Glucagon Amino Groups

EVALUATION OF MODIFICATIONS LEADING TO ANTAGONISM AND AGONISM*

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Using native glucagon and [12-homoarginine]glucagon (analogue A), prepared in high yield and purity by new procedures, we have synthesized the following glucagon analogues by semisynthetic methods: [1-des-histidine][12-homoarginine]glucagon (analogue B); N°-carbamoylglucagon (analogue C); N°, N°-dicarbamoylglucagon (analogue D); [1-N°-carbamoylhistidine, 12-N°-trinitrophenyllysine]glucagon (analogue E); [12-N°-trinitrophenyllysine, 12-homoarginine]glucagon (analogue III); and [1-N°-trinitrophenylhistidine, 12-homoarginine]glucagon (analogue IV).

The introduction of hydrophobic groups at the α- and ε-amino positions of glucagon results in a reduction in potency. The α-position is also involved in biological activity. Carbamylaation of the α-position results in a partial agonist (analogues C and D). The introduction of hydrophobic groups and the neutralization of the positive charge at the α- and ε-amino positions result in glucagon antagonists (analogues II, III, and IV). [1-N°-Trinitrophenylhistidine, 12-homoarginine]glucagon (analogue IV) is the most potent inhibitor tested. Based on its competitive inhibitory action, this analogue appears to have about one-third the affinity of glucagon for the receptor site. These modifications at the ε-amino position cause an increase in the secondary structure of the peptide (as shown by circular dichroism studies) which may be related to their biological activities.

It has been suggested that glucagon may be involved in the pathogenesis of diabetes mellitus (1). Development of glucagon antagonists would aid in understanding the mechanism of glucagon action on the liver. A few glucagon antagonists have recently been synthesized (2, 3) which resulted from chemical modifications of the α- and ε-amino positions of the hormone. In this communication, the importance of these amino groups to biological function is investigated. It is shown that antagonists result from similar group modifications at the α- and/or ε-amino positions which result in a change in the monomeric structure of glucagon. Agonists result from hydrophilic modifications at the α- or ε-amino position, but differing effects on potency and agonistic ability of the analogues occur depending on the modification.

MATERIALS AND METHODS

Crystalline glucagon obtained from Eliaco was purified on DEAE-Sephadex A-25 to remove desamido glucagon. DEAE-Sephadex A-25, SP-Sephadex C-25, and Sephadex G-10 were purchased from Pharmacia. Phenylisothiocyanate and trinitrobenzenesulfonic acid were obtained from Pierce. O-Methylisourea hydrogen sulfate was obtained from Aldrich. Carboxypeptidase A, chymotrypsin, and phosphokinase were purchased from Sigma, and amionopeptidase M from Rohn and Haas. [α-32P]ATP and cyclic [3H]AMP were obtained from New England Nuclear. All other chemicals were of reagent grade.

Liver plasma membranes were prepared as described by Pohl et al. (4). Liver adenylate cyclase activity was determined as previously described (3); purification of cyclic AMP was done according to Salomon et al. (5). Membrane protein was determined by the procedure of Markwell et al. (6).

Ultraviolet-visible measurements were made on a Gilford 240 spectrophotometer. Circular dichroism measurements were performed on a Cary 60 DC spectrophotometer. The peptides were analyzed on a Beckman 120 C following either acid hydrolysis in 6 N HCl for 22 h at 100°C or enzymatic proteolysis. The peptide sample (1 mg/ml) was incubated with (a) aminopeptidase M (1%, w/w) for 24 h at 30°C, (b) carboxypeptidase A (1%, w/w) or (c) chymotrypsin (0.5%, w/w), followed by carboxypeptidase A (1%, w/w) followed by aminopeptidase M (1%, w/w), each step for 24 h at 30°C, all in 20 mM sodium bicarbonate. Several hormone derivatives were purified on a DEAE-Sephadex A-25 column (1.2 × 15 cm). They were solubilized in 7 mM urea, and the column was developed at 5°C with 70 ml of 0.01 M Tris (pH 7.7), followed by 60 ml of 0.5 M NaCl in 0.01 M Tris (pH 7.7), all in 7 mM urea. [HArg]glucagon and [dHis]glucagon were purified and analyzed on a SP-Sephadex C-25 column (2.5 × 18 cm) equilibrated in 10% acetic acid containing 20 mM sodium acetate and 1 mM urea. The column was developed with 100 ml of buffer containing 1 mM urea, followed by a linear gradient from 0.0 to 0.3 M NaCl (400 ml), and finally, 400 ml of 0.5 M NaCl in buffer which eluted [HArg]glucagon from the column.

SYNTHESES

[1-HArg]glucagon (Analogue A)—O-Methylisourea-H2SO4 (3.4 g) was dissolved in 20 ml of H2O. Ba(OH)2 (6 g) was added slowly to the stirring solution. The mixture was filtered through two sheets of Whatman No. 1 filter paper on a Buchner funnel. The supernatant was centrifuged for 15 min at 3000 × g. The clear supernatant was desalted on a Sephadex G-10 column (2.5 × 75 cm) developed with a linear gradient from 0.0 to 0.3 M NaCl (400 ml), and finally, 400 ml of 0.5 M NaCl in buffer which eluted [HArg]glucagon from the column.

The abbreviations used were: [HArg]glucagon, [12-homoarginine]glucagon; [dHis]glucagon; [HArg]glucagon, [1-des-histidine][12-homoarginine]glucagon; [N°-TNB, HArg]glucagon, [1-N°-trinitrophenylhistidine, 12-homoarginine]glucagon; [dHis]glucagon; [N°-TNB, HArg]glucagon, [1-des-histidine][2-N°-trinitrophenylserine, 12-homoarginine]glucagon; [dHis]glucagon; [N°-Pc]glucagon, [1-des-histidine][12-N°-phenylthiocarbamoyl-lysine]glucagon; TNB, trinitrophenyl.
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**RESULTS**

**Amino acid analysis of glucagon analogues**

| Glucagon structure | [HArg<sup>2</sup>]glucagon | [dHis<sup>1</sup>]glucagon | Carboxypeptidase A of [dHis<sup>1</sup>]glucagon |
|--------------------|-----------------------------|---------------------------|-------------------------------------------|
| Asp                | 4                           | 4.08                      | 4.14                                     |
| Thr                | 3                           | 2.82                      | 2.86                                     |
| Ser                | 4                           | 3.86                      | 3.76                                     |
| Glu                | 3                           | 3.03                      | 2.99                                     |
| Gly                | 1                           | 1.04                      | 1.08                                     |
| Ala                | 1                           | 1.05                      | 1.09                                     |
| Val                | 1                           | 0.94                      | 1.04                                     |
| Met                | 1                           | 0.95                      | 0.94                                     |
| Leu                | 2                           | 1.97                      | 2.05                                     |
| Tyr                | 2                           | 2.03                      | 1.92                                     |
| Phe                | 2                           | 2.05                      | 2.02                                     |
| Lys (Orn)          | 2                           | 0.02<sup>a</sup>          | 0.02<sup>a</sup>                         |
| His                | 1                           | 0.93                      | 0.93<sup>a</sup>                         |
| Arg                | 2                           | 2.02                      | 2.02                                     |
| HArg               | 0.99                        | 0.98                      | 0.98                                     |

<sup>a</sup> Expected value in parentheses.

<sup>b</sup> Actually asparagine and glutamine, which co-chromatograph with serine.

<sup>c</sup> Glucagon contains 1 residue of Trp which is lost on acid hydrolysis.

<sup>d</sup> Due to breakdown of HArg to Lys and Arg to Orn during acid hydrolysis. In separate experiments both Arg and HArg standards were hydrolyzed under the same conditions used to hydrolyze glucagon and its analogues. Amino acid analysis before and after hydrolysis showed a 1.0 ± 0.3% breakdown of Arg and HArg to Orn and Lys, respectively.

<sup>e</sup> See “Results” for discussion.

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**Fig. 1.** DEAE-Sephadex chromatography of the products derived from the reaction with potassium cyanate at pH 7.0 (---), pH 9 (-----). Tris buffer, 0.01 M, in 7 M urea (pH 7.7) was used as eluent solvent with a discontinuous NaCl gradient (•••••).
shown). This establishes that analogue C was pure and only modified at the α-position.

Dicarbamoylglucagon (analogue D) was synthesized by raising the pH to 9 in the carbamylation step. This increases reactivity of the ε-amino position. The resultant product was purified on DEAE-Sephadex (Fig. 1). The increase in pH resulted in over 50% of the material eluting with high salt (Peak 2) which, based on previous results (3, 11), indicates that the ε-amino position had been blocked. No His was released by aminopeptidase M. Amino acid analysis of acid hydrolysates gave 90% homocitrulline and 10% Lys. These hydrolysis conditions will cause some of the homocitrulline to break down and form free Lys (13). Thus, the material contained in Peak 2 (Fig. 1) is analogue D.

Trinitrobenzenesulfonic acid reacts rapidly and specifically with the free amino groups of peptides (14, 15). The TNB group was specifically incorporated into analogues A, B, and C which all have only one free amino group. Using its molar extinction coefficient at 350 nm (7) where only TNB absorbs, each of the products obtained in the work reported here was a monosubstituted product. Aminopeptidase M digestion of [Nε-carbamoyl, Nε-TPC]glucagon (analogue II) did not release any His, demonstrating that the α-position had remained carbamylated. Aminopeptidase M digestion of [Nε-TNB, HArgε]glucagon (analogue IV) and [dHisε] (Nε-TNB, HArgε]glucagon (analogue III) did not release any His or Ser, while analogues A and B did. Acid hydrolysis resulted in the significant regeneration of the free amino acid from the TNB-modified amino acid.

At physiological concentrations, glucagon exists in aqueous solution as a monomer with mainly an unordered random coil structure (16-19). The circular dichroism spectrum of analogues A, B, D, and IV are virtually identical with that of glucagon. The only significant change in the spectra occurred when glucagon was modified at the ε-amino position so as to neutralize the positive change and introduce a lipophilic group. As was previously observed with analogue II (7), there is a large increase in the trough at 220 to 208 nm (Fig. 2). The CD spectra for the antagonist I (Fig. 2) also displays an increase in negative ellipticity at 222 nm with a much deeper trough at 205 nm. The CD spectra were obtained below the concentration at which glucagon aggregates, and the results were not changed by dilution. The introduction of a large hydrophobic group onto the ε-amino position causes a large change in secondary structure. Neutralization of ε-amino charge alone was not sufficient to cause a conformational change as was observed in the CD spectra of analogue D. The CD spectra of analogues II and I are not the same (Fig. 2).

This may be due to the size of the modifying group. The TNB group is more compact than the phenylthiocarbamoyl group. The benzene ring in phenylthiocarbamoyl would break the change in the spectra occurred when glucagon was modified at the ε-amino position so as to neutralize the positive change and introduce a lipophilic group. As was previously observed with analogue II (7), there is a large increase in the trough at 220 to 208 nm (Fig. 2). The CD spectra for the antagonist I (Fig. 2) also displays an increase in negative ellipticity at 222 nm with a much deeper trough at 205 nm. The CD spectra were obtained below the concentration at which glucagon aggregates, and the results were not changed by dilution. The introduction of a large hydrophobic group onto the ε-amino position causes a large change in secondary structure. Neutralization of ε-amino charge alone was not sufficient to cause a conformational change as was observed in the CD spectra of analogue D. The CD spectra of analogues II and I are not the same (Fig. 2). This may be due to the size of the modifying group. The TNB group is more compact than the phenylthiocarbamoyl group. The benzene ring in phenylthiocarbamoyl would be situated further away from the ε-amino group of Lys-12 than the benzene ring of TNB, which is attached directly onto the ε-amino position. Thus, the phenylthiocarbamoyl group may not fit into the conformation normally formed by glucagon or analogue II and thus ends up in an intermediate conformation.

Fluorescence studies on a related glucagon derivative, Nε-4-azide-2-nitrophenyl-glucagon, suggested that its monomeric form exists in a folded, nonrandom structure (11). This observation is consistent with CD spectra results we have reported in this study.

Chemical modifications at either the α- or ε-amino position of glucagon result either in a weaker agonist or in an antagonist. The dose-response curve for the stimulation of liver adenylate cyclase by various glucagon agonists is shown in Fig. 3. Effect of glucagon agonists on liver adenylate cyclase activity. The stimulatory effect of glucagon (-·-·), [HArgε]glucagon (analogue A) (-●-●), Nε-carbamoylglucagon (analogue C) (△-△), and Nε, Nε-dicarbamoylglucagon (analogue D) (□-□) on adenylate cyclase activity is expressed as the percentage of activation over basal. 0% activation represents the basal activity (240 pmol/10 min/mg of protein) and 100% is the maximum activity observed with glucagon (1680 pmol/10 min/mg of protein). The results are the mean of two determinations each from triplicate experiments.

### Table II

| Agonist | Maximum stimulation | Relative potency | Reference |
|---------|---------------------|-----------------|-----------|
| Glucagon | 100% | 1.000 | This work |
| A. [HArgε]glucagon | 100% | 0.200 | This work |
| >80%* | 0.110* | 8 |
| B. [dHisε] [HArgε]glucagon | 22% | 0.001 | This work |
| C. Nε-Carbamoylglucagon | 27% | 0.055 | This work |
| >70%* | 0.110* | 8 |
| D. Nε,Nε-Dicarbamoylglucagon | 17% | 0.004 | This work |

* Dose response curve not carried out to maximal hormone concentration.

** Estimated from published dose-response curve.
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Fig. 3. The introduction of the guanidyl group onto the ε-amino group of lysine-12, which maintains the positive charge, only affects the potency, reducing it by 80%. [HArg'ε]glucagon can still effect a maximal adenylate cyclase activity equivalent to that obtained with glucagon. This result is in general agreement with those previously for liver adenylate cyclase (Table II) (8) and the stimulation of lipolysis (9). As we previously observed with [dHis'ε]glucagon, the removal of His from analogue A resulted in a concomitant reduction in potency and agonistic ability (Table II), and a weak partial agonist was obtained. In addition, the carbamoylation of glucagon results in a large reduction in activity. The complete dose-response of purified N'-carbamoylglucagon on liver adenylate cyclase (Fig. 3) again demonstrates the involvement of the 1-position in both binding and subsequent stimulation of activity (transduction). The addition of the small carbamoyl group onto the α-amino group results in a 20-fold reduction in potency (Table II). The maximal adenylate cyclase activity obtained relative to glucagon is also reduced by 73%. The introduction of a second carbamoyl group onto the ε-amino position results mainly in a reduction in potency (Table II), suggesting that the ε-amino position is only involved in the initial binding to the receptor and has little to do with the subsequent activation of adenylate cyclase (transduction).

The chemical modification of either the α- or ε-amino positions by lipophilic groups that neutralize the positive charge result in antagonistic forms of glucagon (Fig. 4). The circular dichroism results showed that the introduction of a phenylthiocarbamoyl or TNB group onto the ε-amino position results in changes to the secondary structure of the hormone. Even very high concentrations of analogues I (5 μM) and II (5 μM) do not stimulate liver adenylate cyclase. These inactive forms of glucagon do interact with the receptor, as evidenced by the shift in the glucagon dose-response curves in their presence (Fig. 4) (3). Using relative inhibitory power (Table III) as a measure for the strength of these antagonists, one observes that except for analogue IV they are relatively weak. Evaluating the effect of these modifications on hormone activity is complicated by the change in conformation they cause. If, as was the case with the agonists, the substitution at ε-amino position resulted only in a loss in potency (probably because the substituted group interferes with binding), then antagonism results from an event subsequent to binding which may be the result of the conformational change.

The introduction of a hydrophobic group onto the α-amino group (analogues III and IV) also generated an antagonist (Fig. 4). The altered conformation of these analogues may be responsible for the resulting antagonistic properties observed (Table III). The relative inhibitory powers of analogues III and IV (Table III) are greater than the relative agonist potency of their respective starting materials, analogues A and B (Table II). This suggests that there is an increase in affinity following the addition of TNB at the N'-position. This may result because the TNB group contributes additional hydrophobic binding energy or because the TNB derivatives exist in a new conformation which overcomes some of the steric restraints imposed at the 1-position by this modification. In any case, our results demonstrate that charge neutralization and the introduction of a hydrophobic group onto the α- or ε-amino groups of glucagon leads to an antagonistic form of the hormone, with analogue IV being the strongest antagonist found to date.

DISCUSSION

The introduction of hydrophilic groups such as trifluoroacetyl (21), acetyl (22), and carbamoyl (8) at the α-amino position of glucagon results in a decrease in activity. The potency and biological activity values we obtained with analogue C do not agree with previously published values (Table II) (8). The results of the previous work suggested that analogue C is a full agonist, whereas in our hands it is a weak partial agonist. The carbamoylation of glucagon can result in a mixture of three components, glucagon and analogues C and D. In the earlier study, (8), the reaction mixture was used as obtained with no additional purification such as the ion exchange column chromatography employed here (Fig. 1). In the earlier study, glucagon was carbamylated at pH 7.0 in the absence of urea (8), and since glucagon is very insoluble at pH 7.0, a significant amount of unreacted glucagon was probably present in the latter preparation. The assay system employed by these workers (2) (liver homogenates) displayed a 500-fold lower sensitivity than the standard (4) liver plasma membrane adenylate cyclase system employed here. The additional proteases present in their crude preparation may have resulted in the generation of significant amounts of active glucagon compounds. All of these factors would provide a product with full biological activity. However, the carefully purified mono-carbamoyl derivative of analogue C which we prepared was only a weak, partial agonist. The additional carbamoylation of the ε-amino position of lysine (analogue D) results primarily in a very large further reduction in potency (Table II), which suggests that the central portion of glucagon is primarily involved in binding. This notion is further supported by the observations of Patterson and Bromer (23) that the introduction of NO2- (mono- or di-) or NH2-groups into tyrosine-13 resulted only in a reduction in potency, with all these deriva-

**Table III**

| Inhibitory powers of glucagon antagonists |  |
|-----------------------------------------|---|
| Antagonist                              | Relative inhibitory strength | Reference |
| I [dHis'] [N'-Ptc]glucagon              | 0.055 | 3 |
| II. [N'-carbamoyl, N'-TNB]glucagon     | 0.010 | This work |
| III. [dHis'] [N'-TNB, HArg']glucagon   | 0.000 | 7 |
| IV. [N'-TNB, HArg']glucagon            | 0.000 | 7 |

*Relative inhibitory strength equals (increase K,)/(concentration antagonist) = (K, (observed) − K, (glucagon alone))/(1 × 10^−6 M), where K, is the glucagon concentration which induces 50% adenylate cyclase activity.
The measured potency and agonistic ability of analogue B to stimulate adenylate cyclase is much lower than that previously obtained with [dHis]glucagon (Table III) (Ref. 3 and 20). This is probably because attachment of the bulky guanidyl group on the ε-amino position of lysine-12 weakens the interaction between the modified hormone and its receptor. This illustrates the importance of histidine-1, not only to the initial binding, but also to a subsequent process which affects the hormone and receptor complex interaction with adenylate cyclase. The observation that the partial agonist activity is only 22% that of glucagon (Table II) while [dHis]glucagon had about 55% partial agonist activity (3, 20) suggests that the relationship of the NH₂-terminal of glucagon to the lysine-12 (or homoarginine-12) residue is important in the transduction process, whereby glucagon activates adenylate cyclase.

A previous study which used crude preparations of analogues II and IV did not find any inhibition of the glucagon-stimulated adenylate cyclase activity (7) for these compounds, which is contrary to our results (Table III). These previous results cannot be properly evaluated and compared with our results since the actual data and hormone concentrations used were not presented (7). Furthermore, in this earlier study, analogues II and IV showed significant biological activity, which suggests that their preparations were contaminated with significant amounts of starting material (agonists). This group has also presented contradictory data as to how they evaluated the integrity of their TNB-modified analogues. In the earlier study by this group (7), the TNB-modified amino acids were reported to be acid-sensitive, with regeneration of the free amino acid (Lys or His) during acid hydrolysis (7). In a subsequent study, they reported that the TNB-modified amino acids were stable to acid hydrolysis (2). We observed that these TNB-modified amino acids were acid-sensitive, which agrees with the established chemistry (14, 15).

Several reports utilizing peptide fragments or fragment analogues of glucagon have shown that making modifications or removing amino acids from the carboxyl end generally only results in loss of binding affinity but not intrinsic activity (20, 24, 25), and that glucagon₁₋₄ alone contains sufficient information for binding and activation of adenylate cyclase (26).

The results presented in this paper demonstrate that for agonists, the ε-amino of lysine-12 is involved mainly in binding and that the histidine-1 position contributes both to binding and biological activity. A model is presented in Fig. 6 to aid in discussing and explaining the mechanisms relating to agonism and antagonism in the glucagon-hepatic adenylate cyclase system. The model is based on the mobile receptor hypothesis (27, 28) and does not attempt to define all the multi-equilibria present in this system. The receptor (R) exists in high and low affinity states. The low affinity state (R) exists in a free state in the membrane. Since glucagon binding displays positive cooperativity (29), we propose that R represents clusters of binding sites. The high affinity sites are represented by R’⁺-AC (AC, adenylate cyclase) and based on various equilibria, would represent a very small amount of the total receptor sites. At the start of the cycle, hormone or hormone analogue (H) binds to free receptor (R). Subsequent to binding, an interaction takes place between the hormone (or a portion of the hormone) and the receptor, and this interaction provides the free energy needed to convert the H-R complex to H'R⁺, resulting in a lower energy state. H'R⁺ has a much higher affinity than R or H-R for AC. We propose that partial agonism occurs when H is unable to convert H-R to the lowest energy conformation (H-R'), but instead leads to a different complex, H-R⁺⁺, H-R⁺⁺ affinity for AC in the lipid matrix is lower than that of H-R' and therefore, at saturation, hormone binding would result in a large amount of adenylate cyclase remaining uncoupled. Since observed adenylate cyclase activity is the summation of AC (basal) and H-R⁺⁺-AC⁺⁺ (active), this results in partial agonism.

One of the limiting factors controlling this cycle is the concentration of adenylate cyclase versus free receptor concentration. There is some indirect evidence that in the hepatic system the glucagon receptors are present in excess of adenylate cyclase (30). Kinetic studies suggest that only 10 to 15% of the receptor sites need be occupied to elicit full adenylate cyclase activity (31). GTP interacts with the nucleotide-binding protein associated with the adenylate cyclase complex to convert AC to AC⁺⁺, which has a much higher affinity for H-R'. Thus, GTP through its binding protein helps drive Step 3 to the right and results in more H-R⁺⁺-AC⁺⁺ (active) at equilibrium. We propose that the main function of the GTP system in the cell membrane is to provide the energy that drives this cycle. At Step 4, the hydrolysis of the GTP bound to the nucleotide-binding protein provides the energy that breaks up the H-R⁺⁺-AC⁺⁺ (active) complex, releasing the hormone and converting H-R⁺⁺-AC⁺⁺ to R⁺⁺-AC. It is proposed that some of this free energy is used to convert R⁺⁺ to a higher energy state (R⁺⁺⁺), R⁺⁺⁺-AC would represent the high affinity binding site. In this high energy state, R⁺⁺ would exist in a form which has a very low affinity for AC. This completes the cycle by driving Step 5, the dissociation of R⁺⁺⁻AC to free uncoupled R and AC. The conversion of R⁺⁺ to the lower energy state (R) during dissociation (Step 5) supplies the free energy needed to overcome the stabilization energy of the complex provided by the interaction between R⁺⁺ and AC.

There are several lines of evidence to support the notion that glucagon stimulation of adenylate cyclase is dependent on the occupied hormone receptor's ability to laterally diffuse through the lipid matrix and to couple with adenylate cyclase. For example, activation of hepatic adenylate cyclase by fluororhodamine yields a linear Arrhenius plot, while activation by glucagon results in biphasic Arrhenius plots (32, 33). Several physical studies have shown that hepatic adenylate cyclase increases in size upon the addition of glucagon (34, 35). Also, treatment of the membrane with phospholipase C abolishes...
the ability of glucagon to activate adenylate cyclase even though it can still bind (36). These results are all consistent with the notion of an occupied receptor diffusing through the lipid matrix to couple with adenylate cyclase to effect an increase in enzyme activity. The introduction of hydrophilic groups onto the α-amino position of lysine-12 only results in lower potency. This shows that residues in the middle of the hormone are involved in the initial binding (Step 1) and contribute little to the conversion of H-R to H-R′. On the other hand, removal of histidine or introduction of a hydrophilic group (carbamoyl) onto the NH2 terminus results both in a loss in potency and agonism. This demonstrates that the N-terminal end of glucagon is involved in both the initial binding (Step 1) and subsequent conversion of H-R to H-R′ (Step 2). Partial agonism results because the interaction of these hormone analogues with the receptor does not provide sufficient stabilization to generate only R′, but instead results in an intermediate higher energy conformation R′*, which has lower affinity for AC* (i.e. equilibrium 3 in Fig. 5 is more to the left). This would explain how an analogue could have a higher potency, but be a weaker agonist. The model also predicts that for weak partial agonists, the binding affinities will be greater than the observed potencies in adenylate cyclase activation. Because the H-R′* that would be generated would have a very low affinity for AC*, a significantly greater number of receptors (R) would have to be occupied before a threshold amount of H-R′*.AC* (active) would be formed.

Other modulators of hepatic adenylate cyclase such as the inhibitory adenosine binding P-site, which inhibits glucagon stimulation in a GTP-independent manner (37, 38), may act mechanistically in a similar manner by altering the affinity of one component for another in the lipid matrix. In several systems, it has been shown that the inhibitory effect of the P-site on hormone-stimulated activities is larger than the effect on basal activity (39, 40). Thus, the P-site may be associated with the adenylate cyclase complex and, in the presence of adenosine, convert the enzyme to a lower activity state and, most importantly, greatly reduce the affinity of AC for H-R′.

The net result would be to effectively uncouple the hormonal response.

In β-adrenergic systems, GTP alters the binding of agonist, but has no effect on the antagonist (41, 42). The binding of a competitive antagonistic form of [dHis'] glucagon, presumably analogue I (3), was also unaffected by GTP (43). GTP had no effect on the binding of N′-4-azido-2-nitrophenyl-glucagon,2 which is inactive (44), and similar in structure to the N′-substituted antagonists tested here. We are proposing that antagonists bind only to R′-AC (Step 5A) and have little affinity for R (Fig. 5). This would explain the inability of GTP to affect binding, and predicts that at saturation the antagonists would not bind to all the receptors. Alternatively, one could propose that the antagonists only bind to R and this binding leads to a complex (H-R′R) which has no affinity for AC′. If this were the case, then we would predict that the antagonists would bind to all the available receptors because the equilibrium for R′-AC lies to the right (Step 5) and would readily break down to R + AC as the antagonist tied up free R. Binding studies are currently underway to distinguish between these two possibilities. Arrhenius plots of adenylate cyclase activity in the presence of an antagonistic form of [dHis'] glucagon (presumably analogue I) (3), are biphasic, while the plot for fluoride stimulation is linear (32). This demonstrates that the receptor, when occupied with antagonist, is associated with adenylate cyclase, and suggests that

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The first condition is true. The introduction of lipophilic groups onto the α- or ε-amino positions results in a glucagon analogue with a different peptide conformation (Fig. 2, Ref. 7) than the native hormone. These antagonistic forms of glucagon may have an altered specificity, being able to bind to only R′-AC. Less likely, but still possible, these antagonists may bind to R and then are unable to interact with R to generate H-R′ because of their altered conformational states.

Our results suggest that replacement of histidine-1 and/or lysine-12 with amino acids or amino acid-like compounds which remove the positive charge and contribute hydrophobic bulk at these positions may result in stronger antagonists. There is some support for this view. For example, gastric inhibitory protein, which has a glucagon-like amino acid sequence through its first 29 amino acids, has been reported to inhibit glucagon binding to adipocytes, glucagon-stimulated cAMP production, and lipolysis (45). Gastric inhibitory protein contains tyrosine-1 and isoleucine-12 residues instead of the histidine-1 and lysine-12 residues found in glucagon, and this may explain its antagonistic properties at the glucagon receptor.

Surprising results have been obtained in the few cases where glucagon antagonists were used in attempts to block glycogenolysis which is thought to be a cyclic AMP-dependent event. No inhibition of the glucagon stimulation was observed (2, 46). These results raise the possibility that glucagon activates glycogenolysis by a cyclic AMP-independent process. Alternatively, only a small elevation in cyclic AMP levels may be needed for full biological response. If this is true, then an inhibitor would have to have a Ks approximately equal to the Ks of glucagon. The antagonists used in these previous studies were relatively weak inhibitors in the in vitro assay. Perhaps similar experiments with the stronger antagonists reported in this paper will provide further insight into the mechanism of glucagon action in vivo.

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