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# (DES-HISTIDINE[1])($N^{\varepsilon}$-PHENYLTHIOCARBAMOYLLYSINE[12])-GLUCAGON: EFFECTS ON GLYCOGENOLYSIS IN PERFUSED RAT LIVER

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SUMMARY: (Des-Histidine[1])($N^{\varepsilon}$-phenylthiocarbamoyllysine[12])-glucagon, synthesized by the one-step Edman degradation procedure is a competitive inhibitor of glucagon action in the rat liver plasma membrane adenylate cyclase system. However, in the perfused rat liver, the compound did not inhibit glucagon stimulated glycogenolysis even when used at a concentration 100-fold in excess of native glucagon. Instead, it showed a weak potency, but full agonist activity, stimulating liver glycogenolysis to 100% of the level obtained by glucagon. These results are discussed in terms of the possible mechanism(s) of glucagon action.

The controversy generated by the proposal (1) that glucagon mediates certain metabolic aberrations of diabetes mellitus which were previously attributed solely to insulin deficiency, has stimulated further research into the nature of the glucagon receptor, structure-function studies of glucagon, and the search for antagonists of the native hormone. Several laboratories have examined various approaches for preparing purified structural analogs of glucagon (2-16), and have examined the effects of these structural modifications of glucagon on its biological activity both in vitro and in vivo with the goal of finding those residues primarily important for binding to the glucagon receptor (binding message) on cell membranes and those essential for the biological activity (biological message). Knowledge of these factors and proper assessment of their importance should permit the design of a glucagon analog which can bind to glucagon receptors, display no intrinsic activity,

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but compete for native hormone binding at the receptor site (an antagonist to the hormone).

Some time ago we demonstrated that highly purified [des-histidine$^{1}$]-glucagon ([DH$^{1}$]-glucagon) was a partial agonist in the adenylate cyclase assay (8). More recently we showed that (des-histidine$^{1}$)(N$^{\varepsilon}$-phenylthiocarbamoy lysine$^{12}$)-glucagon ([DH$^{1}$][N$^{\varepsilon}$-PTC]-glucagon) had essentially no effect on liver plasma membrane adenylate cyclase activity and showed competitive inhibitory action on glucagon stimulated activity in the same assay system (11). These findings have prompted us to extend these studies to examine the effect of [DH$^{1}$][N$^{\varepsilon}$-PTC]-glucagon on glycogenolysis in the perfused rat liver in the presence and absence of glucagon. In this communication we report the results of these studies.

## MATERIALS AND METHODS

Materials: Male Sprague-Dawley rats, 150-200g, were maintained on a standard Purina rat chow diet at 22°C in a temperature-controlled room on a cycle of 12 hrs of light and 12 hrs of darkness. Bovine serum albumin, fraction V, was obtained from Calbiochem and crystalline glucagon was obtained from Elanco Company or Sigma Chemical Company. $MgSO_4 \cdot 7H_2O$, $KH_2PO_4$, $CaCl_2 \cdot 2H_2O$, and NaCl were purchased from Grand Island Biological Co. KCl and $NaHCO_3$ were obtained from Mallinckrodt. (des-His$^{1}$)(N$^{\varepsilon}$-phenylthiocarbamoyl-lysine$^{12}$)-glucagon was synthesized and purified as previously reported (11), and used shortly after purification for biological studies. Glucagon concentrations were determined spectrophotometrically using a Gilford 240 spectrophotometer.

Liver plasma membrane adenylate cyclase assay: Plasma membranes were prepared in the usual manner (17) and the adenylate cyclase assay was run as previously reported (11). Cyclic AMP was determined by the method of Saloman, et al. (18).

Glycogenolysis assay: For the glycogenolysis studies, the rat liver was perfused with modified Krebs-Ringer-bicarbonate buffer (100 ml) containing NaCl, 131 mM; KCl, 4.7 mM; $NaHCO_3$, 25 mM; $KH_2PO_4$, 2.3 mM; $MgSO_4 \cdot 7H_2O$, 2.3 mM; and $CaCl_2 \cdot 2H_2O$, 1.64 mM; glucose, 50 µg/ml; and bovine serum albumin, 2 mg/ml. The perfusion volume was 100 ml recirculating buffer, and all experiments were performed at 37°C. The rats were anesthetized with ether. The abdominal cavity was opened, the portal vein was isolated, and the canula of the perfusion apparatus (grooved 16 gauge SS needle) inserted and tied to the portal vein at the level of the lineal branch. The perfusion was started at 5 mL/min, and the liver transferred from the abdominal cavity to the perfusion apparatus. After the transfer, the perfusion rate was adjusted to a flow rate of 0.10 mL/g/min. The entire procedure took about three min. The perfusion apparatus was filled with perfusion buffer prior to the liver isolation and equilibrated with 95% $O_2$, 5% $CO_2$ at 37°C. About 100 ml of

perfusate was used to clear the liver of red cells. Fresh perfusate (100 ml) was then recirculated through the liver. Since earlier studies showed that the glycogenolytic (19) and gluconeogenic (20) responses of perfused liver to glucagon are exaggerated when basal activity has subsided to low levels following a period of recirculation of perfusate, all experiments described here have been carried out according to the following protocol. Livers were perfused initially for 30 min with recirculating media containing no hormones in order to establish steady basal metabolic rates. Samples of 100 µL were withdrawn at 5, 20, and 30 min intervals. Immediately after the 30 min sample, the hormone, its analog, or a mixture of the two compounds were added depending on the experimental protocol. Further samples were then taken at 40, 50, and 60 min. The circulation of perfusate was stopped at 60 min and the wet weight of the liver was measured. Glucose was determined with a Beckman Glucose Analyzer using the glucose oxidase reaction. The results of an assay using glucagon and following these procedures as shown in Figure 1.

## RESULTS

As previously reported (11), [$DH^1$][$N^{\epsilon}$-PTC]-glucagon had substantially no effect on liver plasma membrane adenylate cyclase activity. In the presence

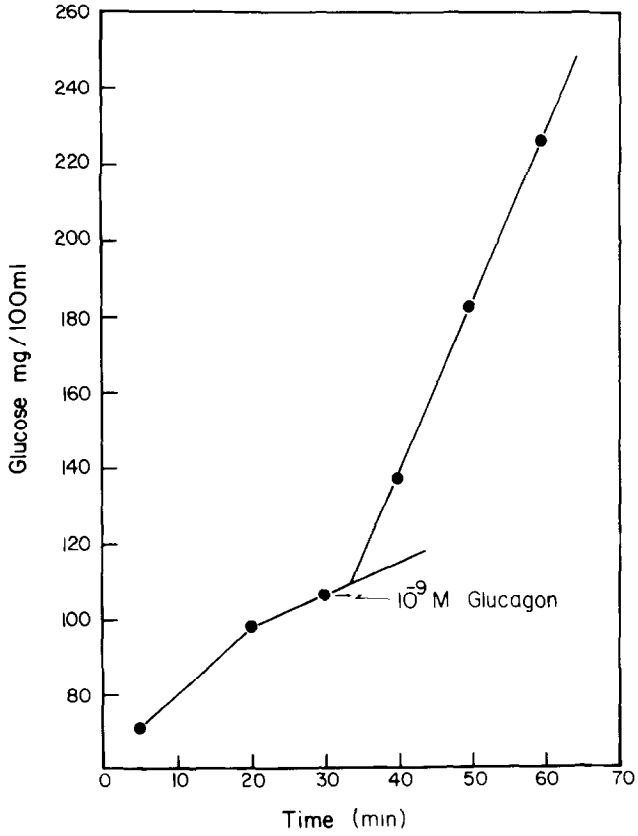


Fig. 1. Glycogenolysis in the isolated recirculating perfused rat liver plotting glucose accumulation versus time in minutes. Perfusion conditions are described in Materials and Methods.

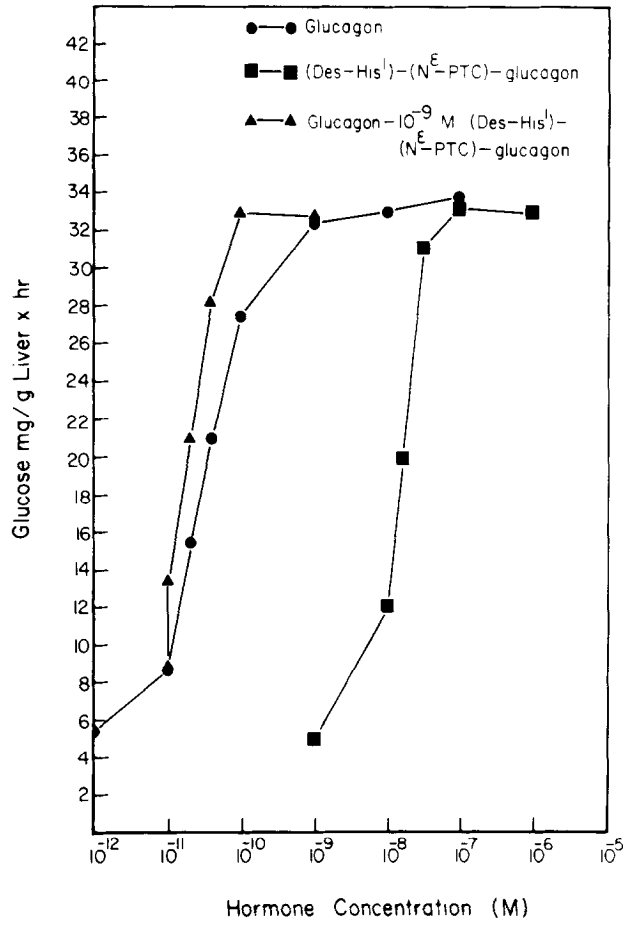


Fig. 2. Dose-response curves for glucagon, (des-His$^1$)(N$^\epsilon$-PTC)-glucagon, and a mixture of glucagon and a constant concentration of (des-His$^1$)(N$^\epsilon$-PTC)-glucagon ($10^{-9}$ M) plotting rates of glucose release by the isolated recirculatory perfused liver versus hormone concentration. Experimental conditions are described in Materials and Methods.

of [DH$^1$][N$^\epsilon$-PTC]-glucagon (1.5 μM), approximately a 25-fold increase in the glucagon was required for 50% activation of adenylate cyclase by the hormone. This analog, therefore, is a fairly potent antagonist to glucagon activity in this assay system, possessing about 1/18th the affinity of glucagon for the receptor site. On the other hand, we find that [DH$^1$][N$^\epsilon$-PTC]-glucagon has a weak but definite ability to stimulate liver glycogenolysis in the perfused liver system (Figure 2). The potency of the analogue was approximately 1/350th that of glucagon, but it was able to stimulate glycogenolysis to the same level as that obtained by glucagon. In addition, [DH$^1$[N$^\epsilon$-PTC]-glucagon

did not have any measurable inhibitory effect on glucagon stimulated glycogenolysis even when it was used in a concentration 100-fold higher than that of glucagon. In contrast, the analog in combination with glucagon appeared to slightly augment glycogenolysis.

## DISCUSSION

Although cyclic AMP (cAMP) mediates hormone action in various tissues, several authors recently have shown that metabolic rates can be altered by cAMP-dependent hormones without detectable changes in cAMP levels in some tissue preparations. For example, gluconeogenesis or glycogenolysis is activated by epinephrine even when the activation of adenylate cyclase by the catecholamine is antagonized by adrenolytic agents in isolated hepatocytes (21), kidney tubules, and perfused liver (19, 22). Addition of cAMP to these preparations results in an activation of metabolic changes. Also, glucagon and epinephrine stimulate glycogenolysis in perfused liver (23) and skeletal muscle (24), respectively at concentrations lower than those required to produce a measurable elevation of tissue cAMP. A similar dissociation of tissue cAMP and metabolic alterations has been noted for isolated fat (25, 26) and adrenal (27) cells. In related studies (28) it has been found that 2',5'-dideoxyadenosine, while producing a significant inhibition of cAMP accumulation due to hormones, did not block hormone-induced increases in hepatic glucose production. Birnbaum and Fain (29) have noted that in contrast to the activation of protein kinase in isolated rat liver cells, the increase in glycogen phosphorylase activity does not always correlate well with changes in cAMP. Thus, there appears to be two distinct conditions under which there is no apparent correlation of cAMP levels and phosphorylase activity: treatment of hepatocytes with low concentrations of glucagon, and stimulation by $\alpha$-adrenergic agents.

In whole animal studies, Okajima, et al. (30), reported that the infusion of glucagon at the rate of 5 ng or more per min per 100g body weight caused an

increase in tissue cAMP in association with rapid breakdown of glucagon. However, at lower rates (0.5 to 1.0 ng per min per 100g body weight) glucagon produced significant glycogenolysis without a detectable increase in tissue cAMP levels. Similar relations were observed with epinephrine infusion. These results might be explained by postulating that measurable increases in cAMP levels following exposure of tissue to the hormone is physiologically unnecessary (26), or equally likely, that small changes in cAMP levels went undetected in these studies. A recent investigation into the mechanism of adrenocorticotropin action on isolated adrenal cells, which used a highly sensitive assay for cAMP, showed that at physiological concentrations the hormone effected a previously undetected increase in cAMP which resulted in a significant occupation of protein kinase receptors (31). The dissociation of the effects of glucagon on adenylate cyclase (32) and glycogenolysis (Fig. 2) may be explained by the presence of highly sensitive cAMP receptor which amplifies the cAMP signal at low hormone concentrations.

In view of the above findings, the disagreement in regards to the inhibitory effect of [$DH^1$][$N^{\epsilon}$-PTC]-glucagon on glucagon activation of plasma membrane adenylate cyclase, and its lack of agonist activity in this system, and the lack of inhibitory effect and weak but significant agonist activity in the perfused liver study reported here may not be completely surprising. It may be that the two systems differ substantially in the activity or sensitivity of the hormone receptors involved, or that cAMP independent-processes play a role under the more nearly physiological conditions of the perfused liver system. In this regard, Cote and Epand (7) have recently reported that $N^{\alpha}$-trinitrophenyl glucagon is inactive and can inhibit glucagon-induced stimulation of adenylate cyclase. However, this derivative was capable of activating glycogenolysis in isolated hepatocytes, though the concentrations required for this activation were considerably higher than those required here. These authors suggested that glucagon may also be able to activate glycogenolysis by a cAMP-independent process. However, several

other explanations may be offered. First it is possible that enzymes present in the whole tissue or whole cell preparations are capable of cleaving the protecting groups ($N^{\varepsilon}$-PTC in the case of our analog, and $N^{\alpha}$-trinitrophenyl in the case of the analog of Cote and Epand) to give a small amount of glucagon or [$DH^{1}$]-glucagon, both of which have agonist activity. Also the possibility cannot be completely excluded that a small amount of some minor contaminant is present which has a low biological activity and only manifest itself in whole cell preparations. We have been careful to purify our analogue just before use to minimize this problem. In any case the possibility that a glucagon analog with inhibitory activity but no agonist activity in vitro may be able to stimulate glycogenolysis by a cAMP-independent process requires further study. This work also suggests that a more potent glucagon inhibitor should be developed to determine whether glucagon-induced glycogenolysis can be completely blocked in vivo.

Acknowledgments

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