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Glycogenolytic effect of pancreastatin in isolated rat hepatocytes is mediated by a cyclic-AMP-independent Ca^{2+}-dependent mechanism

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We have studied the effect of pig pancreastatin on glucose and lactate production in freshly isolated rat hepatocytes. Pancreastatin stimulated the rate of glucose output, whereas, in contrast with glucagon, it failed to modify the rate of lactate production. The effective concentration of pancreastatin was in the range 0.1–100 nM, with half-maximal rate close to 1 nM. The ability of pancreastatin to increase glucose output was abolished by chelation of the calcium in the medium. By itself, pancreastatin did not increase cyclic AMP (cAMP) levels and had no influence on cAMP levels in glucagon-stimulated hepatocytes. Our results point out a possible role of pancreastatin in glycogenolysis. This appears to be mediated by a cAMP-independent Ca^{2+}-dependent mechanism.

INTRODUCTION

Pancreastatin is a 49-amino-acid peptide, initially isolated from pig pancreatic tissue [1], whose physiological role remains to be elucidated. On the basis of sequence similarity, chromogranin A has been proposed to be the precursor of pancreastatin [2]. Immunohistochemical studies have revealed that the peptide occurs in the B and D cells of the pig pancreas [3], and it has even been reported that pancreastatin-like immunoreactivity and insulin are released in parallel [4].

It has been reported that pancreastatin inhibits glucose- as well as arginine- and tolbutamide-induced insulin secretion in isolated pancreas and islets of the rat [1,5,6]. The peptide has been shown to inhibit arginine-stimulated somatostatin release from the perfused rat pancreas [5] and to enhance basal glucagon levels in mice *in vivo* [7]. We have recently reported that pancreastatin has glycogenolytic and hyperglycaemic effects in the rat *in vivo*, without modifying basal plasma insulin or glucagon levels [8]. These considerations point to a possible direct effect of pancreastatin on glycogen metabolism. We aimed to study this possibility by comparing the effects of pancreastatin and the well-known agonist glucagon and vasopressin on glucose output and lactate production in the isolated hepatocyte. In addition, possible mechanisms involved in these metabolic effects were considered through the analysis of the production of cyclic AMP (cAMP) and the dependence on extracellular Ca^{2+}.

MATERIALS AND METHODS

Reagents

Collagenase was purchased from Boehringer Mannheim G.m.b.H. (Mannheim, Germany). Bacitracin and 3-isobutyl-1-methylxanthine were supplied by Sigma Chemical Co. (St Louis, MO, U.S.A.). Pig pancreastatin was obtained from Peninsula Laboratories Europe (Merseyside, U.K.). Pig glucagon was obtained from Novo Biolabs (Bagsvaerd, Denmark) and [8-arginine]vasopressin was from Boehringer Mannheim.

Isolation of hepatocytes

Male Wistar rats weighing 180–250 g were used. The animals were fed on a standard diet *ad libitum*. Hepatocytes were prepared by perfusion of the liver with collagenase as described by Krebs *et al.* [9] and modified by Hems *et al.* [10]. The isolated cells were resuspended in Krebs–Ringer bicarbonate medium (KRB), consisting of 119 mM-NaCl, 3.5 mM-KCl, 1.2 mM-KH_2PO_4, 25 mM-$NaHCO_3$, 1.2 mM-$MgCl_2$, 2.5 mM-$CaCl_2$, 20 mM-glucose, 0.1% BSA, final pH 7.4. Hepatocyte viability was routinely evaluated by the Trypan Blue test. The stain was excluded by 90–95% of cells.

Hepatocyte incubation conditions

Isolated hepatocytes were washed by centrifugation, and portions of the cell suspension, equivalent to 15–30 mg of cell protein, were incubated in 50 ml Erlenmeyer flasks in a total volume of 2.5 ml of KRB (see above) modified as follows: 0.5 mg of bacitracin/ml, 0.5% BSA, 1.5 mM-glucose and either 2 mM-$CaCl_2$ or 2 mM-EGTA as indicated in the legends to Figures. The flasks were incubated at 37 °C with shaking (80 strokes/min) and gassed with O_2/CO_2 (19:1) throughout the incubation. Samples

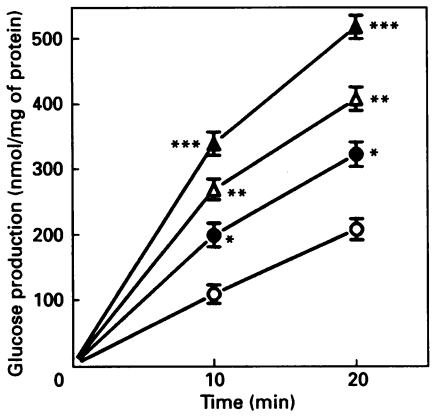

Fig. 1. Time course of hormonal stimulation of glucose output

Hepatocytes from fed rats were preincubated for 2 min at 37 °C. Glucose output was analysed in the supernatants of samples of the cell suspension removed before (zero time) and at 10 and 20 min after addition of 0.1 μM hormone: ●, pancreastatin; △, vasopressin; ▲, glucagon (○, controls). Asterisks denote statistical significance of the differences from controls at the same time: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Abbreviations used: cAMP, 3',5'-cyclic AMP; KRB, Krebs–Ringer bicarbonate.

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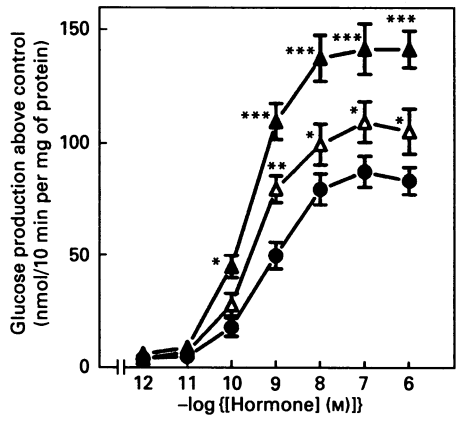

Fig. 2. Concentration-dependence of hormonal stimulation of glucose output

Hepatocytes from fed rats were incubated with different concentrations of pancreastatin (●), vasopressin (△) or glucagon (▲). The values of hormone-stimulated glucose output (nmol/10 min per mg of protein) were calculated by subtraction of the control value obtained in each experiment. The effect of pancreastatin was statistically significant even at 0.1 M. Asterisks denote statistically significant differences from the mean values of pancreastatin experiments: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

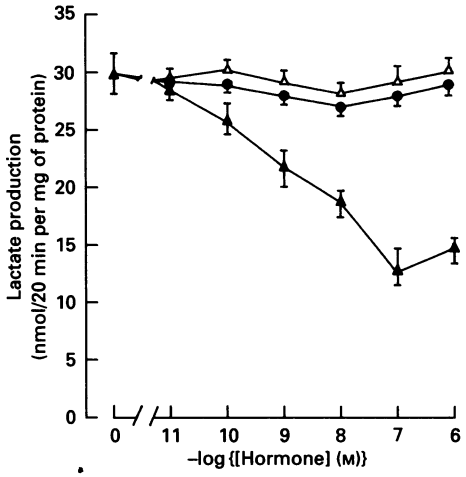

Fig. 3. Concentration-dependence of hormonal inhibition of lactate production

Hepatocytes from fed rats were incubated for 20 min with the indicated concentrations of pancreastatin (●), vasopressin (△) or glucagon (▲). Lactate production is expressed as the difference between final and initial contents of the hepatocyte suspension. Values are means ± S.E.M. The effect of glucagon was very significant even at the 0.1 nM concentration assayed ($P < 0.05$).

of the cell suspension were removed as indicated in the Results section and centrifuged for measurement of glucose and lactate in the supernatant. Hepatocytes were incubated in the same medium to determine cAMP production, but, to avoid cAMP degradation, 0.2 mM-isobutylmethylxanthine was added and the incubation was carried out at 15 °C for 45 min. The choice of these experimental conditions was based on the lower variability observed in preliminary experiments.

Analytical methods

Glucose and lactate were measured by standard procedures [11]. Glucose production is expressed as the difference between final and initial glucose content of the hepatocyte suspension. cAMP was determined by radioimmunoassay (kit from Amersham International, Amersham, Bucks., U.K.) after methanol precipitation of the cell suspension. Hepatocyte protein content was determined by the Bio-Rad assay kit (Bio-Rad Laboratories, Munich, Germany).

Data are means ± S.E.M. from at least four different cell preparations. Differences were analysed by Student's *t* test.

RESULTS

Stimulation of glucose output by pancreastatin

The time course of glucose output was studied in isolated hepatocytes for 20 min in the presence of various glycogenolytic hormones. Pancreastatin (0.1 μM) significantly stimulated the release of glucose, reaching values up to 0.35 μmol/mg of cell protein. The degree of stimulation was relatively lower than that produced by vasopressin and glucagon (Fig. 1). The stimulation of glucose output by the above agonists was dose-dependent at final concentrations in the range 0.1 nM–1 μM. Maximal rate of glucose output was obtained at concentrations close to 0.1 μM of the indicated peptides; higher concentrations failed to modify the rate of glucose release, reaching an apparent saturation pattern in the micromolar range (see Fig. 2). Pancreastatin stimulated hepatic glycogenolysis in a dose-dependent manner, too. The half-maximal effect of pancreastatin, vasopressin and glucagon, obtained by direct extrapolation from lines in Fig. 2, occurred in the nanomolar range. Glucose output produced by vasopressin and glucagon was significantly higher than that caused by pancreastatin.

Concentration-dependence of hormonal inhibition of lactate production

The rate of lactate production by isolated hepatocytes under basal conditions approached 1.5 nmol/min per mg of protein. Pancreastatin and vasopressin did not modify the rate of lactate production, which, as expected, was significantly inhibited by glucagon in a dose-dependent manner (Fig. 3).

Effect of hormones on cAMP levels

Fig. 4 shows that glucagon caused a dose-dependent increase in cAMP levels. However, pancreastatin, like vasopressin, failed to increase cAMP levels. Besides, the addition of 0.1 μM-pancreastatin or -vasopressin did not modify the increase in cAMP induced by different glucagon concentrations.

Hormonal effect on glucose output in the presence of Ca^{2+} or EGTA

The possible dependence of pancreastatin action on extracellular Ca^{2+} was investigated in comparison with glucagon and vasopressin. The increase of glucose output caused by vasopressin was dependent on extracellular Ca^{2+}, whereas the glucagon-mediated increase in glucose production was Ca^{2+}-independent (see Table 1). Like vasopressin, but unlike glucagon, pancreastatin was not effective in stimulating glucose production when hepatocytes were incubated in medium lacking Ca^{2+} and containing 2 mM-EGTA.

DISCUSSION

In agreement with previous observations in experiments *in vivo* [8], we describe here the glycogenolytic effect of pancreastatin in isolated rat hepatocytes and the possible mechanism underlying this phenomenon.

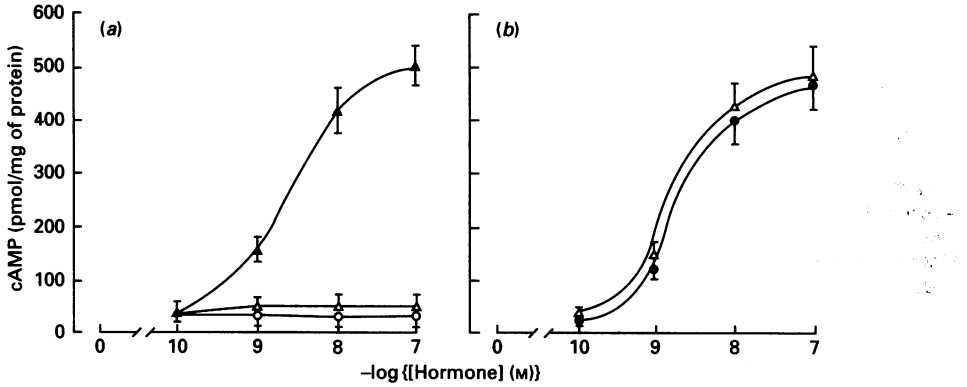

Fig. 4. cAMP production in hepatocytes

Freshly isolated hepatocytes were incubated as indicated in the Materials and methods section in the presence of the indicated hormone concentrations. (*a*) Concentration-dependence of hormone effect on cAMP levels in hepatocytes (○, pancreastatin; △, vasopressin; ▲, glucagon). (*b*) Combined effect of different concentrations of glucagon with either 0.1 μM-pancreastatin (●) or 0.1 μM-vasopressin (△) on cAMP levels in hepatocytes. Values are means ± S.E.M.

Table 1. Effect of Ca^{2+} on hormone-induced glucose output in hepatocytes

Values are means ± S.E.M. of four experiments: [a] $P < 0.05$ versus Ca^{2+}-containing control; [b] $P < 0.05$ versus Ca^{2+}-containing sample of same hormone content. Hepatocytes from fed rats were resuspended in KRB supplemented with either 2 mM-$CaCl_2$ or 2 mM-EGTA (no Ca^{2+}), and incubated at 37 °C (see the Materials and methods section). Samples of the cell suspension were removed at 5 min, the hormones (0.1 μM) were added except to controls, and a second sample was removed after 25 min. The samples were centrifuged and the concentration of glucose in the supernatant was determined. Glucose-output values were calculated from the differences in glucose concentrations at the indicated times.

	Glucose output (nmol/20 min per mg of protein)			
Conditions	Control	Pancreastatin	Glucagon	Vasopressin
Ca^{2+} (2 mM)	225 ± 20	340 ± 26[a]	530 ± 30[a]	405 ± 28[a]
EGTA (2 mM)	175 ± 16	180 ± 23[b]	520 ± 30[a]	190 ± 30[b]

In the studies reported here, the glycogenolytic effect of pancreastatin was compared with those exerted by glucagon and vasopressin. At 0.1 μM, pancreastatin stimulated glycogenolysis by about 55% as much as did glucagon and by about 75% as much as did vasopressin, on the basis of equimolar concentrations. As expected, the effects of glucagon and vasopressin were Ca^{2+}-independent and Ca^{2+}-dependent respectively [12,13]. The effect of pancreastatin, like that of vasopressin, was found to be Ca^{2+}-dependent. Whether pancreastatin exerts its action on hepatocytes by a specific receptor or by the vasopressin receptor remains unknown, but it is worth pointing out that pancreastatin shares the *C*-terminal-Arg-Gly-NH_2 structure with vasopressin, and *C*-terminal fragments of pancreastatin are thought to have full bioactivity [1].

Under our incubation conditions, i.e. 1.5 mM-glucose, pancreastatin and vasopressin failed to stimulate lactate production, which is in agreement with a previous report [10]. However, because of the Ca^{2+}-dependency of pancreastatin and vasopressin, an increase in the glycolytic flux by a fructose 2,6-bisphosphate-dependent mechanism [14], and therefore of lactate production, should be expected. These apparent discrepancies can be explained, since pancreastatin, like vasopressin [14], could hinder the increase of lactate production through a stimulation of pyruvate dehydrogenase.

Pancreastatin has been shown to be increased after a meal containing carbohydrates, protein and fat in pigs [15], reaching 0.15 nM concentration in peripheral blood. A much higher pancreastatin concentration should be expected in the portal vein (although it remains to be determined, but see [4]), which could be in the range used in the present experiments, and thus suggesting a physiological role in hepatic glycogenolysis. In fact, we have shown that the half-maximal effect of pancreastatin in isolated rat hepatocytes occurred at about 1 nM, so the output of glucose induced by pancreastatin could prevent the hypoglycaemia induced by insulin after a protein-rich meal [16]. It is worthwhile to point out that pancreastatin secretion in healthy subjects appears to be dependent on fat and protein stimulation [17,18]. Besides, plasma pancreastatin levels in non-insulin-dependent diabetes mellitus have been found to be significantly increased after a glucose ingestion, compared with healthy controls [17]. So pancreastatin may contribute to the impaired glucose tolerance test that occurs in these patients.

The foregoing results suggest an endocrine effect of pancreastatin exerted in a target cell outside the pancreatic islets. Thus pancreastatin could be a counter-regulatory peptide which may also play a role in pathological disorders such as diabetes and neuroendocrine tumours.

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REFERENCES

1. Tatemoto, K., Efendic, S., Mutt, V., Makk, G., Festner, G. J. & Barchas, J. D. (1986) Nature (London) **324**, 476–478
2. Iancangelo, A. L., Fischer-Colbrie, R., Koller, K. J., Brownstein, M. J. & Eiden, L. E. (1988) Endocrinology (Baltimore) **122**, 2339–2342
3. Ravazzola, M., Efendic, S., Östenson, C. G., Tatemoto, K., Hutton, J. C. & Orci, L. (1988) Endocrinology (Baltimore) **123**, 227–229
4. Östenson, C. G., Efendic, S & Holst, J. J. (1989) Endocrinology (Baltimore) **124**, 2986–2990
5. Efendic, S., Tatemoto, K., Mutt, V., Quan, C., Chang, D. & Östenson, C. G. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7257–7260
6. Silvestre, R. A., Peiro, E., Miralles, P., Villanueva, M. L. & Marco, J. (1988) Life Sci. **42**, 1361–1367
7. Ahren, B., Lindskog, S., Tatemoto, K. & Efendic, S. (1988) Diabetes **37**, 281–285
8. Sanchez, V., Calvo, J. R. & Goberna, R. (1990) Biosci. Rep. **10**, 87–91

9. Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) in Regulation of Hepatic Metabolism (Lundquist, F. & Tygstrup, N., eds.), pp. 726–750, Munksgaard, Copenhagen
10. Hems, D. A., Rodrigues, L. M. & Whitton, P. D. (1978) Biochem. J. **172**, 311–317
11. Bergmeyer, H. U. (1984) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), vol. 6, pp. 588–592, Verlag Chemie, Weinheim
12. Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) Biochim. Biophys. Acta **639**, 243–295
13. Exton, J. H. (1988) Hepatology **8**, 152–166
14. Hue, L. & Rider, M. H. (1987) Biochem. J. **245**, 313–324
15. Bretherton-Watt, D., Ghatei, M. A., Bishop, A. E., Facer, P., Fahey, M., Hedges, M., Williams, G., Valentino, K. L., Tatemoto, K., Roth, K., Polak, J. M. & Bloom, S. R. (1988) Peptides **9**, 1005–1014
16. Cherrington, A. D. & Vranic, M. (1973) Diabetes **22**, 537–543
17. Funakoshi, A., Tateishi, K., Shinozaki, H., Matsumoto, M. & Wakasugi, H. (1990) Regul. Pept. **30**, 159–164
18. Funakoshi, A., Taiteshi, K., Shinozaki, H., Miyasaka, K., Ito, T. & Wakasugi, H. (1990) Dig. Dis. Sci. **35**, 721–725

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