP or amylin antagonized the ability of insulin to inhibit hepatic glucose output. Therefore, our results provide additional evidence that the liver is susceptible to the action of CGRP and amylin as anti-insulin agents. The impairment in the insulin-stimulated glycogen synthesis and in the counteraction of glucagon effects by insulin may contribute to the pathogenesis of NIDDM.

We thank Ms. Catalina Relaño and Ms. Anna Vilalta for their valuable technical assistance. This work was supported by the Fondo de Investigaciones Sanitarias de la Seguridad Social (project 89/490), Spain.

REFERENCES

- Baldwin, D., Terris, S. & Steiner, D. F. (1980) *J. Biol. Chem.* **255**, 4028–4034
- Nyfelér, F., Fasel, P. & Walter, P. (1981) *Biochim. Biophys. Acta* **675**, 17–23
- Akpan, J. D., Gardner, R. & Wagle, S. R. (1974) *Biochem. Biophys. Res. Commun.* **61**, 222–229
- Witters, L. A. & Avruch, J. (1978) *Biochemistry* **17**, 406–410
- Ciudad, C. J., Bosch, F. & Guinovart, J. J. (1981) *FEBS Lett.* **129**, 123–126
- Massagué, J. & Guinovart, J. J. (1977) *FEBS Lett.* **82**, 317–320
- Westermarck, P., Wernstedt, C., Wilander, E. & Sletten, K. (1986) *Biochem. Biophys. Res. Commun.* **140**, 827–831
- Cooper, G. J. S., Willis, A. C., Clark, A., Turner, R. C., Sim, R. B. & Reid, K. B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8628–8632
- Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. (1982) *Nature (London)* **298**, 240–244
- Leffert, J. D., Newgard, C. B., Okamoto, H., Milburn, J. L. & Luskey, K. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3127–3130
- Lukinius, A., Wilander, G. T., Westermarck, U., Engström, U. & Westermarck, P. (1989) *Diabetologia* **32**, 240–244
- Ohsawa, H., Kanatsuka, A., Yamaguchi, T., Makino, H. & Yoshida, S. (1989) *Biochem. Biophys. Res. Commun.* **160**, 961–967
- Ishizuka, J., Greeley, G. H., Jr., Cooper, C. W. & Thompson, J. C. (1988) *Regul. Pept.* **20**, 73–82
- Leighton, B., Foot, E. A., Cooper, G. J. S. & King, J. M. (1989) *FEBS Lett.* **249**, 357–361
- Leighton, B. & Cooper, G. J. S. (1988) *Nature (London)* **335**, 632–635
- Cooper, G. J. S., Leighton, B., Dimitriadis, G. D., Parry-Billings, M., Kowalchuck, J. M., Howland, K., Rothbard, J. B., Willis, A. C. & Reid, K. B. M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7763–7766
- Molina, J. M., Cooper, G. J. S., Leighton, B. & Olefsky, J. M. (1990) *Diabetes* **39**, 260–265
- Kreutter, D., Orena, S. J. & Andrews, K. M. (1989) *Biochem. Biophys. Res. Commun.* **164**, 461–467

19. Yamaguchi, A., Chiba, T., Morishita, T., Nakamura, A., Inui, T., Yamatani, T., Kadowaki, S., Chihara, K., Fukase, M. & Fujita, T. (1990) *Diabetes* **39**, 168–174
20. Carrol, N. V., Longley, R. W. & Roe, J. H. (1956) *J. Biol. Chem.* **220**, 583–593
21. Rodríguez-Gil, J. E., Gómez-Foix, A. M., Ariño, J., Guinovart, J. J. & Bosch, F. (1989) *Diabetes* **38**, 793–798
22. Guinovart, J. J., Salavert, A., Massagué, J. J., Ciudad, C. J., Salsas, E. & Itarte, E. (1979) *FEBS Lett.* **106**, 284–288
23. Gilboe, D. P., Larson, K. L. & Nuttall, F. Q. (1972) *Anal. Biochem.* **47**, 20–27
24. Hue, L., Bontemps, F. & Hers, H. G. (1975) *Biochem. J.* **152**, 105–114
25. Layne, E. (1957) *Methods Enzymol.* **3**, 450–451
26. Blackmore, P. F., Assimacopoulos, F., Chan, T. M. & Exton, J. H. (1979) *J. Biol. Chem.* **254**, 2828–2834
27. DeFronzo, R. A., Simonson, D. & Ferrannini, E. (1982) *Diabetologia* **23**, 313–319
28. Rizza, R., Mandarino, L. J. & Gerich, J. E. (1981) *Diabetes* **30**, 990–995
29. Kanatsuka, A., Makino, H., Ohsawa, Y., Tokuyama, Y., Yamaguchi, T., Yoshida, S. & Adachi, M. (1989) *FEBS Lett.* **259**, 199–201
30. Kubota, M., Moseley, J. M., Butera, L., Dusting, G. J., MacDonald, P. S. & Martin, T. J. (1985) *Biochem. Biophys. Res. Commun.* **132**, 88–91
31. Yamaguchi, A., Chiba, T., Yamatani, T., Inui, T., Morishita, T., Nakamura, A., Kadowaki, S., Fukase, M. & Fujita, T. (1988) *Endocrinology (Baltimore)* **123**, 2591–2596

Received 29 October 1990/24 January 1991; accepted 13 February 1991