Receptor Binding and Adenylate Cyclase Activities of Glucagon Analogues Modified in the N-Terminal Region†

Roberta L. McKee,‡ John T. Pelton,§ Dev Trivedi,§ David G. Johnson,∥ David H. Coy,⊥ Javier Sueiras-Diaz,⊥ and Victor J. Hruby*,‡,§

Departments of Biochemistry, Chemistry, and Internal Medicine, University of Arizona, Tucson, Arizona 85721, and Section of Endocrinology and Metabolism, Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana 70112

Received September 16, 1985

ABSTRACT: In this study, we determined the ability of four N-terminally modified derivatives of glucagon, [3-Me-His[1],Arg[12]]-, [Phe[1],Arg[12]]-, [D-Ala[4],Arg[12]]-, and [D-Phe[4]]glucagon, to compete with ^{125}I-glucagon for binding sites specific for glucagon in hepatic plasma membranes and to activate the hepatic adenylate cyclase system, the second step involved in producing many of the physiological effects of glucagon. Relative to the native hormone, [3-Me-His[1],Arg[12]]glucagon binds approximately twofold greater to hepatic plasma membranes but is fivefold less potent in the adenylate cyclase assay. [Phe[1],Arg[12]]glucagon binds threefold weaker and is also approximately fivefold less potent in adenylate cyclase activity. In addition, both analogues are partial agonists with respect to adenylate cyclase. These results support the critical role of the N-terminal histidine residue in eliciting maximal transduction of the hormonal message. [D-Ala[4],Arg[12]]glucagon and [D-Phe[4]]glucagon, analogues designed to examine the possible importance of a β-bend conformation in the N-terminal region of glucagon for binding and biological activities, have binding potencies relative to glucagon of 31% and 69%, respectively. [D-Ala[4],Arg[12]]glucagon is a partial agonist in the adenylate cyclase assay system having a fourfold reduction in potency, while the [D-Phe[4]] derivative is a full agonist essentially equipotent with the native hormone. These results do not necessarily support the role of an N-terminal β-bend in glucagon receptor recognition. With respect to in vivo glycogenolysis activities, all of the analogues have previously been reported to be full agonists. The partial agonism of [3-Me-His[1],Arg[12]]-, [Phe[1],Arg[12]]-, and [D-Ala[4],Arg[12]]glucagon for adenylate cyclase activity in isolated liver plasma membranes observed in this study is not modulated by changes in the guanosine triphosphate (GTP) concentration. In addition, the receptor binding dose–response curve for [Phe[1],Arg[12]]glucagon is shifted to the right in the presence of GTP to the same extent as that seen with the native hormone. Thus, the partial agonism demonstrated by these three analogues in this study is not due to a lack of modulation by GTP of the receptor binding and adenylate cyclase activities measured on liver plasma membranes. The in vivo degradation rates for glucagon and [D-Phe[4]]glucagon, half-lives of 5.3 and 7.5 min, respectively, were determined in this study. This slightly slower rate of degradation for [D-Phe[4]]glucagon is not sufficient to account for its highly potent glycogenolytic activity seen in vivo. The lack of correlation between the in vitro adenylate cyclase and the in vivo glucose release activities for these compounds is discussed.

The hormone glucagon, a peptide of 29 amino acids, is best known for its key role in maintaining glucose homeostasis in animals. Secreted by the α cells of the pancreas, glucagon exerts its major effects on hepatocytes by stimulating glucose production and release during the hypoglycemic state. Aside from its action at the liver, glucagon stimulates lipolysis in fat cells (Harris et al., 1979; Witters et al., 1979) and has been reported to exert effects on heart and kidney tissue (Glick et al., 1968; Bailly et al., 1980). In addition, it has been suggested that glucagon may be involved in the pathogenesis of diabetes mellitus (Unger, 1978).

The main target of glucagon action is the liver, where it simulates the processes of glycogenolysis and gluconeogenesis. These events are considered to be mediated by a cAMP[1]-dependent mechanism in which binding of the hormone to its membrane-bound receptor activates the enzyme adenylate cyclase, initiating the production of cAMP. It is this increase in the concentration of cAMP that results in the activation of glycogenolytic and gluconeogenic enzymes and consequently a rise in glucose output by the liver.

The precise nature of the interaction between glucagon and its hepatic receptor, and the mechanism(s) by which this leads to glycogenolysis and gluconeogenesis, remains unclear. However, structure–activity studies employing glucagon fragments and analogues have been utilized in an attempt to determine those properties of the molecule that are important for receptor recognition and binding and those that are necessary for transduction of the biological response. From the measurements of the binding and biological activities of nu-

† This research was supported by grants from the U.S. Public Health Service (AM 21085 and AM 30167) and Merck Sharp and Dohme. J.T.P. is the recipient of NIH Postdoctoral Fellowship AM 06936.

‡ Department of Biochemistry, University of Arizona.
§ Department of Chemistry, University of Arizona.
∥ Department of Internal Medicine, University of Arizona.
⊥ Tulane University School of Medicine.

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; PTC, phenylthiocarbamoyl; TNP, trinitrophenyl; CAR, carbamoyl; DEAE, diethylaminoethyl; LHRH, luteinizing hormone-releasing hormone; HPLC, high-performance liquid chromatography. Other abbreviations are those recommended by IUPAC–IUB.

0006-2960/86/0425-1650$01.50/0

merous compounds, it appears that essentially the entire molecule is necessary for receptor recognition and activation of the adenylate cyclase system. Nevertheless, the C-terminal region of glucagon is primarily involved in binding to the receptor while the N-terminal region is critical for transducing the biological message (Hruby, 1982). For example, the partial agonist activities of des-His[1]-glucagon and [N^{α}-CAR-His[1]]glucagon demonstrate the importance of histidine-1 to the bioactivity of the native hormone (Lin et al., 1975; Bregman et al., 1980). Also, while the glucagon fragment glucagon(5–29) possesses considerable binding activity but essentially lacks activity when assayed for adenylate cyclase, receptor binding activity is almost completely lost when sequences of five or six amino acid residues are deleted from the C-terminal region as in des(16–21)- and des(22–26)-glucagon (Frandsen et al., 1981). This result suggests that the C-terminal region of glucagon is critical for binding to the hepatic glucagon receptor. In addition to these two conclusions, other studies have indicated that the positive charge at position 12 (Lys) and the structural relationship between this side chain and the N-terminal region also seem to be important for transduction of the hormonal message. For example, in analogues in which the positive charge of the Lys ϵ-amino group is neutralized, as in several N$^{\epsilon}$-acylated derivatives, the result is partial agonist analogues possessing diminished adenylate cyclase activity (Carrey & Epand, 1982). The weak potency and partial agonist activity of [N^{α}-CAR-His[1]]glucagon is lowered even further when this analogue is also carbamoylated at position 12, and the partial agonist des-His[1]-glucagon is converted to an antagonist when the PTC moiety is introduced at the Lys[12] ϵ-amino position (Bregman et al., 1980; Bregman & Hruby, 1979). Indeed, the most potent antagonist described to date, [N^{α}-TNP-His[1],HArg[12]]glucagon, possesses modifications at both positions 1 and 12, clearly demonstrating the significance of the relationship between these two moieties for transduction of the biological message.

In general, both receptor binding assays as well as biological activity assays are critical to determining the structure–activity relationships of glucagon. Several biological activity assays for this hormone are available. It is possible to measure an analogue's ability to activate adenylate cyclase in liver plasma membranes (Pohl et al., 1971), the second step involved in producing many of the physiological effects of glucagon. Alternatively, assays focusing on overall cellular events stimulated by glucagon such as glucose production by the liver or lipolysis by adipose tissue can be used.

Recently, in an effort to extend our understanding of the structure–activity relationships of the peptide hormone glucagon, a series of analogues with modifications in the N-terminal hexapeptide region were synthesized and tested for their effects on glucose levels in normal rats (Sueiras-Diaz et al., 1984). On the basis of reduced potencies but full activities of [3-Me-His[1],Arg[12]]- and [Phe[1],Arg[12]]glucagon relative to glucagon, it was concluded that neither the histidine residue nor its imidazole nitrogen atoms are essential for biological activity. Rather, since [Phe[1],Arg[12]]glucagon is nearly a full agonist, although less potent, in the in vivo glucose release bioassay, these results and previous studies (Hruby, 1982) suggest that the aromatic and steric properties of the position-1 residue may be critical to full agonist activity. Analogues designed to test the hypothesis that a β-bend occurs in the N-terminal region, like that seen in luteinizing hormone-releasing hormone (LHRH) and other glycine-containing peptides (Monahan et al., 1973), were synthesized by replacing the glycine residue at position 4 with D-amino acids (Sueiras-Diaz et al., 1984). [D-Ala[4],Arg[12]]glucagon was found to be fully active although somewhat less potent than the native hormone in stimulating in vivo glucose release. Replacement of glycine by D-phenylalanine resulted in an analogue that was found to be highly potent, stimulating glucose release some 6.6 times that of glucagon.

These conclusions concerning structure–activity relationships of glucagon are based on activities observed at the in vivo physiological level. In an effort to more precisely define these relationships, we have examined the binding properties and adenylate cyclase activities of four analogues of glucagon modified in the N-terminal region of the hormone: [3-Me-His[1],Arg[12]]-, [Phe[1],Arg[12]]-, [D-Ala[4],Arg[12]]-, and [D-Phe[4]]-glucagon. We have found that the pattern of biological activities for these analogues as measured at the level of the rat liver plasma membrane is quite different from that seen in the whole animal. The results of these studies, along with their implications for the structure–function relationships of glucagon, are presented.

Experimental Procedures

Materials

Crystalline porcine glucagon was purchased from Elanco (lot 7PE64B). Sephadex G-25M gel filtration resin and DEAE-Sephadex A-25 ion-exchange resin were obtained from Pharmacia Fine Chemicals, and Dowex AG50W-X4 cation-exchange resin was from Bio-Rad. Carrier-free Na^{125}I in 0.1 N NaOH and ACS scintillation fluid came from Amersham, [^{3}H]cAMP from New England Nuclear, and [α-^{32}P]ATP from ICN. Cellulose acetate filters (1.0 μm pore size) were purchased from Millipore Corp. Sodium metabisulfite was obtained from Mallinckrodt, and chloramine T, Tris-HCl (reagent grade), bovine serum albumin (fraction V), barbital, chromatographic alumina (type WN3, neutral), cAMP, ATP, GTP, EDTA, phosphocreatine, and creatine phosphokinase were from Sigma. Male Sprague-Dawley rats came from the University of Arizona College of Medicine, Division of Animal Resources. Wistar rats were obtained from Harlan Industries. All rats were fed ad libitum until used.

Methods

Isolation of Liver Plasma Membranes. Partially purified liver plasma membranes were prepared from male Sprague-Dawley rats weighing between 200 and 250 g according to the method of Neville (1968) with modifications as described by Pohl et al. (1971). The final membrane pellet was resuspended with 10 mL of 25 mM Tris-HCl buffer, pH 7.5 at 25 °C. Aliquots containing approximately 1–2 mg of protein, as determined by the Lowry method as modified for membrane proteins (Markwell et al., 1978), were stored in liquid nitrogen for use within 3 months.

Preparation of Radioiodinated Glucagon and [D-Phe[4]]-glucagon. Glucagon and [D-Phe[4]]glucagon were radioiodinated with Na^{125}I in the presence of chloramine T by a modification of the method of Hunter and Greenwood (1962) as previously described for glucagon by Rodbell et al. (1971a,b). All steps were performed at room temperature. Carrier-free Na^{125}I, 1.5 mCi in 0.1 N NaOH, was added to a small conical test tube containing 10 μL of a 10^{-4} M solution of peptide (1 nmol). At zero time, 10 μL of a chloramine T solution (3.5 mg/mL in 0.6 M sodium phosphate buffer, pH 7.4) was added followed by immediate mixing. After 10 s, the reaction was stopped by addition of 50 μL of a sodium metabisulfide solution (2.4 mg/mL in phosphate buffer) followed by dilution with 150 μL of 25 mM Tris-HCl, pH 7.5

Table I: Binding Affinities and Adenylate Cyclase Activities of N-Terminal Derivatives of Glucagon

peptide	receptor binding IC_{50} (nM)[a] (95% confidence limits)	adenylate cyclase EC_{50} (nM)[b] (95% confidence limits)	% maximal adenylate cyclase activity relative to glucagon	Hill number
glucagon	5.85[c] (5.11–6.65)	6.87[c] (5.89–8.06)	100	0.82
[3-Me-His^1,Arg^{12}]glucagon	2.75 (1.74–4.65)	30.6 (24.4–39.7)	85.3	0.98
[Phe^1,Arg^{12}]glucagon	19.7 (14.8–27.2)	34.9 (20.7–61.4)	47.6	0.72
[D-Ala^4,Arg^{12}]glucagon	18.8 (13.4–24.2)	28.7 (18.8–57.7)	81.1	0.77
[D-Phe^4]glucagon	9.06 (6.65–14.2)	7.96 (5.50–11.6)	100	0.85

[a] Determined from data in Figure 1. [b] Determined from data in Figure 2. [c] Determined from six separate assays.

at 25 °C, containing 1% BSA.

Labeled glucagon and [D-Phe^4]glucagon were purified by the procedure of Thorell and Johansson (1971). Briefly, the reaction mixture was immediately transferred to a Sephadex G-25 (medium) column and eluted with 0.075 M sodium barbital buffer, pH 8.6. The first peak of radioactivity was collected, aliquoted, and stored at −20 °C for use within 10 days.

Synthesis, Purification, and Storage of Peptides. Glucagon was purified on DEAE-Sephadex A-25 to remove deamidoglucagon following the procedure described by Bromer et al. (1972). [3-Me-His^1,Arg^{12}]glucagon, [Phe^1,Arg^{12}]glucagon, [D-Ala^4,Arg^{12}]glucagon, and [D-Phe^4]glucagon were synthesized and purified as previously described (Sueiras-Diaz et al., 1984). Purified glucagon and glucagon analogues were initially dissolved in 2 mM sodium carbonate to a concentration of 10^{-4} M determined by using the 278-nm molar absorptivity of 8310 M^{-1} cm^{-1} (Gratzer et al., 1968). Dilutions were made with 25 mM Tris-HCl, pH 7.5 at 25 °C, containing 1% BSA, to concentrations desired for adenylate cyclase and receptor binding assays. Aliquots of the diluted solutions were then lyophilized and stored at −20 °C.

Receptor Binding Assay. The binding of glucagon and glucagon analogues to liver plasma membranes was assayed by a method similar to that of Lin et al. (1975). The incubation medium had a final volume of 500 μL consisting of liver plasma membrane containing 50 μg of protein, 150 000 cpm of ^{125}I-glucagon, and unlabeled glucagon or glucagon analogues at concentrations ranging from 0 to 10 μM, all in 25 mM Tris-HCl, pH 7.5 at 25 °C, containing 1% BSA. When testing the effect of GTP on the binding of partial agonist analogues, the medium also contained GTP at a concentration of 100 μM. The mixture was incubated for 10 min at 30 °C followed by immediate cooling in an ice bath and then filtered through 1.0-μm cellulose acetate filters previously soaked for 12 h in the Tris–BSA buffer. Filters were washed 4 times with 1 mL of ice-cold 25 mM Tris-HCl, pH 7.5 at 25 °C, transferred to test tubes, and counted on an LKB 1275 Minigamma counter. Nonspecific binding, measured in the presence of excess unlabeled peptide (1024 nM), was typically 15%–20% of the total binding and was subtracted from the total to give specific binding. Results are expressed as the percent inhibition of ^{125}I-glucagon specific binding. Triplicate determinations were made in all binding experiments, and each experiment was carried out at least twice.

Adenylate Cyclase Assay. Adenylate cyclase activity was determined by the procedure of Lin et al. (1975) with the standard incubation medium containing the following in a final volume of 0.1 mL: 1 mM [α-^{32}P]ATP (about 50 cpm/pmol); 5 mM $MgCl_2$; 1 mM cAMP plus 10 000 cpm [^{3}H]cAMP; 10 μM GTP; 25 mM Tris-HCl, pH 7.5 at 25 °C; 1% BSA; 1 mM EDTA; 35 μg of liver plasma membrane protein; and an ATP regenerating system consisting of 20 mM phosphocreatine and 0.72 mg/mL (100 units/mL) creatine phosphokinase. For experiments testing the modulation by GTP of partial agonist analogues, the incubation medium was the same except for the concentration of GTP, which ranged from 0 to 100 μM. Labeled cAMP was determined by the method of Salomon et al. (1976) using Dowex 50 and alumina chromatography. Results are expressed as the percentage of stimulation of cAMP production above basal. Triplicate determinations of each point were obtained at each peptide concentration, and all experiments were carried out at least twice.

Degradation of Glucagon and [D-Phe^4]glucagon in Vivo. The methodology used in these experiments was adapted from a similar procedure described by Jaspan et al. (1981). Following administration of sodium pentobarbital anesthesia (65 mg/kg, ip), two catheters were inserted, one being placed into the femoral artery and another into the femoral vein of a male Wistar rat. At zero time, radioiodinated peptide was injected as a bolus into the femoral vein, and blood samples were taken from the femoral artery at 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, and 30 min. The plasma was separated within 10 min, and radioiodinated peptide was extracted from the plasma according to the method of Walter et al. (1974) by treatment with acetone. The extracts were then lyophilized and analyzed by HPLC. From the amount of intact peptide remaining at each time point, the half-lives of glucagon and [D-Phe^4]glucagon were calculated.

Data Analysis. Adenylate cyclase dose–response and liver plasma membrane receptor binding curves for glucagon and glucagon analogues were constructed by using the methods described by Bowman and Rand (1980) for the statistical analysis of the relationship between dose and response. Treatment of the data considers that the response to hormone is directly proportional to the logarithm of its concentration when between 20% and 80% of the maximal response. The regression lines of the linear region of the dose–response curves of glucagon and all analogues were calculated with the data from replicate experiments (two to six assays per peptide with triplicate determinations for each point). From these, the concentrations required for half-maximal response and their 95% confidence limits were calculated. In cases where the regression lines for an analogue and that for glucagon were shown to be parallel, a test for coincidence by using Student's *t* test was also performed.

RESULTS

Receptor Binding Studies of Glucagon and Glucagon Analogues. Glucagon has been shown to bind with high affinity to specific sites on isolated liver plasma membranes as determined by its ability to compete with the binding of ^{125}I-glucagon (Rodbell et al., 1971a). The binding curves for the compounds tested are shown in Figure 1, and the concentrations of peptide required for half-maximal displacement of ^{125}I-glucagon bound to the membrane (the IC_{50} values) are given in Table I. For glucagon, half-maximal displacement occurred at 5.85 nM, which is in good agreement with the

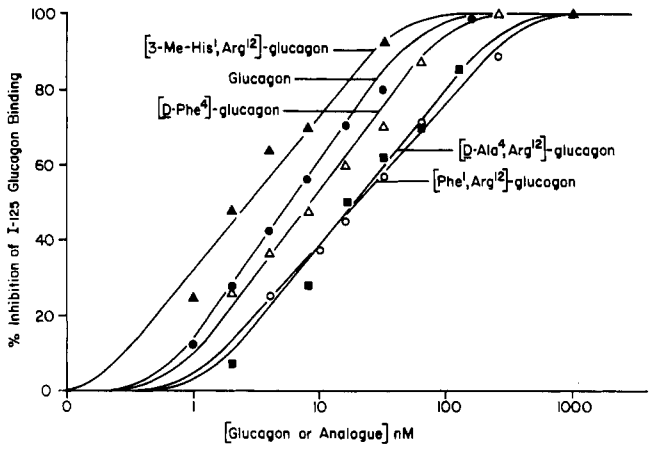

FIGURE 1: Percent inhibition of ^{125}I-glucagon binding to rat liver plasma membranes by (●) glucagon, (▲) [3-Me-His^1,Arg^{12}]glucagon, (○) [Phe^1,Arg^{12}]glucagon, (■) [D-Ala^4,Arg^{12}]glucagon, and (△) [D-Phe^4]glucagon. Plasma membranes, radiolabeled glucagon, and unlabeled glucagon or analogues at concentrations ranging from 0 to 10 μM were incubated for 10 min at 30 °C as detailed under Methods. The values in the figure are corrected for nonspecific binding measured in the presence of excess unlabeled peptide, and the curves are constructed as described under Data Analysis.

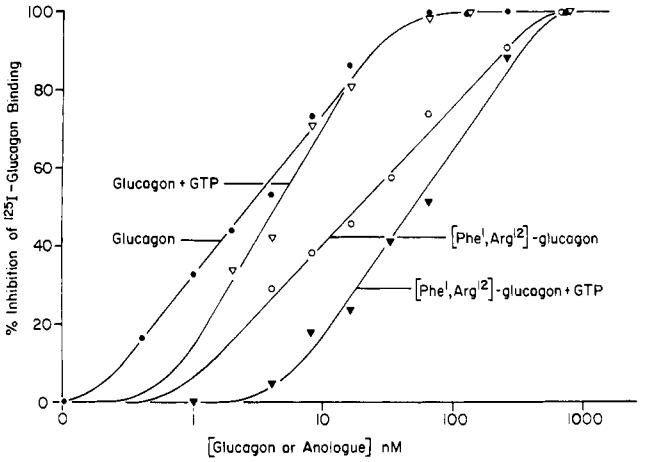

FIGURE 2: Effect of GTP on the percent inhibition of ^{125}I-glucagon binding to rat liver plasma membranes by glucagon and [Phe^1,Arg^{12}]glucagon. Incubations were carried out as described under Figure 1, except for the addition of 100 μM GTP where indicated. The values in the figure are corrected for nonspecific binding for each set of conditions, and the curves are constructed as described under Data Analysis. (●) Glucagon; (▽) glucagon + 100 μM GTP; (○) [Phe^1,Arg^{12}]glucagon; (▼) [Phe^1,Arg^{12}]glucagon + 100 μM GTP.

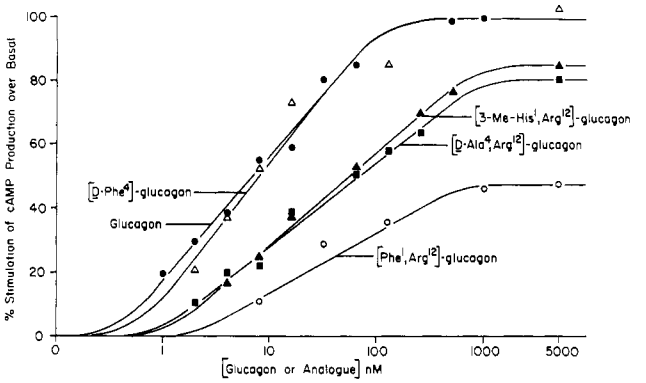

FIGURE 3: Stimulation of cAMP production resulting from activation of rat liver plasma membrane adenylate cyclase by (●) glucagon, (▲) [3-Me-His^1,Arg^{12}]glucagon, (○) [Phe^1,Arg^{12}]glucagon, (■) [D-Ala^4,Arg^{12}] glucagon, and (△) [D-Phe^4]glucagon. Plasma membranes were incubated for 10 min at 30 °C with 1 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 1 mM [^{3}H]cAMP, 10 μM GTP, 1 mM EDTA, and glucagon or glucagon analogues at concentrations ranging from 0 to 5 μM as detailed under Methods. The data points in the figure represent the percentage of cAMP accumulation over basal relative to that produced by 1 μM glucagon which stimulates 3–5 times over basal activity. The curves are constructed as described under Data Analysis.

reported by other laboratories (Lin et al., 1975; England et al., 1982). Two of the glucagon derivatives tested had IC_{50} values similar to that of the native hormone. The affinity of [3-Me-His^1,Arg^{12}]glucagon for glucagon receptors in the liver was found to be somewhat greater than that of the native hormone with an IC_{50} value of 2.75 nM, whereas [D-Phe^4]-glucagon binding was slightly weaker having an IC_{50} value of 9.06 nM. In contrast, the other two analogues, [Phe^1,Arg^{12}]glucagon and [D-Ala^4,Arg^{12}]glucagon, bound more weakly with half-maximal binding occurring near 20 nM. When each of the curves was tested for coincidence with glucagon, all were found to have regression lines significantly different from that of glucagon at the 95% confidence level except [D-Phe^4]glucagon. Analysis of the data for this compound showed that its binding curve and IC_{50} value are not significantly different from that of glucagon. The Hill coefficients calculated from the binding data are listed in Table I.

The effects of GTP on the receptor binding activities of glucagon and [Phe^1,Arg^{12}]glucagon, a partial agonist in the adenylate cyclase assay (see below), were also examined (Figure 2). In the presence of 100 μM GTP, the receptor binding dose–response curve for glucagon was shifted to the right 1.6-fold. This value was similar to that seen for [Phe^1,Arg^{12}]glucagon in which its dose–response curve was shifted approximately 2.5-fold when GTP is present. These values agree well with that previously reported for glucagon by Rodbell et al. (1971b).

Activation of Adenylate Cyclase by Glucagon and Glucagon Analogues. The dose–response curves for the activation of rat liver adenylate cyclase by glucagon and analogues are shown in Figure 3. The concentrations of peptide required for half-maximal activity of the various analogues relative to glucagon are listed in Table I. [D-Phe^4]glucagon had a half-maximal activation value of 7.96 nM, which is close to that determined for native glucagon (6.78 nM), and was the only analogue found to be a full agonist. Although [3-Me-His^1,Arg^{12}]glucagon and [D-Ala^4,Arg^{12}]glucagon were able to stimulate the system to greater than 80% that of glucagon, their EC_{50} values were more than 4 times larger, being 30.6 and 28.7 nM, respectively. In contrast, [Phe^1,Arg^{12}]glucagon was clearly a partial agonist. At concentrations at 1 and 10 μM, where glucagon is maximally stimulating, this derivative gave only 47.6% of the maximal activity of the native hormone and had an EC_{50} concentration of 34.9 nM. When tested for coincidence, the curves for glucagon and [D-Phe^4]glucagon were shown to be statistically identical.

The effect of GTP on the adenylate cyclase activity of the three partial agonist analogues, [3-Me-His^1,Arg^{12}]-, [Phe^1,Arg^{12}]-, and [D-Ala^4,Arg^{12}]glucagon, is shown in Figure 4. At GTP concentrations below 10 μM, stimulation of adenylate cyclase activity by the analogues is reduced relative to glucagon. At GTP levels between 10 and 100 μM, stimulation by the analogues is maximal, but the analogues still are unable to stimulate cAMP production to the same extent as the native hormone.

In Vivo Degradation of Glucagon and [D-*Phe*^{4}]*glucagon.* The degradation of glucagon and [D-Phe^4]glucagon was determined in whole animals by measuring the disappearance of the respective intact radiolabeled peptide in plasma over a period of 30 min (Figure 5). From the plot of the logarithm

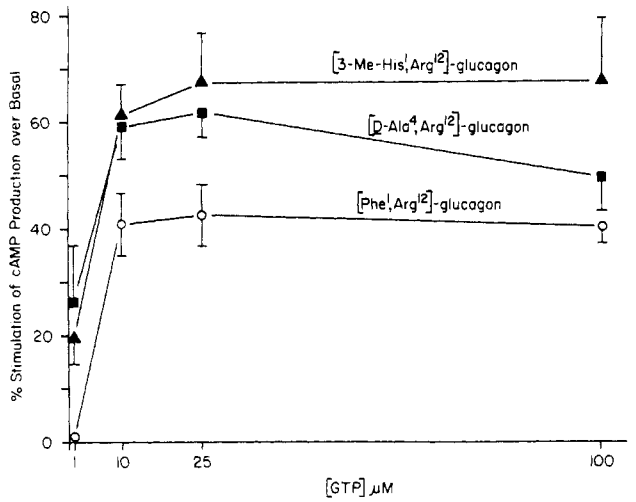

FIGURE 4: Effect of GTP on the stimulation of cAMP production resulting from the activation of rat liver plasma membrane adenylate cyclase by [3-Me-His^1,Arg^{12}]-, [Phe^1,Arg^{12}]-, and [D-Ala^4,Arg^{12}]-glucagon. Plasma membranes were incubated for 10 min at 30 °C with 1 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 1 mM [^{3}H]cAMP, 1 mM EDTA, 1024 nM glucagon analogues, and GTP at concentrations ranging from 0 to 100 μM. Values in the figure indicate the percentage of cAMP accumulation over basal relative to that produced by 1 μM glucagon at the same GTP concentration.

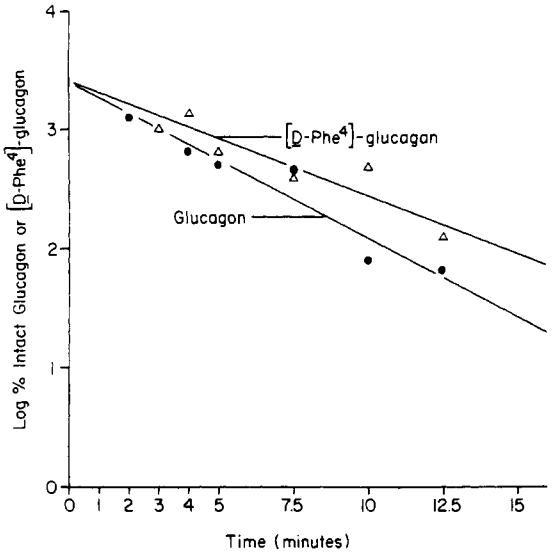

FIGURE 5: In vivo degradation of glucagon and [D-Phe^4]glucagon in rat plasma. Radiolabeled glucagon or [D-Phe^4]glucagon was injected into the femoral vein of an anesthesized rat, and blood samples were taken from the femoral artery at times indicated. Intact peptide was extracted from the plasma as described under Methods. From the amount of intact peptide remaining at each time point, the half-lives of glucagon and [D-Phe^4]glucagon were calculated to be 5.3 and 7.5 min, respectively. (●) Glucagon; (△) [D-Phe^4]glucagon.

of the intact peptide in plasma vs. time, the half-lives for glucagon and [D-Phe^4]glucagon were determined to be 5.3 and 7.5 min, respectively.

Discussion

The results obtained in these experiments are useful in clarifying the conclusions drawn from structure–activity studies based on the in vivo glucose release assay previously reported (Sueiras-Diaz et al., 1984). Specifically, two major conclusions based on in vivo results need to be modified in light of the in vitro data of this study. The first concerns the critical role of glucagon's N-terminal histidine residue in the transduction of biological activity. This notion was first suggested when

Table II: Relative Binding, Adenylate Cyclase, and Glycogenolytic Potencies at N-Terminal Derivatives of Glucagon

peptide	relative binding potency (%)[a]	relative adenylate cyclase potency (%)[b]	relative glycogenolytic potency (%)[c]
glucagon	100	100	100
[3-Me-His^1,Arg^{12}]glucagon	213	22	30
[Phe^1,Arg^{12}]glucagon	30	20	31
[D-Ala^4,Arg^{12}]glucagon	31	24	44
[D-Phe^4]glucagon	69	86	655

[a] Relative binding potency = [(receptor binding EC_{50} for glucagon)/(receptor binding EC_{50} for glucagon analogue)] × 100. [b] Relative adenylate cyclase potency = [(adenylate cyclase EC_{50} for glucagon)/(adenylate cyclase EC_{50} for glucagon analogue)] × 100. [c] Glycogenolytic activity measured in vivo from Sueiras-Diaz et al. (1984).

removal of this amino acid resulted in an analogue that could only activate the adenylate cyclase system to 60% of its maximal activity (Lin et al., 1975). Results presented in this study provide additional support for this premise. Although replacement of histidine by phenylalanine in [Arg^{12}]glucagon results in an analogue that still contains the structural features necessary for binding to liver plasma membranes and nearly full stimulation of glucose release, the adenylate cyclase results clearly show that this analogue is a partial agonist, capable of stimulating the adenylate cyclase system to less than 50% of the maximal response. Thus, while the [Phe^1,Arg^{12}]-glucagon analogue is only slightly less potent than glucagon in receptor binding, adenylate cyclase, and glucose release assays (Table II), the information required for full biological activation of the adenylate cyclase system has been lost.

Methylation of the imidazole nitrogen of histidine yields a somewhat similar result. This modification results in a partial agonist that is able to stimulate the adenylate cyclase system to only 85% of the maximal activity. Despite this analogue's minimal reduction in potencies for the adenylate cyclase and glycogenolytic responses and its enhanced receptor binding, the properties necessary for maximal transduction of the full adenylate cyclase biological response again appear to be lacking in this derivative.

GTP is an important modulator of both glucagon receptor binding and stimulation of adenylate cyclase (Rodbell et al., 1971b; Lin et al., 1977; Rodbell, 1980). In an effort to explain the differences between the partial agonist activity seen in vitro and the full agonist activity seen in vivo for [3-Me-His^1,Arg^{12}]-, [Phe^1,Arg^{12}]-, and [D-Ala^4,Arg^{12}]glucagon, these analogues were assayed for adenylate cyclase activity at various concentrations of GTP ranging from 1 to 100 μM. As expected, stimulation dropped when the GTP concentration was less than 10 μM. On the other hand, no change in the maximal stimulation by these analogues was observed despite increasing the concentration of GTP from 10 to 100 μM. All three still exhibit partial agonism. In addition, the GTP-induced shift in the dose–response curve for [Phe^1,Arg^{12}]glucagon was similar to that seen for glucagon itself. Hence, the partial agonist activities observed for these analogues in this in vitro study, in contrast to their full agonist activity observed in vivo, are not due to a lack of modulation by GTP.

Without considering these measurements of binding and adenylate cyclase activities, the glycogenolytic activities of the histidine-modified peptides suggested that neither the histidine residue nor its imidazole nitrogen atoms are critical for glucagon agonist activity. Rather our results suggest that the imidazole ring is necessary for full agonist efficacy, but it appears that aromaticity at this position will still allow partial

transduction of the hormonal message, since derivatization or replacement of the N-terminal histidine residue resulted in analogues that retain partial agonist activity. Although possible, it is quite unlikely that substitution of arginine for lysine at position 12 is responsible for the partial agonist adenylate cyclase activity seen in these analogues. [Arg^{12}]-glucagon conserves the positive charge in the side chain at this position, which appears to be necessary for maximal stimulation of the adenylate cyclase system (Hruby, 1982). Indeed, the analogues [$HArg^{12}$]glucagon and [N^{ϵ}-acetamido-Lys^{12}]-glucagon, which both conserve charge at position 12, can also maximally activate the adenylate cyclase system of liver plasma membranes (Bregman et al., 1980; Wright & Rodbell, 1980).

Another conclusion, which is based on results obtained from the glycogenolytic assay, concerned the putative role of a β-bend at position 4 in glucagon and its importance as a conformational requirement for biological activity. It has been suggested that a β-bend may be an active conformation in several glycine-containing peptides such as LHRH (Monahan et al., 1973; Freidinger et al., 1980) and secretin (Konig et al., 1977). For example, substitution of glycine with D-alanine or D-phenylalanine in LHRH that might favor a β-bend conformation results in analogues with considerable enhanced activity (Monahan et al., 1973; Coy et al., 1976). Given that glucagon is a glycine-containing peptide, the possibility that a β-bend is also an important conformational feature of the N-terminal region of this hormone was investigated by examining analogues with substitutions of D-amino acids for their binding and biological activities. Results obtained in these experiments do not necessarily support that this conformation is important to the activity of glucagon. In the case of [D-Ala^4,Arg^{12}]glucagon, this analogue demonstrated both reduced binding and adenylate cyclase potencies relative to the native hormone in agreement with its reduced potency in its glycogenolytic activity. More importantly, this analogue is a partial agonist with respect to adenylate cyclase activity, suggesting that this putative conformation may somehow inhibit expression of the full biological message in some cases.

In the case of [D-Phe^4]glucagon, the results are markedly different. Although this analogue has a slightly lower binding potency than the native hormone, its adenylate cyclase potency is not statistically different from that for glucagon, and in addition, [D-Phe^4]glucagon is clearly a full agonist. It is evident that the binding and adenylate cyclase activities for this analogue do not predict the high in vivo potency manifested by this compound at the level of hepatic glucose release, nor do they necessarily provide support for the role of the β-bend in glucagon receptor recognition. Furthermore, the similar half-lives of glucagon (5.3 min) and [D-Phe^4]glucagon (7.5 min) indicate that the two peptides are equally susceptible to in vivo degradation and that replacement of glycine at position 4 of glucagon with D-phenylalanine does not confer added stability. Our results are consistent with similar studies that have been made on the metabolism of [D-Phe^4]glucagon in canine hepatocytes by Hagopian and Tager (personal communication).

The Hill coefficients for glucagon and analogues calculated from the binding data are listed in Table I. The Hill number observed for glucagon in this study is slightly less than 1, suggesting that the native peptide binds differently to liver plasma membranes than the iodinated compound. This conclusion is reasonable given that the ^{125}I-glucagon used is not homogeneous but rather a mixture of mono- and multi-iodinated species, though it has been shown by Hagopian and Tager (1984) that all have the same binding patterns with only different potencies. The Hill numbers for the N-terminally modified derivatives were all found to be similar to that for glucagon, suggesting that these compounds bind in a fashion analogous to that of the native peptide. Since all the values are statistically the same, this suggests that the partial agonism of [3-Me-His^1,Arg^{12}]glucagon, [Phe^1,Arg^{12}]glucagon, and [D-Ala^4,Arg^{12}]glucagon is not due to an alteration in their binding mode but rather is a result of modification of glucagon properties critical for message transduction.

In general, many of the conclusions drawn from structure–function studies utilizing physiological assay systems are supported by results obtained the adenylate cyclase bioassay. This is particularly true for studies involving full agonists of glucagon. Although minor discrepancies may occur (i.e., slightly greater glycogenolytic or lipolytic actvity of an analogue than expected from its corresponding adenylate cyclase activity), the qualitative relationships are basically the same (Hruby, 1982; Hruby et al., 1985).

This has not been the case for partial agonists and antagonists of glucagon. In particular, partial agonist activity at the level of adenylate cyclase is clearly not manifested when assaying for overall cellular events. For example, when des-His^1-glucagon is tested for glycogenolytic activity, its partial agonism is not seen and its glucose mobilizing activity is greater than what is expected from its adenylate cyclase activity (Hruby et al., 1981). On the other hand, [N^{α}-CAR-His^1]-glucagon demonstrates partial agonist activity with respect to adenylate cyclase but is devoid of any lipolytic and glycogenolytic activities in vivo (Grande et al., 1972). In addition, antagonists of glucagon such as des-His^1, [N^{ϵ}-PTC-Lys^{12}]-glucagon and [N^{α}-TNP-His^1,$HArg^{12}$]glucagon, which bind to liver plasma membrane receptors, are inactive in the adenylate cyclase bioassay system, and can antagonize the stimulation of adenylate cyclase by glucagon (Bregman et al., 1980), were found to be weak but full agonists when tested for glycogenolytic activity in the perfused rat liver (Khan et al., 1980) and isolated hepatocyte systems (Corvera et al., 1984). It is possible that the apparent contradictions described above may be explained by a differential interaction of these analogues with the proposed high- and low-affinity receptor sites for glucagon (Musso et al., 1984). It has also been suggested that under some conditions glucagon may mediate its activity by a cAMP-independent mechanism (Exton et al., 1971; Okajima & Ui, 1976; Birnbaum & Fain, 1977; Fain & Shephard, 1977; Cote & Epand, 1979; Khan et al., 1980; Corvera et al., 1984).

It is clear from the results reported here and the studies described above that comprehensive structure–activity analysis requires assessment of hormone activity at various stages from the receptor binding event to the overall cellular response. Studies are in progress in our laboratories to further assess these relationships.

Added in Proof

Recent experiments in our laboratories (D. G. Johnson, unpublished results) have shown that [D-Phe^4]glucagon, administered in vivo to Wistar rats, is equipotent with glucagon in its ability to elevate blood glucose levels.

Acknowledgments

We sincerely thank Dr. Howard Tager for providing us with unpublished data and the reviewers for their meaningful comments. In addition we thank Brenda Howland for her technical assistance in the receptor binding and adenylate cyclase assays, Inger Johnson and Martha Elkind for their assistance with the degradation experiments, and Lourdes

Gallegos for typing the manuscript.

Registry No. GTP, 86-01-1; cAMP, 60-92-4; [D-Phe^4]glucagon, 88200-46-8; [3-Me-His^1,Arg^{12}]glucagon, 88200-40-2; [Phe^1,Arg^{12}]-glucagon, 88200-42-4; [D-Ala^4,Arg^{12}]glucagon, 88200-43-5; porcine glucagon, 16941-32-5; adenylate cyclase, 9012-42-4.

REFERENCES

Bailly, C., Imbert-Teboul, M., Chabardes, D., Hus-Citharel, A., Montegut, M., Clique, A., & Morel, F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3422–3424.

Birnbaum, M. J., & Fain, J. N. (1977) *J. Biol. Chem. 252*, 528–535.

Bowman, W. C., & Rand, M. J. (1980) *Textbook of Pharmacology* (Bowman, W. C., & Rand, M. J., Eds.) pp 41.1–41.48, Blackwell Scientific Publications, Oxford.

Bregman, M. D., & Hruby, V. J. (1979) *FEBS Lett. 101*, 191–194.

Bregman, M. D., Trivedi, D., & Hruby, V. J. (1980) *J. Biol. Chem. 255*, 11725–11731.

Bromer, W. W., Boucher, M. E., Patterson, J. M., Pekar, A. H., & Frank, B. H. (1972) *J. Biol. Chem. 247*, 2581–2585.

Carrey, E. A., & Epand, R. M. (1982) *J. Biol. Chem. 257*, 10624–10630.

Corvera, S., Huerta-Bahena, J., Pelton, J. T., Trivedi, D., Hruby, V. J., & Garcia-Sainz, J. A. (1984) *Biochim. Biophys. Acta 804*, 434–441.

Cote, T. E., & Epand, R. M. (1979) *Biochim. Biophys. Acta 582*, 295–306.

Coy, D. H., Vilchez, J. A., Coy, E. J., & Schally, A. V. (1976) *J. Med. Chem. 19*, 423–427.

England, R. D., Jones, B. N., Flanders, K. C., Coolican, S. A., Rothgeb, T. M., & Gurd, R. S. (1982) *Biochemistry 21*, 940–950.

Exton, J. H., Lewis, S. B., Ho, J., Robison, G. A., & Park, C. R. (1971) *Ann. N.Y. Acad. Sci. 185*, 85–100.

Fain, J. N., & Shepherd, R. E. (1977) *J. Biol. Chem. 252*, 8066–8070.

Frandsen, E. K., Gronvald, F. C., Heding, L. G., Johansen, N. L., Lundt, B. F., Moody, A. J., Markussen, J., & Volund, A. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 665–678.

Freidinger, R. M., Veber, D. F., Perlow, D. S., Brooks, J. R., & Saperstein, R. (1980) *Science (Washington, D.C.) 210*, 656–658.

Glick, G., Parmley, W. W., Wechsler, A. S., & Sonnenblick, E. H. (1968) *Circ. Res. 22*, 789–799.

Grande, F., Grisolia, S., & Diederich, D. (1972) *Proc. Soc. Exp. Biol. Med. 139*, 855–860.

Gratzer, W. B., Beaver, G. H., Rattle, H. W. E., & Bradbury, E. M. (1968) *Eur. J. Biochem. 3*, 276–283.

Hagopian, W. A., & Tager, H. S. (1984) *J. Biol. Chem. 259*, 8986–8993.

Harris, R. A., Mapes, J. D., Ochs, R. S., Crabb, D. W., & Stropes, L. (1979) *Adv. Exp. Med. Biol. 111*, 17–42.

Hruby, V. J. (1982) *Mol. Cell. Biochem. 44*, 49–64.

Hruby, V. J., Agarwal, N. S., Griffen, A., Bregman, M. D., Nugent, C. A., & Brendel, K. (1981) *Biochim. Biophys. Acta 674*, 383–390.

Hruby, V. J., Krstenansky, J. L., McKee, R., & Pelton, J. T. (1985) in *Hormonal Regulation of Gluconeogenesis* (Kraus-Friedmann, N., Ed.) CRC Press, Boca Raton, FL.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Jaspan, J. B., Polonsky, K. S., Lewis, M., Pensler, J., Pugh, W., Moosa, A. R., & Rubenstein, A. H. (1981) *Am. J. Physiol. 240*, E233–E244.

Khan, B. A., Bregman, M. D., Nugent, C. A., Hruby, V. J., & Brendel, K. (1980) *Biochem. Biophys. Res. Commun. 93*, 729–736.

Konig, W., Geiger, R., Wissmann, H., Bickel, M., Obermier, R., Teetz, W., & Uhmaan, R. (1977) *Gastroenterology 72*, 797–800.

Lin, M. C., Wright, D. E., Hruby, V. J., & Rodbell, M. (1975) *Biochemistry 14*, 1559–1563.

Lin, M. C., Nicosia, S., Lad, P. M., & Rodbell, M. (1977) *J. Biol. Chem. 252*, 2790–2792.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.

Monahan, H. W., Amoss, H. S., Anderson, H. A., & Vale, W. (1973) *Biochemistry 12*, 4616–4620.

Musso, G. F., Assoian, R. K., Kaiser, E. T., Kézdy, F. J., & Tager, H. S. (1984) *Biochem. Biophys. Res. Commun. 119*, 713–719.

Neville, D. M., Jr. (1968) *Biochim. Biophys. Acta 154*, 540–552.

Okajima, F., & Ui, M. (1976) *Arch. Biochem. Biophys. 175*, 549–557.

Pohl, S. L., Birnbaumer, L., & Rodbell, M. (1971) *J. Biol. Chem. 246*, 1849–1856.

Rodbell, M. (1980) *Nature (London) 284*, 17–22.

Rodbell, M., Krans, H. M. J., Pohl, S. L., & Birnbaumer, L. (1971a) *J. Biol. Chem. 246*, 1861–1871.

Rodbell, M., Krans, H. M. J., Pohl, S. L., & Birnbaumer, L. (1971b) *J. Biol. Chem. 246*, 1872–1876.

Salomon, Y., Londos, C., & Rodbell, M. (1976) *Anal. Biochem. 58*, 541–548.

Sueiras-Diaz, J., Lance, V. A., Murphy, W. A., & Coy, D. H. (1984) *J. Med. Chem. 27*, 310–315.

Thorell, J. E., & Johansson, B. G. (1971) *Biochim. Biophys. Acta 251*, 363–369.

Unger, R. H. (1978) *Metab., Clin. Exp. 27*, 1691–1709.

Walter, R. M., Dudl, R. J., Palmer, J. P., & Ensinck, J. W. (1974) *J. Clin. Invest. 54*, 1214–1220.

Witters, L. A., Kowaloff, E. M., & Avruch, J. (1979) *J. Biol. Chem. 254*, 245–248.

Wright, D. E., & Rodbell, M. (1980) *Eur. J. Biochem. 111*, 11–16.