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Absence of insulinotropic glucagon-like peptide-I(7–37) receptors on isolated rat liver hepatocytes

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The effects of glucagon and the glucagon-like peptide GLP-1(7–37) were compared in rat liver hepatocytes. Glucagon elevated cAMP, elevated intracellular free calcium ($[Ca^{2+}]_i$), activated phosphorylase and stimulated gluconeogenesis, whereas GLP-1(7–37) was without effect on any of these parameters. GLP-1(7–37) did not block any of the actions of glucagon. The glucagon analog, des $His^1[Glu^9]$ glucagon amide, was a partial agonist in liver, but also was an effective antagonist of glucagon actions in liver but not those of GLP-1(7–37) in islet B cells. It was concluded that in the rat, GLP-1(7–37) is a potent insulin secretagogue [1] but is without effect on liver.

Glucagon-like peptide-I; Hepatocyte receptor; Pancreatic B-cell; Insulinotropic; Cyclic AMP

1. INTRODUCTION

Glucagon-like peptide-1(7–37) (GLP-1(7–37)) is a member of the glucagon family of peptides that stimulates insulin release [1–4]. The peptide is processed from proglucagon specifically in the intestinal neuroendocrine L-cells [5] wherein it is released into the circulation in response to oral nutrients [6] and binds to and activates cAMP-coupled receptors on the pancreatic B-cells [7–9]. Also GLP-1(7–37) partially inhibits glucagon release indirectly from pancreatic A-cells and directly promotes somatostatin release from pancreatic D-cells [10,11]. These effects of GLP-1(7–37) on A-cells are probably indirectly due to suppression by insulin and somatostatin through paracrine mechanisms; B and D-cells have receptors for GLP-1(7–37) whereas A-cells do not [12].

There are apparently conflicting data as to the actions of GLP-1(7–37) on hepatic metabolism. In fish hepatocytes GLPs stimulate gluconeogenesis, but they do not employ cAMP as an intracellular messenger [13]. In rat liver the larger amino terminally-extended peptide GLP-1(1–37) does not alter glucagon-mediated production of cAMP [14] nor does it stimulate insulin release at all [1] or only weakly [15]. Because the short form of GLP-1, GLP-1(7–37), has potent insulinotropic action [1,2] we sought to evaluate its metabolic actions in isolated rat liver hepatocytes. We also evaluated the capabilities of a synthetic peptide antagonist of glucagon [16] to inhibit glucagon actions in rat hepatocytes. This is an important point because des $His^1[Glu^9]$ glucagon-amide is shown not to inhibit GLP-1(7–37) actions on pancreatic B-cells. However we show that des $His^1[Glu^9]$ glucagon amide does inhibit glucagon effects in B-cells. These observations suggest that distinct receptors exist for GLP-1(7–37) and glucagon on B-cells.

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2. MATERIALS AND METHODS

2.1. *Isolation and incubation of hepatocytes*

Hepatocytes were isolated from male Sprague–Dawley rats by collagenase digestion of the liver as previously described [17]. The cells (30–40 mg wet-wt/ml) were suspended in Krebs–Henseleit bicarbonate buffer and were incubated in 25 ml Erlenmeyer flasks with constant gassing with O_2/CO_2 (95:5).

2.2. *Pancreatic tumor B-cell line (BTC1)*

BTC1 cells [18] were passaged and cultured in Dulbecco's Modified Eagle's Medium containing 25 mM glucose, 15% horse serum, 2.5% fetal bovine serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) as described previously [8]. Trypsinized cells were added to multiwell plates and allowed to grow to 80–90% confluence. Before beginning the peptide stimulation experiments the cells were gently washed three times with the above media modified to contain 1% bovine serum albumin rather than serum (control medium). Then either control media or medium containing the glucagon antagonist des $His^1[Glu^9]$ glucagon amide was added. After incubation at 37°C for 15 min the agonist peptides, GLP-1(7–37) and/or glucagon were added at one-tenth volume of 10-times the desired final concentration

and the cells were incubated for an additional 15 min. At the end of the incubation the cells were immediately extracted with trichloroacetic acid and frozen at −70°C for subsequent assay for cAMP.

2.3. *Phosphorylase a measurements*

Aliquots of cells (0.5 ml) were removed after incubation with various agents and immediately frozen in liquid N_2. Phosphorylase *a* activity was measured in cell homogenates as described [17]. Activity is expressed as units of [U-^{14}C]glucose 1-phosphate incorporated into glycogen per g of wet-wt of tissue per min.

2.4. *cAMP determinations*

cAMP was measured by a radioimmunoassay in diluted trichloroacetic acid extracts of hepatocyte suspensions by the method of Steiner et al. [19] as modified by Harper and Brooker [20]. This method has been successfully adapted to measure cAMP in rat liver hepatocytes [21]. Cyclic AMP levels in extracts of BTC1 cells were measured using a radioimmunoassay as described previously [7].

2.5. *Gluconeogenesis determination*

The rate of gluconeogenesis was determined by measuring the conversion of 5 mM [^{14}C]lactate to [^{14}C]glucose during a 20 min incubation [22]. The data (see section 3) are expressed as % of control rate of [^{14}C]glucose formation and are means ± SEM from 3 separate experiments.

2.6. *Free intracellular calcium measurements*

The changes in $[Ca^{2+}]_i$ were measured as previously outlined [23] utilizing fura 2 as the Ca^{2+} indicator.

3. RESULTS AND DISCUSSION

Glucagon-like peptide-1(7–37) does not increase cAMP levels when added at concentrations ranging between 10^{-10} to 10^{-7} M (Fig. 1). Glucagon, however, elevates cAMP (Fig. 1). The inclusion of 10^{-8} M GLP-1(7–37) as a potential antagonist of the actions of glucagon had no effect on the action of glucagon to elevate cAMP (Fig. 1). These data show that GLP-1(7–37) is neither an agonist nor an antagonist of glucagon actions in hepatocytes when cAMP levels are measured.

To further determine that GLP-1(7–37) was without effect in hepatocytes, the effectiveness of GLP-1(7–37) to activate phosphorylase was measured. Consistent with the data in Fig. 1, GLP-1(7–37) did not activate phosphorylase when added at concentrations ranging between 10^{-11} to 10^{-7} M (data not shown). Glucagon did increase phosphorylase *a*, with a maximum twofold increase observed with approximately 10^{-9} M. However the inclusion of 10^{-8} M GLP-1(7–37) with glucagon (10^{-11} to 10^{-7} M) had no effect on the ability of glucagon to elevate phosphorylase *a* (data not shown).

Glucagon has been shown to stimulate calcium influx $[Ca^{2+}]_i$ [24,25], although the precise mechanism by which it does this is not known. We find that GLP-1(7–37) at either 10^{-7} M or 10^{-8} M does not elevate $[Ca^{2+}]_i$ in conditions in which glucagon 10^{-7} M elevated $[Ca^{2+}]_i$ to a level similar to that obtained with 10^{-9} M vasopressin (Fig. 2).

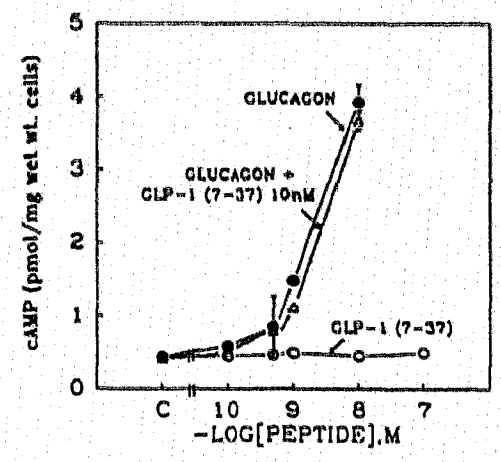

Fig. 1. Dose-response of GLP-1(7–37), glucagon, and glucagon in the presence of GLP-1(7–37) on cAMP levels. Hepatocytes were incubated for three min with the appropriate concentrations of GLP-1(7–37) and glucagon. Aliquots of 0.5 ml were removed after 3 min into 0.5 ml of ice-cold 10% TCA. After removal of protein by centrifugation, the amount of cAMP was measured by radioimmunoassay (see section 2 for details). The data shown (mean ± SEM) are from a representative experiment performed in triplicate and assayed in duplicate.

An important metabolic effect of glucagon in the liver is to stimulate gluconeogenesis [26]. GLP-1(7–37) 10^{-8} M was unable to stimulate gluconeogenesis (106 ± 11% of control). Glucagon (10^{-8} M) produced a substantial increase in gluconeogenesis (262 ± 46% of control). The ability of 10^{-8} M glucagon to stimulate gluconeogenesis was not modified by the inclusion of 10^{-7} M GLP-1(7–37) in the incubation (246 ± 35% of control). To determine whether the lack of GLP-1(7–37) effects on the hepatocytes was not due to loss of activity during handling of the sample, an aliquot from the stock GLP-1(7–37) solution that was us-

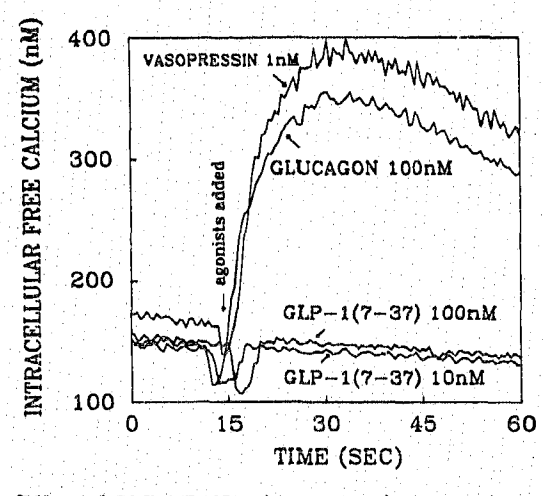

Fig. 2. Effect of GLP-1(7–37), glucagon, and vasopressin on $[Ca^{2+}]_i$ in hepatocyte suspensions. Peptides were added to fura 2-loaded hepatocytes 15 s after data collections were started. Calibration was performed as detailed previously [23].

ed in the above described experiments was assayed in the rat perfused pancreas as described previously [1,4]. The stimulation of insulin secretion at 10^{-9} M concentration was similar to the effect obtained from the freshly prepared GLP-1(7–37) stock solution (G. Hendricks and G. Weir, personal communication).

To confirm that the actions of glucagon could be blocked by an antagonist at the receptor level, the glucagon analog des His^1[Glu^9] glucagon amide, which has been reported to be a glucagon antagonist [16], was utilized. We found that 1 μM des His^1[Glu^9] glucagon amide is a partial agonist because it elevated cAMP levels from 473 ± 29 to 1091 ± 131 fmol/mg. However, it was also a very good antagonist of the effects of glucagon on cAMP levels, especially when the hormone was present at 10^{-9} M and 10^{-8} M (Fig. 3). When lower concentrations of des His^1[Glu^9] glucagon amide were utilized (e.g., 100 nM), there was very little inhibition of glucagon effects (data not shown). The glucagon antagonist des His^1[Glu^9] glucagon amide (1 μM) also activated phosphorylase *a* by itself (approx. 50% of that observed with 10^{-9} M glucagon) and antagonized the effects of glucagon in the concentrations 10^{-10} M to 10^{-8} M (data not shown). At a concentration of 10^{-7} M glucagon, there was no inhibition of phosphorylase *a* activity by the des His^1[Glu^9] glucagon amide (data not shown).

In contrast to the absence of any effects of GLP-1(7–37) on the rat liver hepatocytes, it has been shown that the peptide potently stimulates the pancreatic B-cells to release insulin, increase cAMP formation and elevate proinsulin gene expression [1,4,7–9]. Importantly, the glucagon antagonist, des His^1[Glu^9] glucagon amide (10^{-7} M) does not antagonize the GLP-1(7–37) (10^{-9} M) stimulated formation of cAMP in pancreatic B-cells (Fig. 4). The effect of glucagon (10^{-8} M) on cAMP formation in B-cells, however, is abrogated by the glucagon antagonist (10^{-6} M), although the antagonist has agonist activity at this concentration (Fig. 4). Inasmuch as glucagon is approximately 100-fold less potent than GLP-1(7–37) in releasing insulin in the perfused rat pancreas [4] and in stimulating formation of cAMP in B-cells [4,7], it was necessary to use 10^{-8} M glucagon and 10^{-6} M des His^1[Glu^9] glucagon amide to achieve the antagonistic effect. Further, GLP-1(7–37) (10^{-8} M) and glucagon

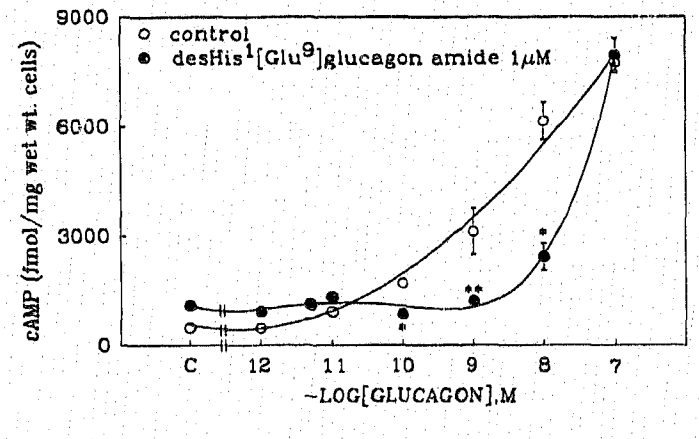

Fig. 3. Dose-response of glucagon to elevate cAMP in the presence and absence of des His^1[Glu^9] glucagon amide. Hepatocytes were incubated for 3 min with the appropriate concentrations of glucagon in the presence and absence of 1 μM des His^1[Glu^9] glucagon amide (added simultaneously). Aliquots were removed for the measurement of cAMP levels as detailed in legend to Fig. 1. Data shown are the means ± SEM from 3 separate incubations, assayed in duplicate. Significant differences are indicated by * $P < 0.05$ and ** $P < 0.01$ (analysis by Student's *t*-test for paired observations).

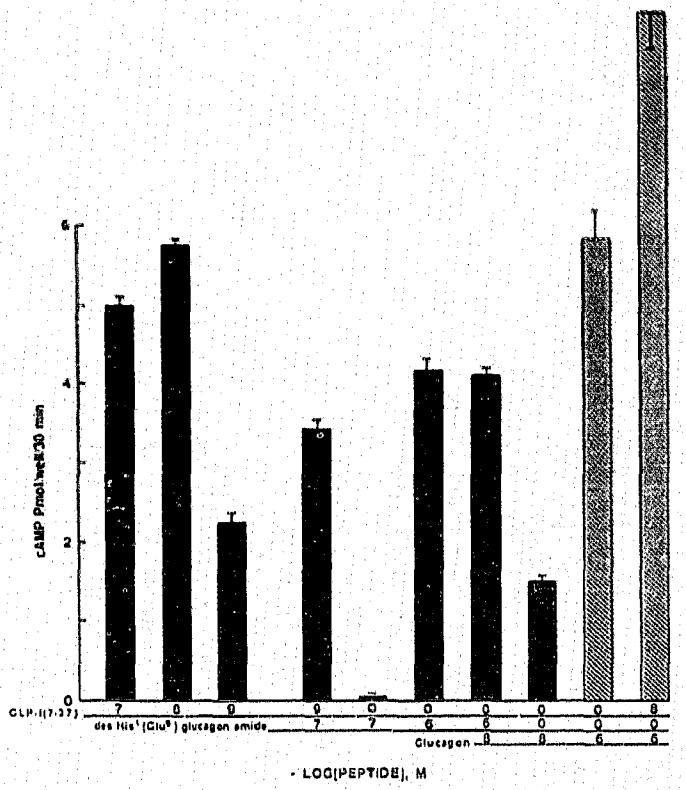

Fig. 4. Effects of GLP-1(7–37), glucagon and the glucagon antagonist des His^1[Glu^9] glucagon amide on the formation of cAMP in pancreatic B-cells. Mouse BTC1 cells were treated with either control media or media plus antagonist, des His^1[Glu^9] glucagon amide, for 15 min and then agonist, GLP-1(7–37) or glucagon were added. After 15 min, cells were extracted and assayed for cAMP. A typical dose-response for GLP-1(7–37) is shown on the left (black bars). The glucagon antagonist (10^{-6} M) prevents stimulation of cAMP by glucagon (10^{-8} M) but at 10^{-7} M does not inhibit stimulation of cAMP by GLP-1(7–37) (10^{-9} M) (middle, shaded bars). GLP-1(7–37) 10^{-8} M is additive with glucagon (10^{-6} M) in stimulating cAMP formation (right, cross-hatched bars). Note that the lower level of cAMP at 10^{-7} M compared to 10^{-8} M GLP-1(7–37) is a result of some homologous desensitization of the receptor [9]. The experiment was done twice, each time using quadruplicate wells of cells and assay of cAMP in cell extracts prepared from each of the four wells was done in quadruplicate. The values shown are means ± SEM of the average cAMP levels for the 4 wells of cells.

(10^{-6} M) were partially additive in the stimulation of cAMP formation in B-cells (Fig. 4). GLP-1(7–37) at 10^{-8} M has been shown to maximally stimulate cAMP formation in B-cells [7,9]; higher concentrations of the peptide result in less cAMP generation due to a homologous desensitization of the B-cell receptor [9].

The data presented in this study clearly show that GLP-1(7–37) does not stimulate, in the rat, hepatic glycogenolysis (measured by phosphorylase activation) or gluconeogenesis (measured by the conversion of [^{14}C]lactate to [^{14}C]glucose). GLP-1(7–37) also does not increase the intracellular second messengers cAMP and Ca^{2+} in rat liver. These data are not consistent with those found in fish where GLP-1 functions as a metabolic hormone [27] and activates hepatic gluconeogenesis [13]. These discrepancies in observations may be more apparent than real. There exists a large family of peptides related in their structure to glucagon including among others, vasoactive intestinal peptide, gastric inhibitory peptide, secretin, growth hormone releasing hormone, the salivary gland peptides (helospectins, helodermins), as yet uncharacterized brain peptides and the glucagon-like peptides 1 and 2. The fish glucagon-related peptides co-encoded with glucagons in the two nonallelic genes have structures that differ considerably from the mammalian GLP-1s. Inasmuch as there must exist corresponding receptors for all of these peptides, it is reasonable to propose that certain of the peptides will cross-react with the receptors of other peptides, especially when crossing species, e.g. rat and fish and liver hepatocytes. Alternatively since there is a conservation of amino acid sequence between fish GLP-1s and mammalian GLP-1(7–37) [28] the discrepancies between our results and those obtained with fish hepatocytes may also be explained by differences in glucose metabolism between fish and mammals.

In conclusion it appears from our findings that the pancreatic B-cell is a major and important target for GLP-1(7–37) and the role of the peptide is to stimulate the secretion of insulin and to thereby suppress the secretion of glucagon. Therefore, GLP-1(7–37) is an anabolic hormone and as such it is physiologically relevant that it not have receptors on hepatocytes that would be glucagon-like in their catabolic actions.

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