A new role for enteric glucagon-37: acute stimulation of glucose absorption in rat small intestine

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Received 10 April 1997; revised version received 13 May 1997

Abstract Glucagon-37 is secreted by intestinal L-cells following carbohydrate uptake. It is known to inhibit gastric acid secretion (hence also named oxyntomodulin) and appears to increase intracellular cyclic AMP concentrations. Since cyclic AMP could enhance intestinal glucose absorption, a possible stimulatory effect of glucagon-37 on glucose transport was examined. Glucagon-37 acutely increased glucose absorption in the isolated, vascularly perfused small intestine and in isolated enterocytes of the rat. In these cells the stimulation by glucagon-37 could be completely blocked by the cAMP antagonist Rp-cAMPS and was therefore mediated by cAMP. The stimulation of intestinal glucose absorption by glucagon-37 appears to be a major new physiological function.

Key words: Glucagon-37; Oxyntomodulin; Glucose absorption

1. Introduction

The glucagon gene is expressed in the A-cells of the endocrine pancreas and in the so called L-cells of the small intestine. It encodes for a polyprotein of 160 amino acids, that is processed by limited proteolysis in a tissue-specific manner. In the A-cells the polyprotein is cleaved to yield pancreatic glucagon-29 (amino acids 33–61), glucagon-related pancreatic polypeptide (GRPP) (amino acids 1–30) and the major proglucagon fragment (amino acids 64–160); in the L-cells it is split to yield mainly enteric glucagon-37 (amino acids 33–69, i.e. glucagon extended by a C-terminal octapeptide), glucagon-like peptide 1 (amino acids 72–108) and glucagon-like peptide 2 (amino acids 126–158) [1]. Pancreatic glucagon-29 is released in the postabsorptive state; its major target tissue is the liver in which it has long been known to stimulate glycogenolysis and gluconeogenesis by a cyclic AMP-mediated mechanism in order to maintain glucose supply to the glucose-dependent organs such as the brain and the erythrocytes. Enteric glucagon-37 is released in the postprandial, absorptive state; its major target tissue is the stomach in which it has been reported to antagonize the pentagastrin- or histamine-stimulated acid secretion [hence its alternative name 'oxyntomodulin'] at least partially through somatostatin release [2–4]. In the somatostatin-secreting cell line RIN-T3 glucagon-37 stimulated somatostatin release, enhanced adenylate cyclase activity and increased cAMP [4].

In the small intestine glucose is absorbed via the sodium-dependent glucose transporter 1 (SGLT1) at the apical, brush border membrane and the glucose transporter 2 (GLUT2) at the basolateral membrane. It is generally believed that the process is controlled only by long-term adaptation of its capacity to metabolic changes such as lactation, pregnancy, diabetes mellitus or hypo- and hyperinsulinaemia rather than by short-term regulation of its activity [5–8]. However, there are some indications from work with freshly excised small intestine, isolated enterocytes and brush border membrane vesicles that cAMP might acutely stimulate glucose absorption; yet the physiological stimulus triggering cAMP formation has remained unknown [9]. Since enteric glucagon-37 is a hormone of the absorptive phase and may increase cAMP, it was the aim of the present study to evaluate whether it could acutely activate glucose absorption. Using the isolated perfused small intestine and villus tips enterocytes of the rat it was found that glucagon-37 indeed stimulated glucose absorption by a cyclic AMP-mediated mechanism. Thus, besides the stomach the small intestine appears to be another target organ for glucagon-37.

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade and from commercial sources Enzymes were purchased from Boehringer (Mannheim, Germany), glucagon-37, dextran and bovine serum albumin from Appli Chem (Darmstadt, Germany), dibutyryl adenosine 3′,5′-cyclic monophosphate from Sigma (Deisenhofen, Germany) and adenosine 3′-5′-cyclic monophosphothioate-Rp from Calbiochem-Novabiochem GmbH (Bad Soden, Germany).

2.2. Animals

Male Wistar rats were obtained from Harlan-Winkelmann (Borchen, Germany). They were subjected to a 12-h day-night rhythm with free access to food and water (standard diet of Ssniff, Soest, Germany). Preparation of the organ perfusion and isolation of enterocytes were started at 9 a.m. Treatment of the animals was in accordance with the German Law on Protection of Animals.

2.3. Isolated, non-recirculating perfusion of the small bowel

The operation followed the procedures described before for the joint perfusion of the isolated intestine and liver [10,11] with the exception that the liver was not included in the present experiments. In brief, rats (300–350 g) were anaesthetised by intraperitoneal injection of pentobarbital (60 mg/kg). The abdomen was opened and the superior mesenteric artery (SMA) and coeliac trunk (CT) were cannulated. Immediately afterwards the inferior vena cava (IVC) was opened by an incision and the non-recirculating perfusion of intestine was started at a hydrostatic pressure of 120 cmH_2O and a total vascular perfusion rate of about 33 ml/min. Flow in the SMA was measured using a Transonic T 106 Flowmeter (Transonic Systems Inc., Ithaca, NY, USA). A catheter was introduced through the pylorus into the lumen of the duodenum and fixed. Following the incision of the coecum the intestinal content was washed out with a warmed saline solution. Afterwards the portal vein (PV) was cannulated for the vascular outflow and total vascular flow was quantitated by fractionating the effluate of the PV into calibrated tubes. Flow of the CT was defined as the difference between the flow in PV and SMA. At last the entire

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PII S0014-5793(97)00628-5

intestine was prepared free from the body and transferred into an organ bath filled with a warmed saline solution. Experiments were started after a preperfusion period of 20 min.

The intestinal lumen was not continuously perfused. A glucose bolus of 1 g in 1.5 ml 0.9% NaCl was infused during a 1 min application period via the pyloric sphincter. Therefore, luminal flow and thus transit were due to a small net water secretion into the lumen and the physiological intestinal peristalsis. The time interval from application of the carbohydrate bolus to appearance of the carbohydrate in the small intestinal effluate was taken as the transit time.

The perfusion medium consisted of a Krebs-Henseleit buffer containing 5 mM glucose, 2 mM lactate, 0.2 mM pyruvate, 1 mM glutamine, 3% dextran and 1% bovine serum albumin, equilibrated with a gas mixture of O_2/CO_2 (19 : 1). Metabolites were measured using standard enzymatic techniques [10,11].

2.4. Isolation of enterocytes

Enterocytes were isolated by a standard low-temperature method [12]. Since the SGLT-1 is expressed mainly in mature enterocytes located on the villus tips, only these cells were used for the present investigations. In brief, the proximal one third of the small intestine of anaesthetised rats was removed and immediately washed with ice-cold Hanks' balanced salt solution (HBSS). It was everted, cut into small pieces and stirred on ice for 5 min in oxygenated HBSS containing 0.5 mM dithiothreitol. The intestinal sheets were then transferred into 150 ml of an oxygenated calcium-chelate buffer (27 mM Na_3-citrate, 5 mM Na_2HPO_4, 96 mM NaCl, 8 mM KH_2PO_4, 1.5 mM KCl, 20 mM D-sorbitol, 20 mM sucrose, 2 mM glutamine, 1.5 mM dithiothreitol, 1 mg/ml hyaluronidase) and stirred on ice for another 20 min. The separated enterocytes were collected by centrifugation ($1000 \times g$, 6 min at 4°C) and washed twice in the incubation medium (80 mM NaCl, 100 mM mannitol, 20 mM Tris, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 2 mM glutamine, 1 mg/ml BSA, according to [13]). Viability of the cells was measured with the trypan blue exclusion method and was found to be greater than 90%. Cells were suspended in incubation medium to a final concentration of $2 \cdot 10^6$ cells·ml^{-1}. Light microscopy and alkaline phosphatase activity were used to proof the purity of the villus tips enterocyte fraction since alkaline phosphatase activity is highest in the upper villus zone and almost absent in the crypts [14].

For carbohydrate transport studies 200 µl of the stock suspension of cells was distributed into several 20 ml glass flasks each containing 100 µl incubation medium. The flasks were then incubated in a thermostated water bath (37°C) with moderate shaking to ensure adequate mixing and oxygenation of the cell suspensions. Following an incubation period of 10 min with or without effector substances the experiments were started by adding 0.6 µCi ^{14}C-glucose (specific radioactivity 3.28 mCi·mmol^{-1}; NEN, Bad Homburg, Germany) diluted in 100 µl incubation medium to a final glucose concentration of 0.45 mM. The experiment in each vial was stopped after another 10 min by quantitative transfer of the content onto a Whatman filter (Whatman, Maidstone, UK; pore size < 0.45 µm) placed on a fritted-glass disk under negative pressure. The cell pellet, retained by the filter was washed with 10 ml of ice-cold incubation medium to remove radioactivity adhering to the cell surface. The filters were dried at 100°C for 15 min and then directly transferred into scintillation fluid (LSC Hydroluma, Baker, Deventer, Netherlands) and counted for radioactivity.

2.5. Statistical analysis

All results are presented as means ± S.E.M. for the indicated number of observations. Data were analysed by Student's *t*-test for unpaired data. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Increase in intestinal glucose absorption by glucagon-37 in the isolated perfused small intestine

Following the first glucose bolus into the intestinal lumen the portal glucose concentration as a result of intestinal glucose absorption was increased from 4.9 ± 0.2 mmol/l to a peak value of 6.7 ± 0.3 mmol/l (Fig. 1). Basal glucose absorption amounted to 706.2 ± 72.6 µmol or 12.8% of the applied bolus as indicated by the corresponding area under the portal glucose concentration curve (AUC 21.4 ± 2.2 µmol·ml^{-1}·min) multiplied by the portal flow (33 ml·min^{-1}) (Fig. 1). With infusion of glucagon-37 (final concentration in the perfusion medium 0.5 nM, a slightly supraphysiological concentration [2]) into the superior mesenteric artery (SMA) the portal glucose concentration following the second glucose bolus was raised to a peak value of 10.8 ± 0.4 mmol/l. Thus, glucagon-37 increased glucose absorption significantly by 3.3-fold to 2369.4 ± 481.8 µmol or 43.1% of the applied bolus (AUC = 71.8 ± 14.6 µmol·ml^{-1}·min)·(flow = 33 ml·min^{-1}) (p 0.01). When as a control, the second glucose bolus was given without arterial infusion of glucagon-37 the maximum increase in portal glucose concentration as well as the total amount of glucose absorbed were not different from glucose absorption following the first glucose bolus (Fig. 1). With infusion of glucagon-29 (final concentration in the perfusion medium 0.5 nM) there was no change in basal glucose absorption (data not shown). Lactate release by the small intestine during the entire experiment was at a very low, constant level and did not increase during infusion of glucagon-37 (data not shown). The vascular flow of the perfusion system remained essentially constant during the whole experimental period, i.e. it was not altered by glucagon-37 (Fig. 1). There was a mean small effluate from the intestinal lumen of about 0.31 ml/min, due to a net movement of water from the blood vessels to the lumen of less than 2% of vascular flow. This water movement, measured by fractionated sampling of the luminal outflow, was not altered by glucagon-37 (data not shown). The intestinal transit time, i.e. motility, did not differ significantly between the first glucose bolus without and the second glucose bolus with glucagon-37 infusion (Fig. 1); it was 21 ± 6 and 23 ± 7 min, respectively.

3.2. Stimulation by glucagon-37 of glucose uptake via cAMP in villus tips enterocytes

In addition to the organ perfusion experiments the stimulatory effect of glucagon-37 on glucose transport was examined in isolated enterocytes. Cellular uptake of ^{14}C-glucose (0.45 mM) was almost linear with time for 10 min; unstimulated uptake of ^{14}C-glucose amounted to about 70 pmol/10^6 cells·min. Uptake in control experiments performed at 37°C was taken as 100%. Accumulation of ^{14}C-glucose was catalyzed by a specific transport system, since it was reduced to 30% by addition of phlorizin (1 mM), an inhibitor of the intestinal brush-border glucose transporter SGLT-1 [15] and by low temperature incubation (4°C) (Fig. 2). Glucagon-37 significantly stimulated ^{14}C-glucose uptake into enterocytes to 220 ± 36% ($p < 0.01$). In an additional series of experiments with ^{14}C-3-*O*-methyl-glucose, a glucose analogon which can not be metabolized, glucagon-37 stimulated glucose uptake into the enterocytes to a similar extent which excludes a potential effect of glucagon-37 on glucose metabolism within the enterocytes (data not shown). In order to examine the putative involvement of cAMP in the glucagon-37 stimulated increase in glucose uptake, additional experiments were performed in the presence of a cAMP antagonist, the *R*-stereoisomer of 3′,5′-cyclic adenosine monophosphothioate (Rp-cAMPS) [16]. Rp-cAMPS [10 µM] completely blocked the stimulatory action of glucagon-37 on enterocytic glucose uptake. In control experiments Rp-cAMPS alone did not show any influence on ^{14}C-glucose uptake into the enterocytes (Fig. 2). This clearly indicated that glucagon-37

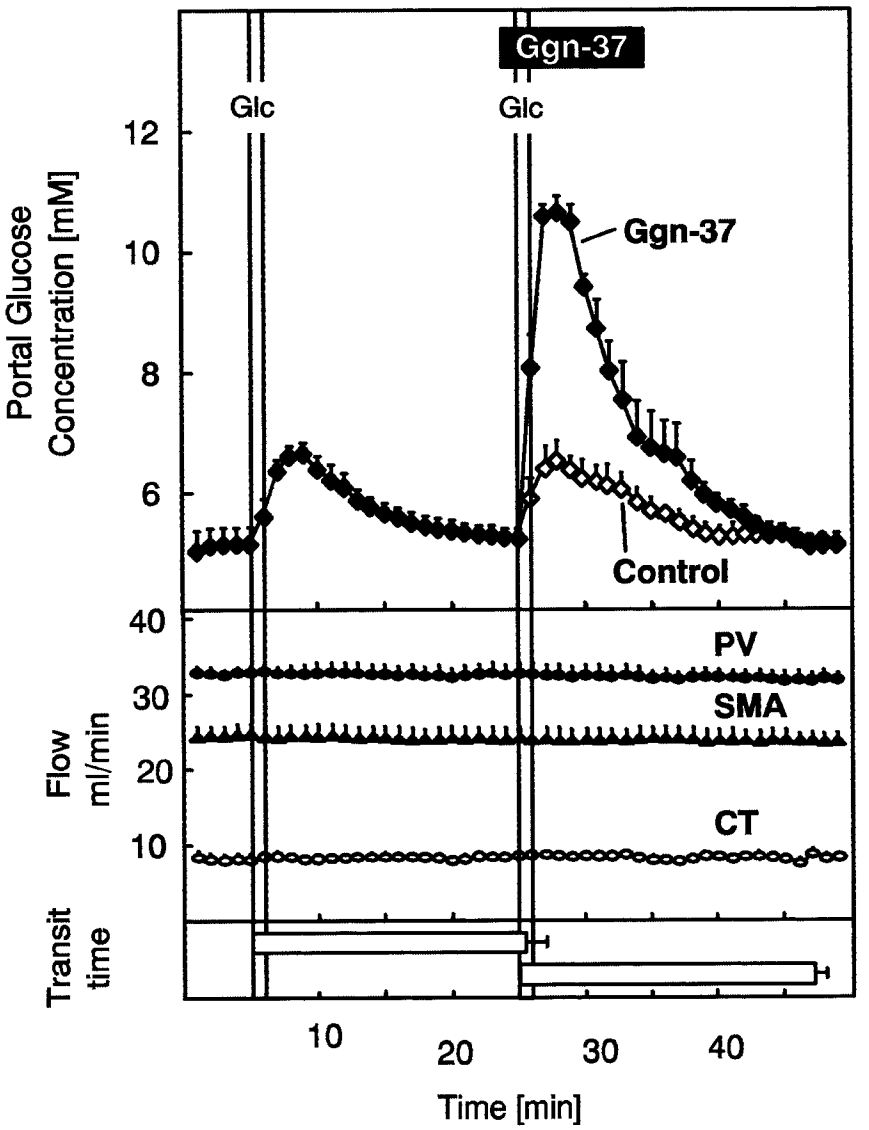

Fig. 1. Increase by glucagon-37 in intestinal glucose absorption (upper panel) and constancy of flow rates and transit time (lower panel) in the isolated perfused small bowel of the rat. Following the intraluminal bolus infusion of 5.5 mmol glucose in the 6th minute glucose was absorbed as indicated by the increase in portal glucose concentration. From the 24th to the 35th minute of the experiment glucagon-37 (0.5 nM, Ggn-37) was infused into the superior mesenteric artery (SMA). Glucose was applied again in the 25th minute. Values are means ± S.E.M. of 4 experiments. Flow in the afferent SMA was measured by a flow meter and in the efferent portal vein (PV) by fractionated sampling. Flow in the coeliac trunk (CT) was the difference between SMA and PV flow. Transit time was the time interval from carbohydrate bolus infusion to appearance in the intestinal effluate.

increased glucose absorption by a cAMP-dependent mechanism.

4. Discussion

Glucagon-37 stimulated intestinal glucose absorption in isolated perfused small intestine and in isolated enterocytes of the rat. This increase in glucose absorption was mediated by cAMP.

4.1. Perfusion of the isolated rat intestine

The vascularly perfused small intestine of the rat and the application of a sugar bolus have been used in several investigations to study intestinal carbohydrate absorption. Unstimulated intestinal glucose absorption and vascular flow rates of the present investigation were comparable with previous results [10,11,17,18]. The stable physiological function was monitored in each used organ preparation: (i) there was only little lactate release and no increase during the entire experiment indicating sufficient oxygen supply; (ii) flow in the CT and SMA did not decrease reflecting deterioration of the preparation due to tissue oedema or development of microembolisms [17,19]; (iii) intestinal motility was smooth and spastic contractions were absent.

4.2. Stimulation by glucagon-37 of glucose absorption in the perfused small intestine

Adaptations of the intestinal absorptive capacity to long-term changes of the metabolic state have been demonstrated. Pregnancy [5], lactation [5], streptozotocin-induced diabetes mellitus [6], experimental hypo- and hyperinsulinaemia [7] and high-carbohydrate diets [8] in rats have been found to

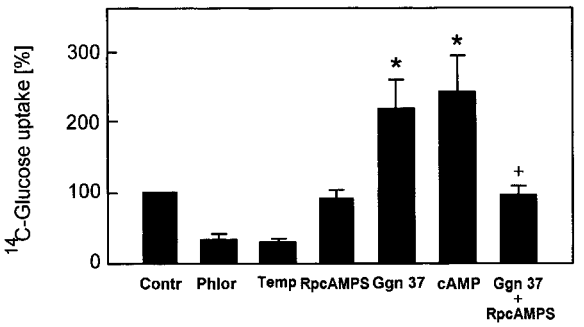

Fig. 2. Increase by glucagon-37 and db-cAMP in glucose uptake and inhibition by Rp-cAMPS of the glucagon-37 stimulated uptake in isolated enterocytes. Enterocytes were isolated by a collagenase-free chelate buffer and then incubated in single glass flasks in a thermostated water bath (37°C, except for analysis of temperature dependency [Temp] at 4°C). In the controls no effector was added and ^{14}C-glucose uptake was taken as 100%. Phlorizin (1 mM, Phlor), Rp-cAMPS (10 μM), glucagon-37 (1 nM, Ggn-37), db-cAMP (10 μM, cAMP) and glucagon-37 plus Rp-cAMPS (10 μM, Ggn-37+RpcAMPS) were given 10 min prior to starting the experiments by adding 0.45 mM ^{14}C-glucose (0.6 μCi) into the flasks. The experiments were stopped by quantitative transfer of the content onto a Whatman filter and the retained cell pellet counted for radioactivity. $^{*}p < 0.01$ vs. Contr; $^{+}p < 0.01$ vs. Ggn 37. Data represent the means ± S.E.M. of 4–5 experiments, each.

increase intestinal glucose absorption; the underlying mechanism was an alteration in the number of intestinal glucose transporters. However, the rapid onset of the increase in glucose absorption in the present investigation (Fig. 1) excludes the possibility of enhancing the carbohydrate absorption by de-novo synthesis of intestinal glucose transporters.

The increase in intestinal carbohydrate absorption could have been caused by three other general unspecific mechanisms: changes in total vascular perfusion rate, changes in effective mucosal flow due to an intestinal redistribution of total flow and changes in intestinal motility. However, there was no change in the total vascular flow nor in SMA and CT flow (Fig. 1). Also, an undetected redistribution of total flow within the intestinal mucosa is unlikely to have occurred since effects of glucagon-37 on vascular flow rates have not been described so far. Intestinal motility, as measured by the transit time of a glucose load, was not significantly altered by glucagon-37 (Fig. 1). In conclusion, general unspecific mechanisms are unlikely to explain the increase by glucagon-37 of intestinal absorption of glucose. A specific stimulation by glucagon-37 of intestinal glucose absorption must be considered.

4.3. *Stimulation by glucagon-37 of glucose uptake into enterocytes*

Enteric glucagon-37 and pancreatic glucagon-29 have been shown to increase intracellular cAMP concentrations in intact RIN T3 cells (a somatostatin secreting cell line) and to stimulate adenylate cyclase activity in RIN T3 cell membranes; glucagon-37 was 10–30 times more potent than glucagon-29 [4]. The two hormones stimulated adenylate cyclase activity also in rat liver plasma membranes, however, conversely glucagon-37 was less potent than pancreatic glucagon-29 [20]. Moreover, in isolated oxyntic glands of the rat glucagon-37 increased the intracellular cAMP concentration with a 20-fold higher potency than glucagon-29 [21]. Finally, [^{125}I]glucagon-37 had a 10-fold higher binding affinity to RIN T3 cell membranes than [^{125}I]glucagon-29 [4,22]. Apparently, glucagon-37 increases cAMP after binding to a specific receptor. The present finding that the stimulatory action of glucagon-37 on ^{14}C-glucose uptake into isolated enterocytes could be completely prevented with the cAMP antagonist Rp-cAMPS and was therefore mediated by an increase in intracellular cAMP concentrations, is in line with expectations.

The cAMP-mediated action of glucagon-37 on isolated enterocytes shows that its effects in the perfused intestine were specific. The involvement of cAMP is in accordance with previous observations: In mice lacking the cAMP-stimulated Cl^- secretion due to targeted gene disruption, a cAMP-stimulated, glucose-dependent increase in Na^+ absorption could be detected under voltage-clamp conditions [23]. In the human rectal adenocarcinoma cell line HRT-18 under basal conditions no glucose-dependent electrogenic Na^+ movement could be measured; however, following cAMP a short circuit current was detected that was glucose-dependent [24]. In brush border membrane vesicles cAMP moderately enhanced glucose uptake by 30% and in the brush border membrane it slightly increased the membrane potential by 10 mV [9].

4.4. *A new function for glucagon-37*

So far, the major role for glucagon-37 in short-term regulation was the inhibition of gastric acid secretion [3]. In the present study evidence for a new role of glucagon-37 in short-term regulation is presented: Since enteric glucagon-37 was found to be secreted in the absorptive state following food intake in man [25] and in rats [2], its acute stimulation of intestinal glucose absorption could be a major physiological function. This action is clearly different from that of pancreatic glucagon which in the postabsorptive state stimulates hepatic glucose release.

Acknowledgements: We wish to thank B. Döring, A. Hunger and F. Rhode for their excellent technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (SFB 402, Molekulare und Zelluläre Hepatogastroenterologie, Teilprojekt B 3).

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