Peptides, Vol. 10, pp. 1171–1177. © Pergamon Press plc, 1989. Printed in the U.S.A.

0196-9781/89 $3.00 + .00

Biological Activities of des-His[1][Glu[9]]Glucagon Amide, a Glucagon Antagonist[1]

CECILIA G. UNSON, ELLEN M. GURZENDA AND R. B. MERRIFIELD

The Rockefeller University, 1230 York Avenue, New York, NY 10021

Received 3 May 1989

UNSON, C. G., E. M. GURZENDA AND R. B. MERRIFIELD. *Biological activities of des-His[1][Glu[9]]glucagon amide, a glucagon antagonist.* PEPTIDES **10**(6) 1171–1177, 1989.—Hyperglycemia in diabetes mellitus is generally associated with elevated levels of glucagon in the blood. A glucagon analog, des-His[1][Glu[9]] glucagon amide, has been designed and synthesized and found to be an antagonist of glucagon in several systems. It has been a useful tool for investigating the mechanisms of glucagon action and for providing evidence that glucagon is a contributing factor in the pathogenesis of diabetes. The in vitro and in vivo activities of the antagonist are reported here. The analog bound 40% as well as glucagon to liver membranes, but did not stimulate the release of cyclic AMP even at 10^6 higher concentration. However, it did activate a second pathway, with the release of inositol phosphates. In addition, the analog enhanced the glucose-stimulated release of insulin from pancreatic islet cells. Of particular importance were the findings that the antagonist also showed only very low activity (<0.2%) in the in vivo glycogenolysis assay, and that at a ratio of 100:1 the analog almost completely blocked the hyperglycemic effects of added glucagon in normal rabbits. In addition, it reduced the hyperglycemia produced by endogenous glucagon in streptozotocin diabetic rats. Thus, we have an analog that possesses properties that are necessary for a glucagon antagonist to be potentially useful in the study and treatment of diabetes.

Glucagon analog Diabetes mellitus Blood glucose Receptor binding Adenylate cyclase Cyclic AMP
Phospholipase C Inositol trisphosphate

GLUCAGON is a 29 residue peptide hormone produced by the A cells of pancreatic islets. It is primarily responsible, together with insulin, for maintaining normal levels of glucose in man and in animals. It exerts its major effects mostly in liver cells as an insulin-counteracting hormone by stimulating glycogenolysis and gluconeogenesis when an organism is in need of glucose. Most of glucagon's actions are mediated by the second messenger cAMP produced upon activation of adenylate cyclase. It is this increase in intracellular cAMP upon activation of the cyclase that results in glycogen hydrolysis and glucose synthesis, with a consequent rise in glucose output by the liver. There is sustained interest in the hormone glucagon because its physiological and pathogenic role in diabetes mellitus and diabetic ketoacidosis is still under debate. It has been observed that in insulin-deficient diabetic patients, plasma levels of glucagon are often abnormally high and may contribute to impaired glucose tolerance and other metabolic derangements (9). Thus, according to the bihormonal hypothesis (20,21) regarding diabetes mellitus, the hyperglycemic state may be brought about not only by glucose underutilization due to a deficiency or lack of insulin, but also by overproduction of glucose by elevated physiological concentrations of glucagon. It is reasonable to assume that antagonists of the hormone will suppress the actions of this endogenous circulating glucagon, and will provide evidence that glucagon is a contributing factor to diabetes mellitus. Johnson *et al.* (11) have shown that semisynthetic [1-N^{α}-trinitrophenylhistidine, 12 homoarginine] glucagon (THG) is an antagonist of glucagon and will reduce blood glucose. Such antagonists could be potentially useful drugs as adjuncts to or as alternatives to insulin therapy. Glucagon can also exert effects independent of intracellular cAMP concentration. Wakelam *et al.* (24) reported that glucagon binds to another receptor in the hepatocyte membrane that is coupled via an as yet unidentified G-protein to phospholipase C, which upon stimulation causes the breakdown of phosphatidylinositol 4,5 bisphosphate to produce the second messengers inositol trisphosphate and 1,2 diacyglycerol. The complex interplay (10) between the two signaling systems remains to be elucidated and emphasizes the importance of selective analogs of the hormone that may inhibit one or both of these pathways.

One of our approaches was to synthesize a secretin-glucagon hybrid (1), the idea being that, since secretin and glucagon belong to the same family and have very similar sequences, these peptides may have evolved into their present sequences possessing different biological roles, from a common precursor by a series of intermediates, one or more of which may have retained some affinity for the glucagon receptor while losing the ability to transduce the signal. One of the analogs that resulted from this series of peptides

[1]This work was supported by U.S. Public Health Service grant DK 24039.

was des-His[1][Glu[9]]glucagon amide. It was recently shown (22) to be a pure glucagon antagonist with a binding affinity for the glucagon receptor 41% as high as glucagon itself. It had an inhibition index of 12 and a pA_2 value of 7.2, and did not activate adenylate cyclase even at concentrations 10^6 greater than required for glucagon.

Since our goal in glucagon structure-function studies is to identify features of the peptide that are important in receptor binding and biological activity, it is necessary to test its physiological effects in living animals. We report here results of biological activity measurements in several systems of des-His[1][Glu[9]]glucagon amide relative to natural glucagon.

METHOD

Synthesis and Purification

des-His[1][Glu[9]]glucagon amide was synthesized several times and purified as described previously (22). The integrity of the sample was verified consistently by analytical HPLC, amino acid analysis and mass spectral analysis which determined the $(M + H)^+$ peaks (3359.2) to be within ± 0.3 Da of theory.

Receptor Binding and Adenylate Cyclase Activity

Rat liver membranes were prepared from male Sprague-Dawley rats (Charles River Laboratories) by the Neville procedure as described by Pohl (18) and were stored under liquid nitrogen until use. The receptor binding assay was performed according to the procedure of Wright and Rodbell (25) in 1% bovine serum albumin, 1 mM dithiothreitol, 25 mM Tris-HCl buffer, pH 7.2. The amount of radioiodinated glucagon displaced from receptor sites by increasing concentrations of antagonist was measured. Binding affinity was expressed as the ratio of the concentration of natural glucagon to that of the antagonist required to displace 50% of receptor-bound labeled glucagon. Activation of adenylate cyclase in rat liver membranes was measured using a procedure described by Salomon *et al.* (19) in an assay medium containing 1% bovine serum albumin, 25 mM $MgCl_2$, 2 mM dithiothreitol, 25 mM GTP, 5 mM ATP, 0.9 mM theophylline, 17.2 mM creatine phosphate and (1 mg/ml) creatine phosphokinase. The cAMP released was determined using a commercial kit from Amersham in which unlabeled cAMP was allowed to compete with [8-^{3}H]cAMP for a high-affinity cAMP binding protein.

Glycogenolysis

New Zealand rabbits (2.8–3.8 kg) were fasted for 18 hours and injected intravenously with 1 ml saline solution of des-His[1][Glu[9]]glucagon amide at a dose of 3.6–4.0 μg/kg body weight. Blood samples (1–2 ml) were withdrawn immediately before injection, then at 3–5-minute intervals for 40 minutes. The same procedure was repeated at ten times and one hundred times that dose on the same rabbit, giving the animal at least a one-week recovery period between assays. Natural glucagon was injected into the same animals for comparison. All blood samples were collected in vacutainer tubes containing EDTA and NaF, and were centrifuged. Glucose content was determined on 25 microliters of serum samples, using an assay kit based on the glucose oxidase method (Sigma) wth spectrophotometric measurement of oxidized o-dianisidine at 425 nm. The color intensity is proportional to the glucose concentration. The blood glucose level (mg/dl) was plotted against time.

Inhibition of Glycogenolysis

Doses of natural glucagon of 3.6–4.0 μg/kg body weight, together with ten, fifty, and one hundred times that amount of des-His[1][Glu[9]]glucagon amide in saline were injected into rabbits, blood samples were withdrawn before injection and at 5-minute intervals, and the glucose content was measured. The mg/dl glucose versus time curve was compared to the response with natural glucagon alone in the same animal after a one-week recovery period.

Lowering of Hyperglycemia of Diabetic Rats

Male Wistar rats weighing 350–400 mg were made diabetic by an IP injection of streptozotocin (Sigma, St. Louis, MO) 65 mg/kg body weight, freshly prepared as a 1.4% solution in 0.05 M citrate, pH 4.5. After 10–14 days, the rats were sufficiently hyperglycemic, with plasma glucose levels stable at around 350–400 mg/dl. Individual rats were anesthetized with Nembutal (Abbott Laboratories) 30–40 mg/kg b.wt., IP. A single 0.2 ml injection of a solution of des-His[1][Glu[9]]glucagon amide in saline, 1 mg/kg, was administered intravenously through the tail vein. Blood samples were withdrawn by nicking the tail before IV injection and at 3-minute intervals after injection for 21 minutes, then every ten minutes for an hour. The rat was kept sedated throughout the procedure by inhalation of small doses of metofane (Pitman-Moore Inc., Washington Crossing, NJ).

In a second group of experiments, an anesthetized rat was injected with an initial 0.2 ml bolus of des-His[1][Glu[9]]glucagon amide in saline (1 mg/kg), followed immediately by a continuous infusion (17 μl/min) of the antagonist, 33 μg/kg/min, for 60 minutes using a constant flow rate infusion pump. Blood samples were withdrawn as described above in tubes containing heparin. Glucose levels were determined on 25 microliters of blood serum using the Sigma glucose assay colorimetric kit.

Phospholipase C Activity

Rat hepatocytes were prepared from male Sprague-Dawley rats (200–250 g) using the collagenase digestion method based on the procedure of Berry and Friend (4), which allowed for at least 90% viability determined by trypan-blue exclusion. The rat was first anesthetized with intraperitoneal nembutal. Following cannulation of the portal vein and inferior vena cava above the diaphragm, liver perfusion was initiated with Dulbecco's modified Eagle medium (DMEM) containing 5.5 mM glucose, and 10 mM EDTA (pH 7.4, 37°) at 40 ml/min for 5 min. Perfusion was continued with 500 ml DMEM containing 10 mM glucose, 5 mM *myo*-inositol, 1% bovine serum albumin, and 0.5 mg/ml collagenase for 10 minutes, after which the liver was removed and gently teased apart in the perfusion buffer containing collagenase. The digest was transferred into conical tubes, washed twice and resuspended in Krebs-Ringer bicarbonate solution (KRB). Tritium-labeled inositol was incorporated into the intracellular phospholipids as follows. After isolation, the cells (5–7 mg dry weight/ml) were preincubated in O_2/CO_2 (95/5%) at 37° in KRB buffer containing 1% BSA, 10 mM glucose and 5 μCi/ml [2-^{3}H]*myo*-inositol for 90 min. Then the cells were washed and resuspended in the same buffer in the absence of inositol, incubated 10 min more, followed by the addition of LiCl (10 mM) and incubation was continued for another 15 min.

The glucagon-stimulated hydrolysis of the phosphatidyl inositol bisphosphate was assayed as follows. The cells (1 ml suspension, 5–8 mg dry weight) were then distributed into plastic vials containing glucagon or des-His[1][Glu[9]]glucagon amide, capped and incubated for 30 min at 37° 5% CO_2. The assay was terminated by the addition of 1 ml $CHCl_3$:CH_3OH (1:2) mixture. Water (0.5 ml) and chloroform (0.5 ml) were added and the

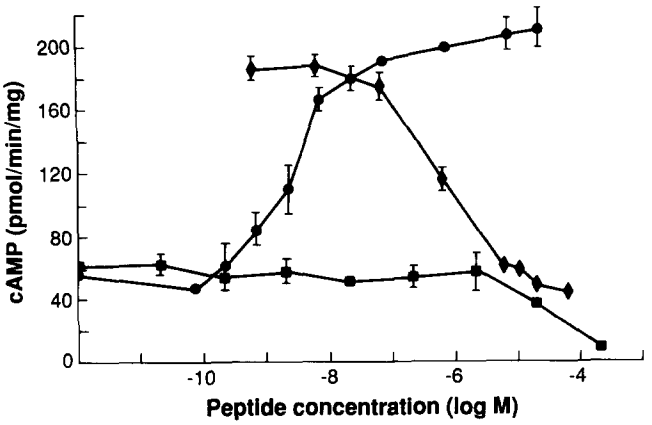

FIG. 1. Adenylate cyclase activation. Production of cAMP in rat liver membranes by natural glucagon ● and des-His[1][Glu[9]]glucagon amide ■ tested up to 2.09×10^{-4} M, and inhibition of 2.78×10^{-8} M glucagon by increasing amounts of antagonist ◆. From the inhibition curve the inhibition index and pA_2 values could be calculated.

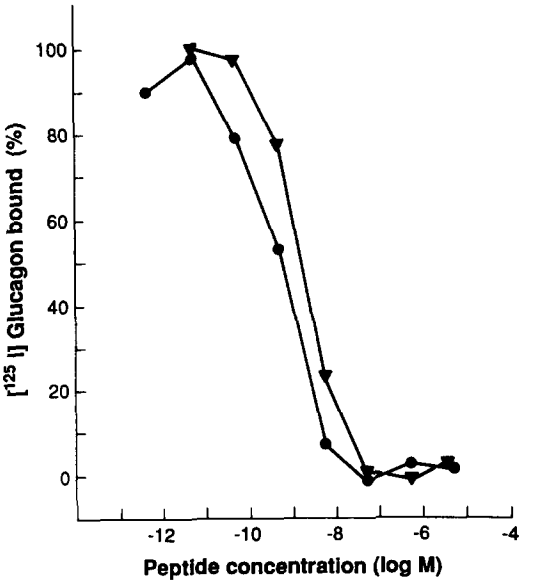

FIG. 2. Binding assay. Displacement of [^{125}I] glucagon from rat liver membranes by natural glucagon ● and synthetic antagonist, des-His[1][Glu[9]]glucagon amide ▼.

emulsion was centrifuged to generate two phases. The water layer was pipetted off and the organic phase washed once with 1 ml water. The radioactivity in the total inositol phosphates was determined by batch ion-exchange chromatography (2) of the combined aqueous layers on Bio-Rad AG 1-X8 formate resin. The percent increase in inositol phosphates above basal, expressed in cpm, stimulated by des-His[1][Glu[9]]glucagon amide was compared to that increase produced by the same concentration of natural glucagon.

RESULTS

In the cAMP assay on isolated rat liver membranes, des-His[1][Glu[9]]glucagon amide did not activate membrane-bound adenylate cyclase up to a concentration of 2×10^{-4} molar (Fig. 1). This is 10^6 times the amount of glucagon required to give a measurable response, indicating the analog to be <0.0001% active. The standard error of the mean in these assays is approximately ±7%. However, the analog bound 40% as well as natural glucagon in the hepatocyte membrane receptor binding assay (Fig. 2). Thus, the analog was a pure antagonist. In a competitive cAMP assay vs. natural glucagon, complete inhibition of the glucagon response was achieved, and an inhibition index of 14 and a pA_2 value of 7.25 were calculated. These results on the new synthetic preparation of des-His[1][Glu[9]]glucagon amide are very similar to the values 12 and 7.2 found previously (22), and indicate good reproducibility in these assays.

A number of studies have shown that some glucagon analogs that exhibit good binding affinity for the glucagon receptor but are devoid of adenylate cyclase activity are still capable of stimulating glycogenolysis in vivo (7,12) and therefore are not useful antagonists in animals. Since our ultimate goal is to find antagonists of the hormone that are of potential therapeutic use, it was necessary to test des-His[1][Glu[9]]glucagon amide in in vivo assays. Figure 3 shows that in rabbits, 4 μg glucagon per kg, IV, caused a 50% increase in blood glucose in 10 to 20 min (standard error of the mean from four similar experiments was ±9%), whereas the analog gave only a marginal 10% rise in glucose levels at 5 min and returned to baseline by 10 min when the same animals were injected with up to 400 μg of analog/kg, indicating a relative activity of less than 0.2%. ANOVA of the glucose values with time as covariate showed significant differences for the response, $F(2,58) = 22.93$, $p<0.0001$. By Duncan's multiple range test, significant differences between treatment with natural glucagon and antagonist at a 100-fold higher concentration were found at all time points between 5 and 20 min, $p<0.05$. This assay on natural or crystalline synthetic glucagon has been carried out many times. The maximum response is always at 15±5 min and it is proportional to concentration over the range 1–5 μg/kg (17). The analog has only been tested on a few animals, but very little response, even at high concentrations, has been found during the first 20 minutes following injection. A small gradual increase in blood sugar, as shown in Fig. 3, was observed with the analog between 10 and 60 min, but a similar rise was also seen with the buffer blank. By ANOVA, no significant differences in glucose levels were detected between treatment with saline and 400 μg/kg

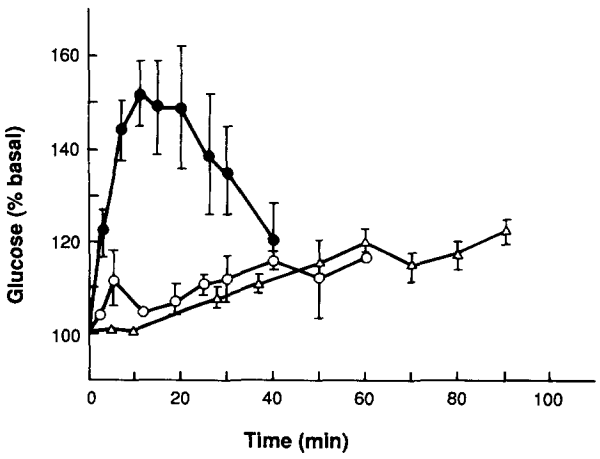

FIG. 3. Blood glucose concentration in fasted rabbits following IV injection of 4 μg/kg natural glucagon ●, 400 μg/kg of des-His[1][Glu[9]] glucagon amide ○, and saline control △.

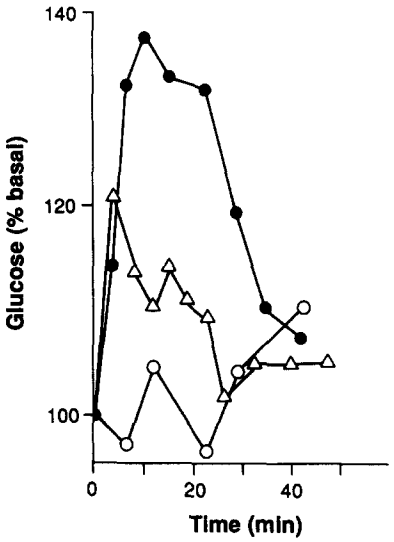

FIG. 4. Inhibition of glucagon by the synthetic analog. Increase in blood glucose of rabbits by 4.5 μg/kg natural glucagon ●, by a (1:50) mixture △, or a (1:100) mixture ○ of glucagon and des-His[1][Glu[9]]glucagon amide.

antagonist, even at the 5-min time point. The apparent rise with time may be of significance but may be due simply to repeated handling of the animals.

In competitive experiments it was found that the hyperglycemic response of glucagon in rabbits was suppressed by simultaneous IV injection of des-His[1][Glu[9]]glucagon amide. At a ratio of 50:1 the response to glucagon was reduced to 58%, giving an inhibition index of approximately 60. At a ratio of 100:1 the hyperglycemic response to glucagon was almost fully suppressed. Figure 4 shows the curves of experiments with a single animal. When repeated with other animals similar results were obtained. ANOVA for glucose levels with time as covariate showed significant differences between responses to glucagon and glucagon plus inhibitor, $F(2,79)=25.18$, $p<0.0001$. Duncan's test revealed significant differences in glucose levels between glucagon and glucagon plus a 100-fold excess of des-His[1][Glu[9]]glucagon amide at time points between 5 min and 20 min ($p \leq 0.05$).

Even more encouraging was the rapid decrease in blood glucose when streptozotocin diabetic rats were treated intravenously with des-His[1][Glu[9]]glucagon amide. Within five minutes after the single administration of 1 mg/kg of a saline solution of the inhibitor, the hyperglycemic effect was reduced by 70 percent (Fig. 5). The glucose level returned to baseline in about twenty minutes, but decreased again in a cyclical manner during the next 40 min. When the initial bolus of the glucagon antagonist was followed immediately by a continuous infusion of the antagonist, at a dose of 33 μg/kg/min, glucose lowering was sustained at a level approximately 60% below baseline for at least 90 minutes without returning to the hyperglycemic baseline, indicating a continuous inhibition of endogenous glucagon. The average data for two such experiments are shown in Fig. 6. The mean hyperglycemic level of glucose before treatment with the antagonist was 386 with a standard deviation of ±68. The mean glucose value after the initial bolus followed by continuous infusion was 214 with a standard deviation of ±47. This difference was found to be statistically significant, $p<0.0001$. Clearly, much more work is required to arrive at appropriate dose levels, and to explore the mechanism of the response.

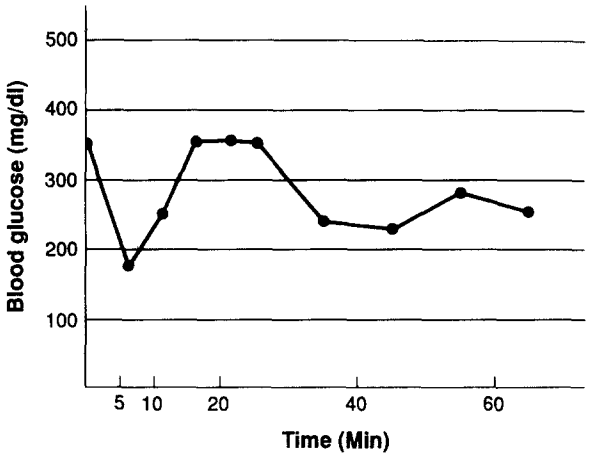

FIG. 5. Effect of an IV 1 mg/kg bolus of the antagonist des-His[1][Glu[9]]glucagon amide on the blood glucose of streptozotocin diabetic rats. Normal blood glucose in untreated rats was 98 ± 12 mg/dl.

In a series of perifusion experiments on mouse pancreatic islets recently carried out by Kofod *et al.* (15) it was shown that 3×10^{-7} M des-His[1][Glu[9]]glucagon amide, in the presence of 10 mM D-glucose, potentiated the release of about 10% as much insulin as did an equal amount of natural glucagon. The response occurred within one minute and the insulin concentration was maintained at a nearly constant level (± 14%) for the full 30-min test period. Removal of the peptide resulted in an immediate return to the level of insulin released by D-glucose alone.

While des-His[1][Glu[9]]glucagon amide did not activate adenylate cyclase, it did trigger the breakdown of inositol phospholipids by phospholipase C in hepatocytes. The isolated viable liver cells were first incubated with tritiated *myo*-inositol to produce labeled phosphoinositides. Phosphatidylinositol 4,5 bisphosphate constitutes only 1% of the total liver inositol lipid pool (16) and it is possible that it is not the only inositol phospholipid that undergoes phospholipase C hydrolysis when receptors are stimulated. Some hydrolysis of phosphatidylinositol 4-phosphate, though most likely not of phosphatidylinositol, may also occur (8,13). The cells were then incubated in the presence of glucagon or analog. The hormonal signal at the receptor was transduced, via a membrane G-protein, and phospholipase C was activated (5). This enzyme

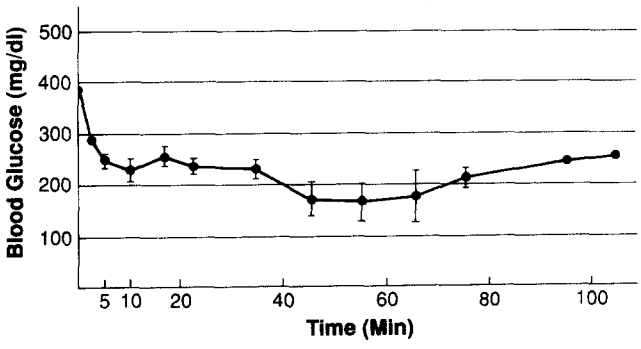

FIG. 6. Effect of an initial IV bolus of 1 mg/kg antagonist des-His[1][Glu[9]]glucagon amide followed immediately by continuous infusion of 33 μg/kg/min for 60 min on the blood glucose of streptozotocin diabetic rats.

TABLE 1

UPTAKE AND RELEASE OF [^{3}H]INOSITOL IN RAT HEPATOCYTES

Component	Distribution of [^{3}H] (Total cpm)		
Labeling of 10^6 cells [2-^{3}H]*myo*-inositol	3,300,000		
Uptake by cells	36,020		
After incubation	Blank	Glucagon (0.97×10^{-9} M)	Des-His^1[Glu^9] Glucagon Amide (1.09×10^{-9} M)
Lipid fraction	5,898	6,101	6,159
Aqueous fraction	9,613	10,891	10,301
Inositol phosphates	312	388	338

catalyzed the cleavage of the phosphatidylinositol phosphates into diacylglycerol and inositol 1,4,5 trisphosphate (3). Since the latter rapidly equilibrates with other isomeric derivatives, the entire mixture of inositol phosphates was isolated chromatographically and counted for total [^{3}H]inositol. The radioactivity data from one of these experiments are summarized in Table 1. The results are expressed as the percentage increase in radioactivity in inositol phosphates above that of the basal value. The increase in radioactivity in total inositol phosphates released upon incubation of hepatocytes with des-His^1[Glu^9]glucagon amide amounted to 8.3% above the control value at a concentration of 1.09×10^{-9} M, while a similar concentration of natural glucagon, 0.97×10^{-9} M, achieved a 24.4% stimulation of inositol phosphate production over basal, the ratio of the two responses being 0.34. Although these values depend on a small difference in cpm against a large endogenous background of labeled lipids, they were quite reproducible.

The dependence on hormone concentration was also estimated. For this purpose data were pooled from experiments using four different cell preparations (Fig. 7). The responses to glucagon or to des-His^1[Glu^9]glucagon amide varied in an approximately parallel way and we conclude that both peptides cause an increase in the production of inositol phosphates in intact hepatocytes. The potency, which is the amount of glucagon relative to analog required to produce a given response, was approximately 12%. The observations that des-His^1[Glu^9]glucagon amide can elicit a phosphatidylinositol response but not activate adenylate cyclase confirm the idea that glucagon exerts its action on the liver via two receptor systems.

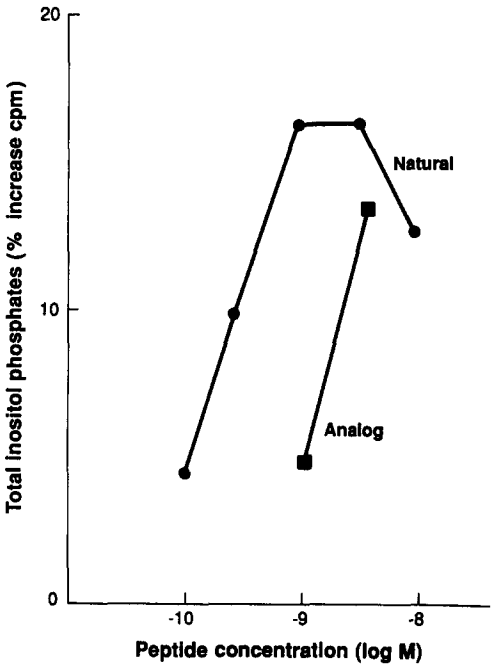

FIG. 7. Dose-response curve of the effect of natural glucagon ● and des-His^1[Glu^9]glucagon amide ■ on the breakdown of inositol phospholipids in isolated rat hepatocytes.

DISCUSSION

des-His^1[Glu^9]glucagon amide is a potent inhibitor of the effects of glucagon on liver membrane adenylate cyclase (22). It remains an intriguing observation that a conservative replacement of an aspartic acid at position 9 in the natural sequence by a glutamic acid residue resulted in an analog that exhibited low cyclase activity but substantial membrane binding and that additional removal of histidine-1 and conversion of the C-terminus to an amide gave an analog that exhibited no adenylate cyclase activity and yet retained 40% of the affinity for the glucagon receptor. Indeed, other recent sequence modifications in combination with des-His^1[Glu^9]glucagon amide have also resulted in pure inhibitors of the hormone (23), suggesting that the transduction properties are centered around the Asp^9 residue. Thus, the analogs des-His^1[Glu^9,Lys^{29}] and des-His^1[Glu^9,Orn^{12}]glucagon amides are also antagonists of the hormone with inhibition indices of 14 and 10, respectively, in in vitro cAMP assays (23).

To further explore the potential of des-His^1[Glu^9]glucagon amide we have tested the biological activities of this antagonist in other in vitro assays and in in vivo assays. des-His^1[Glu^9]glucagon amide did not stimulate appreciable glycogenolysis in fasted rabbits during the normal 20-min assay period (17) at doses of up to 400 μg/kg body weight, a concentration more than 400 times the concentration of glucagon required for a measurable effect. However, when the analog was administered intravenously in a mixture with the natural hormone at a ratio of 100:1 it was able to suppress almost completely the elevation of blood glucose caused by metabolic effects of glucagon. At a ratio of 50:1 the antagonist was able to reduce glucagon-induced glycogenolysis to 58% of the amount of glucose produced when the rabbit was injected with glucagon alone. This corresponds to an inhibition index of approximately 60, which is only 5 times higher than that observed in the in vitro assay, under different conditions. The gradual rise of blood glucose observed both with the antagonist and the saline blank after 30 min or longer is not understood. It may be a real effect or simply a result of repeated handling of the animal. The fact that des-His^1[Glu^9]glucagon amide was able to strongly block glycogenolysis in vivo demonstrates that it is a potent glucagon antagonist and has properties not shared by the other inhibitors reported to be antagonists in vitro, because they were still capable of activating glycogenolysis and even augmenting the effects of natural glucagon in intact hepatocytes (6).

The dramatic lowering of blood glucose concentrations in streptozotocin-induced diabetic rats to 60–70% below the hyperglycemic baseline also demonstrates the inhibitory potency of des-His^1[Glu^9]glucagon amide and supports the hypothesis that part of the elevated blood glucose in diabetic animals is due to glucagon. The data suggest that the analog may be a potentially useful agent in combination with insulin in controlling some of the metabolic abnormalities that accompany diabetes mellitus. It seems reasonable that such an agent would reduce the level of insulin required to lower blood glucose to a normal level, but these

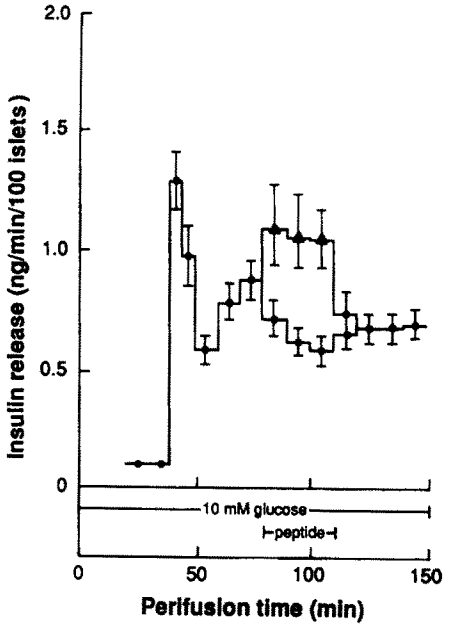

FIG. 8. Insulin release from pancreatic islets in response to 10 mM D-glucose ●, and 10 mM glucose with the addition of des-His[1][Glu[9]]glucagon amide ▲. Adapted from Kofod *et al.* (16).

experiments remain to be done.

A desirable feature of such a glucagon antagonist would be a lack of effect on the glucose-induced release of insulin by pancreatic islet cells. Fortunately, the des-His[1][Glu[9]]glucagon amide analog did not block the increase in insulin release caused by glucose. In addition, it actually promoted a further increase in insulin release to 65% above the 10 mM glucose baseline response (Fig. 8). In this regard the analog was about 10% as effective as glucagon itself (15). It is interesting to recall that a more secretin-like peptide [Asp[3],Glu[9],Arg[12]]glucagon maintained full insulin-releasing ability in islets (14) while retaining only 2% binding affinity to the glucagon receptor in liver membranes. The data on both peptide analogs suggest that the affinity and structural requirements of the hormone binding sites in liver differ from those in pancreatic islets.

It is known that glucagon can activate a second receptor (24) in liver membranes to generate two other second messengers, inositol trisphosphate and diacylglycerol. Wakelam *et al.* (24) have shown that the glucagon effect is small but real, and our data are in agreement with this finding. The conclusion that a second receptor is present was based on a semisynthetic analog of natural glucagon. The present data with totally synthetic des-His[1][Glu[9]]glucagon amide conform to the belief that there are two glucagon effector systems on the hepatocyte. The conclusion is based on the observations 1) that this analog binds well to the receptor that normally leads to adenylate cyclase activation by glucagon, but does not activate the cyclase and 2) the analog binds to a receptor that does lead to activation of phospholipase C and release of inositol phosphates. The simplest explanation is that there are two distinct glucagon receptors on the hepatocyte. Since this is a complex system, it is possible that there is only one binding site but two G proteins, and it is their activation by the hormone-receptor complex that is different and is responsible for the difference in the release of the two second messengers in response to the analog. It has been suggested that these two receptors trigger events that attenuate each other's cellular function (10). Thus, occupancy of receptors coupled to phospholipase C at glucagon concentrations of 10^{-10} M led to desensitization of glucagon-stimulated adenylate cyclase activity. At elevated glucagon concentrations ($\sim 10^{-9}$ M), the functioning of receptors coupled to adenylate cyclase predominates and led to G_s protein-mediated inhibition of glucagon-stimulated inositol phosphate production.

Thus, des-His[1][Glu[9]]glucagon amide, which has been shown to be a pure antagonist in vitro, is also a potent inhibitor of glucagon in vivo and maintains the ability to stimulate glucose-induced release of insulin in islets. It is therefore potentially useful in the study of the pathogenesis of diabetes. Since adenylate cyclase is insensitive to the peptide analog up to micromolar concentrations, while phospholipase C is activated at nanomolar levels, the antagonist should be very useful in the study of the cAMP independent physiological role of glucagon.

ACKNOWLEDGEMENTS

The technical assistance of Sandra Fuentes of the Laboratory Animal Resesarch Center in the in vivo experiments is gratefully acknowledged. We also thank Dr. Mary Jeanne Kreek, Dr. Henrik Albeck and Dr. Banvir Chaudhary of the Rockefeller University for the statistical analysis of our data. This work was supported by Public Health Service grant DK 24039.

REFERENCES

1. Andreu, D.; Merrifield, R. B. Glucagon antagonists: synthesis and inhibitory properties of Asp[3]-containing glucagon analogs. Eur. J. Biochem. 164:585–590; 1987.
2. Berridge, M. J.; Downes, C. P.; Hanley, M. R. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem. J. 206:587–595; 1992.
3. Berridge, M. J.; Irvine, R. F. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315–321; 1984.
4. Berry, M. N.; Friend, D. S. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J. Cell Biol. 43:506–520; 1969.
5. Cockcroft, S.; Gomperts, B. D. Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature 314:534–536; 1985.
6. Corvera, S.; Huerta-Bahena, J.; Pelton, J. T.; Hruby, V. J.; Trivedi, D.; Garcia-Sainz, J. A. Metabolic effects and cyclic AMP levels produced by glucagon, (1-N[α]-trinitrophenylhistidine, 12-homoarginine) glucagon and forskolin in isolated rat hepatocytes. Biochim. Biophys. Acta 804:434–441; 1984.
7. Cote, T. E.; Epand, R. M. An inhibitor of glucagon-stimulated cyclic AMP production and its effects on glycogenolysis. Biochim. Biophys. Acta 582:295–306; 1979.
8. Downes, C. P.; Wusteman, M. M. Breakdown of polyphosphoinositides and not phosphotidylinositol accounts for muscarinic agonist-stimulated inositol phospholipid metabolism in rat parotid glands. Biochem. J. 216:633–640; 1983.
9. Genrich, J. E.; Lorenzi, M.; Bier, D. M.; Schneider, V.; Tsalikian, E.; Karam, J. H.; Forsham, P. H. Prevention of human diabetic ketoacidosis by somatostatin: evidence for an essential role of glucagon. N. Engl. J. Med. 292:985–989; 1975.
10. Houslay, M. D.; Anderson, N.; Murphy, G. J.; Pyne, N. J.; Wakelam, M. J. O.; Wilson, A. Glucagon desensitization identifies "cross-talk" between two distinct receptor-signaling pathways. In: Current communications in molecular biology. Cold Spring Harbor: Cold Spring Harbor Laboratory; 1987:99–105.
11. Johnson, D. G.; Goebel, C. U.; Hruby, V. J.; Bregman, M. D.; Trivedi, D. Hyperglycemia of diabetic rats decreased by a glucagon receptor antagonist. Science 215:1115–1116; 1982.
12. Khan, B. A.; Bregman, M. D.; Nugent, C. A.; Hruby, V. J.; Brendel, K. (Des-histidine[1])(N[ε]-phenylthiocarbamoyllysine[12])-glucagon: effects on glycogenolysis in perfused rat liver. Biochem. Biophys. Res. Commun. 93:729–736; 1980.
13. Kirk, C. J.; Creba, J. A.; Downes, C. P.; Michell, R. H. Hormone-stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. Biochem. Soc. Trans. 7:377–379; 1981.
14. Kofod, H.; Andreu, D.; Thams, P.; Merrifield, R. B.; Hedeskov, J.;

Hansen, B.; Lernmark, A. Insulin release by glucagon and secretin: studies with secretin-glucagon hybrids. Endocrinol. Metab. 17:E454–E458; 1988.
15. Kofod, H.; Unson, C. G.; Merrifield, R. B. Potentiation of glucose-induced insulin release in islets by des-His[1][Glu[9]]glucagon amide. Int. J. Pept. Protein Res. 32:436–440; 1988.
16. Michell, R. H.; Hawthorne, J. N.; Coleman, R.; Karnovsky, M. L. Extraction of polyphosphoinositides with neutral and acidified solvents: a comparison of guinea-pig brain and liver, and measurements of rat liver inositol compounds which are resistant to extraction. Biochim. Biophys. Acta 210:86–91; 1970.
17. Mojsov, S.; Merrifield, R. B. Solid-phase synthesis of crystalline glucagon. Biochemistry 20:2950–2956; 1981.
18. Pohl, S. L. The glucagon receptor in plasma membranes prepared from rat liver. In: Blecher, M., ed. Methods in receptor research. New York: Marcel Dekker; 1976:160–164.
19. Salomon, Y.; Londos, C.; Rodbell, M. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58:541–548; 1974.
20. Unger, R. H. Diabetes and the alpha cell. Diabetes 25:136–151; 1976.
21. Unger, R. H.; Orci, L. The essential role of glucagon in the pathogenesis of diabetes mellitus. Lancet 1:14–16; 1975.
22. Unson, C.G.; Andreu, D.; Gurzenda, E. M.; Merrifield, R. B. Synthetic peptide antagonists of glucagon. Proc. Natl. Acad. Sci. USA 84:4083–4087; 1987.
23. Unson, C. G.; Gurzenda, E. M.; Iwasa, K.; Merrifield, R. B. Glucagon antagonists: contribution to binding and activity of the amino-terminal sequence 1–5, position 12, and the putative α-helical segment 19–27. J. Biol. Chem. 264:789–794; 1989.
24. Wakelam, M. J. O.; Murphy, G. J.; Hruby, V. J.; Houslay, M. D. Activation of two signal-transduction systems in hepatocytes by glucagon. Nature 323:68–71; 1986.
25. Wright, D. E.; Rodbell, M. $Glucagon_{1-6}$ binds to the glucagon receptor and activates hepatic adenylate cyclase. J. Biol. Chem. 254:268–269; 1979.