Noncooperative Receptor Interactions of Glucagon and Eleven Analogues: Inhibition of Adenylate Cyclase†

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ABSTRACT: Glucagon and 11 glucagon derivatives were characterized and compared with respect to the cooperativity of their receptor interactions and their ability to elicit a biphasic (activation–inhibition) response from the adenylate cyclase system of rat liver plasma membranes. Slope factors were evaluated from two sets of experimental data, binding to hepatocyte receptors and activation of adenylate cyclase. The results are consistent with noncooperative binding to a single affinity state of the glucagon receptor for all derivatives, irrespective of the modification and the agonist properties of the derivatives. High-dose inhibition of adenylate cyclase activity was observed for native glucagon and all of the derivatives which were examined at high concentrations ($>10^{-5}$ M). Partial agonism of some low-affinity glucagon derivatives is not caused by high-dose inhibition. Several mechanisms which might give rise to high-dose inhibition such as receptor cross-linking or multivalent receptor binding are discussed in relationship to the glucagon–receptor interaction. These phenomena indicate that significant differences exist between the glucagon system and the β-adrenergic system.

The role of glucagon in the activation of adenylate cyclase has received considerable attention in the decade since Rodbell and co-workers initiated their investigations of the hepatic adenylate cyclase system (Pohl et al., 1971). Much attention has been directed toward the development of specifically modified glucagon derivatives useful for investigating the structure–function relationships of the hormone (Hruby, 1982). Relatively few of these derivatives have been extensively purified or assayed for their binding ability and biologic potency under similar conditions, primarily because of the difficulty of their preparation and the inherent physical characteristics of this particular peptide hormone (Hruby, 1982). More recently, a number of models have been proposed which attempt to integrate a number of hormones which activate adenylate cyclase into a single unifying concept (Rodbell, 1980; Swillens & Dumont, 1980; Lefkowitz et al., 1982; Stadel et al., 1982). Such modeling can only improve with the incorporation of reliable data and additional phenomenological information concerning the hormone–receptor interaction as well as the transduction of this binding signal into adenylate cyclase activation.

We report here that native glucagon fails to show cooperativity effects when its binding is examined in the absence of GTP and that the apparent slight negative cooperativity of adenylate cyclase activation analysis is unlikely to be a real phenomenon. Eleven carefully prepared, highly purified derivatives mimic native glucagon in their failure to show cooperativity on analysis of binding and activation data, implying that lack of cooperativity is independent of whether the modification is present in the amino-terminal region, the central region, or the carboxyl-terminal binding region. The lack of cooperativity is independent of the agonist character since it is the same for full and partial agonists. Furthermore, glucagon and all derivatives tested inhibit adenylate cyclase at concentrations greater than those which produce maximum activation of the enzyme.

Experimental Procedures

Materials and Methods. The preparation and characterization of most of the carboxyl-terminal glucagon derivatives and the sources of all materials used for their study have been previously reported by England et al. (1982). The preparation and characterization of N^{ϵ}-acetimidoglucagon and N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon were reported by Flanders et al. (1982), and the preparation and characterization of (oxindolylalanine^{25})glucagon and (methionine sulfoxide^{27})-glucagon were reported by Coolican et al. (1982). The preparation of N^{α}-maltoglucagon has also been described (Coolican, 1982). All peptides were purified by reverse-phase HPLC[1] or ion-exchange chromatography and were determined to be of sufficient purity that biological activity could not be accounted for by trace contamination.[2] Biological activity was measured in an adenylate cyclase assay conducted essentially according to Wright & Rodbell (1979) using partially purified plasma membranes of rat liver (England et al., 1982). Binding affinities were determined by the displacement of purified mono[^{125}I]iodoglucagon according to England et al. (1982).

Data Analysis. Experimental data were fit to the appropriate mathematical expression by unweighted nonlinear least-squares regression using the Gauss–Newton algorithm of the BMDX85 program (Dixon, 1970). All calculations and plotting were performed on a CDC 6600 computer connected to a Versatec 1200 plotter. Figures represent tracings of computer-generated plots.

Results

Activation of Adenylate Cyclase by Native Glucagon. Experimental data for the activation of adenylate cyclase over a wide range of concentrations of native glucagon are shown in Figure 1. The data were analyzed by curve fitting to a four-parameter equation (De Lean et al., 1978) of the form

$$A = A_{min} + \frac{A_{max} - A_{min}}{1 + (K_{0.5}/H)^{s}} \quad (1)$$

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[1] Abbreviations: HPLC, high-pressure liquid chromatography; Hse, homoserine.

[2] The possibility that trace contamination could be responsible for the partial agonist behavior of some of the derivatives is considered in detail elsewhere (Flanders et al., 1982).

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Table I: Statistical Analysis of Slope Factors and Receptor Affinities for Glucagon Binding and Activation

peptide	binding assay		adenylate cyclase assay		
	slope factor	p value[a]	slope factor	p value	$K_{0.5}$[b] (nM)
native glucagon	0.98 ± 0.11[c]	>0.20	0.91 ± 0.04	<0.05	1.9 ± 0.1
(oxindolylalanine[25])glucagon	1.11 ± 0.08	<0.20	1.02 ± 0.07	>0.20	1.6 ± 0.1
(Met sulfoxide[27])glucagon	1.06 ± 0.11	>0.20	0.82 ± 0.04	<0.005	3.9 ± 0.1
[des-Thr[29]]glucagon	ND[d]		0.85 ± 0.06	<0.05	5.6 ± 0.5
N^{ϵ}-acetimidoglucagon	1.15 ± 0.09	<0.10	1.03 ± 0.07	>0.20	6.6 ± 0.4
[des-Asn[28],Thr[29]](Hse[27])glucagon	0.92 ± 0.03	<0.20	0.69 ± 0.10	<0.05	62 ± 11
(*S*-methyl-Met[27])glucagon	0.80 ± 0.10	<0.20	0.87 ± 0.05	<0.10	101 ± 6
N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon	1.01 ± 0.05	>0.20	1.28 ± 0.20	>0.20	105 ± 15
[des-Asn[28],Thr[29]](Hse lactone[27])glucagon	1.05 ± 0.11	>0.20	0.88 ± 0.08	<0.20	109 ± 11
[des-Asn[28],Thr[29]]glucagon	1.01 ± 0.21	>0.20	0.82 ± 0.04	<0.005	115 ± 8
N^{α}-glycosylated glucagon	1.19 ± 0.12	<0.20	1.05 ± 0.10	>0.20	186 ± 21
[des-Asn[28],Thr[29]](*S*-methyl-Met[27])glucagon	0.90 ± 0.08	>0.20	0.94 ± 0.04	<0.20	546 ± 29

[a] Probability of unit slope based on the F test ratio for the sum of squares of the residuals. [b] Half-maximal activation concentration determined from eq 1. [c] Results are reported ± the standard error. [d] Not determined.

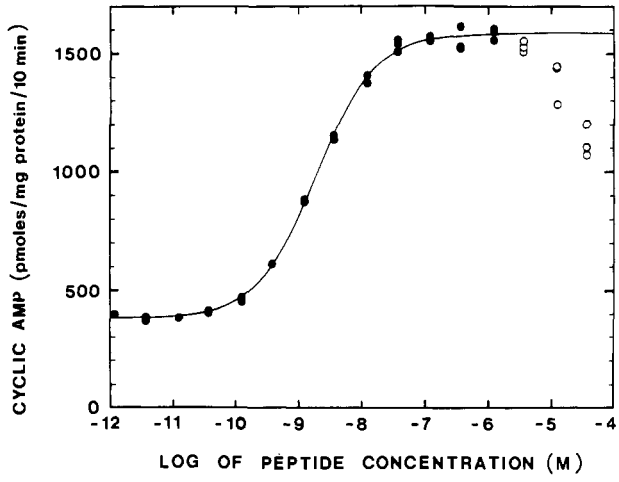


FIGURE 1: Activation of adenylate cyclase by native glucagon. Adenylate cyclase activity was assayed in the presence of 1 μM GTP under standard assay conditions using 2 mM [α-^{32}P]ATP as the substrate (England et al., 1982). The solid line represents the best fit of the data to eq 1 of the text. Data points not included in the fitting are denoted by open circles.

where A is the observed adenylate cyclase activity, H is the hormone concentration, A_{min} is the minimal (basal) activity, A_{max} is the maximal activity, $K_{0.5}$ is the concentration of hormone causing half-maximal activation, and s is a "slope factor" mathematically equivalent to the Hill coefficient of the Hill equation (Hill, 1910) and directly proportional to the slope of the activation curve at its midpoint when activity is plotted vs. the logarithm of the hormone concentration.[3] At high concentrations glucagon is observed to inhibit the activation of adenylate cyclase (Figure 1). Since eq 1 does not allow for a reduction in activity at high concentrations of hormone, for purposes of curve fitting to eq 1 all data points beyond the maximally activating concentration of hormone were excluded. For the data of Figure 1, $K_{0.5}$ was found to be 1.9 ± 0.1 nM and s was determined to be 0.91 ± 0.03. The data were also fit to eq 1 by a weighted regression procedure as advocated by Rodbard et al. (1976) to compensate for nonuniformity in variance. The replicate data (n = 3) of Figure 1 were averaged and weighted inversely proportional to the value of a quadratic function of the data variance. With this analysis $K_{0.5}$ values and slope factors were essentially unchanged, 1.8 ± 0.1 nM and 0.91 ± 0.03, respectively.

The averaged data for native glucagon were replotted according to Brown & Hill (1922) and fit by unweighted linear least-squares regression to verify that the slope factor determined from eq 1 adequately described the slope of the Hill plot throughout the normal (10–90%) dose–response range. Throughout this range the Hill plot would be expected to be very nearly linear with the maximum (or minimum) Hill coefficient occurring at the point of half-maximal response in the absence of complex allosteric interactions or a number of possible artifacts (Cornish-Bowden & Koshland, 1975). With this analysis, the Hill plot for native glucagon was highly linear (exclusive of the high-dose inhibition) and symmetrical about the half-maximal response point, indicating an absence of complex cooperativity or systematic error (England, 1982). Values of $K_{0.5}$ and s were found to be 1.8 ± 2.1 nM and 0.88 ± 0.02, respectively.

Analysis of Slope Factors for the Receptor Binding Interactions of Glucagon and Glucagon Derivatives. Table I gives the slope factors for the binding and adenylate cyclase activation curves of native glucagon and 11 glucagon derivatives. In several cases the slope factor differs substantially from 1, being less than 1 in most of these cases. In order to assess the statistical significance of the slope factors different from 1, the data were reanalyzed with the slope factor set equal to 1 and the results were evaluated on the basis of residual variance, by the use of the "extra sum of squares" principle (Draper & Smith, 1981) as utilized by De Lean et al. (1980). Accordingly, the residual sums of squares for the two fittings (s = 1 and $s \neq 1$) were compared by the F test to determine whether the better fit obtained with a slope other than unity was statistically significant. The probability (p) values corresponding to the F ratio statistic determined for each data set are indicated in Table I.

For the displacement binding assay curves the F tests for native glucagon and all the derivatives were nonsignificant (p > 0.05), indicating that the slope factor for binding is essentially 1. It would appear, therefore, that under the conditions of the assay, glucagon and all other derivatives in this study bind with a single equilibrium constant and do not bind cooperatively.

The slope factors for adenylate cyclase activation given in Table I are less than 1 (p < 0.05) for native glucagon and four of the derivatives. The data for the most extreme case [des-Asn[28],Thr[29]]glucagon are shown in Figure 2. By visual inspection the difference in slopes appears to be essentially

[3] The proportionality constant is $0.25(A_{max} - A_{min})$ log 10. As pointed out by De Lean et al. (1978), a value for the slope factor different from 1 is not necessarily an indication of cooperativity since the slope of a dose–response curve may be influenced by other effects.

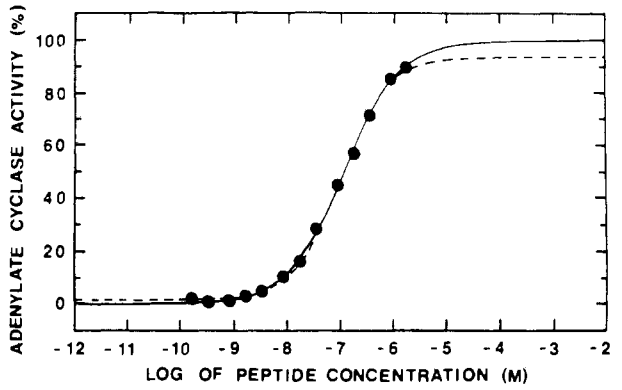


FIGURE 2: Comparison of the adenylate cyclase curves for [des-Asn^{28},Thr^{29}]glucagon. The solid curve was analyzed by fitting to eq 1 and has a slope value of 0.82. The dashed line represents the best fit to eq 1 with the slope factor set equal to 1. The circles are experimental data points.

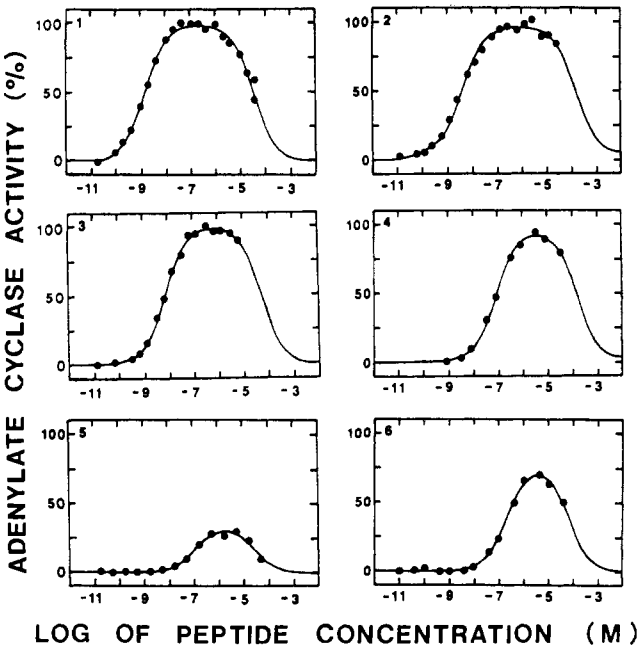


FIGURE 3: Inhibition of adenylate cyclase by glucagon derivatives. Solid lines are theoretical curves derived from fitting all data points to eq 2 of the text. Circles are experimental data points. 1, (oxindolylalanine^{25})glucagon; 2, (Met sulfoxide^{27})glucagon; 3, N^{ϵ}-acetimidoglucagon; 4, (*S*-methyl-Met^{27})glucagon; 5, N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon; 6, N^{α}-maltoglucagon.

negligible with the major difference between the curves being the levels of maximal and minimal response which were poorly defined by the data. These data could be interpreted as indicating that approximately half the receptors have a dissociation constant of 38 ± 9 nM and the other half a dissociation constant of 310 ± 70 μM, in contrast to a single dissociation constant of 115 nM (Donner et al., 1980). Alternatively the data could be taken as an indication of negative cooperativity since, as has been pointed out, it is not possible to distinguish between receptor heterogeneity and true negative cooperativity on the basis of equilibrium data alone (De Meyts et al., 1973).

High-Dose Inhibition of Adenylate Cyclase Activity. A striking feature of the activation data shown for six derivatives in Figure 3 is that for each of the derivatives inhibition of adenylate cyclase occurs at high hormone concentrations, as shown previously for native glucagon in Figure 1. The inhibitory phase was also evident for four of the other derivatives as shown in Table II but was less well defined as a result of less experimental data at the high hormone concentrations

Table II: Calculated Parameters for High-Dose Inhibition by Glucagon and Ten Glucagon Derivatives

peptide	K_A (nM)	K_I (μM)	A_{max} (%)
(oxindolyl-alanine^{25})glucagon	1.6 ± 0.1[a]	40 ± 4	99 ± 1
native glucagon	1.9 ± 0.1	62 ± 4	100 ± 1
(Met sulfoxide^{27})-glucagon	3.4 ± 0.3	190 ± 50	97 ± 1
[des-Thr^{29}]glucagon	4.8 ± 0.4	180 ± 460	96 ± 2
N^{ϵ}-acetimidoglucagon	6.9 ± 0.3	53 ± 10	101 ± 1
[des-Asn^{28},Thr^{29}]-(Hse^{27})glucagon	44 ± 8	2600 ± 2600	91 ± 4
(*S*-methyl-Met^{27})glucagon	92 ± 7	210 ± 40	97 ± 2
N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon	150 ± 20	35 ± 7	34 ± 2
[des-Asn^{28},Thr^{29}]-(Hse lactone^{27})-glucagon	99 ± 9	790 ± 860	97 ± 2
N^{α}-glycosylated glucagon	200 ± 20	72 ± 9	78 ± 2
[des-Asn^{28},Thr^{29}]-(*S*-methyl-Met^{27})-glucagon	540 ± 30	1500 ± 3600	99 ± 2

[a] Parameter estimates are reported ± their standard error.

required. Data for [des-Asn^{28},Thr^{29}]glucagon was not extended to supramaximal concentration. As shown, inhibition was observed with both full and partial agonists as well as with amino-terminal, central, and carboxyl-terminal region modifications.

For the purpose of characterizing this inhibition more fully the activation data were fit to a biphasic equation of the form used to describe competitive inhibition by high substrate concentration in enzyme systems (Dixon & Webb, 1979):

$$A = A_{min} + \frac{A_{max} - A_{min}}{1 + K_A/H + H/K_I} \quad (2)$$

A is the observed adenylate cyclase activity, H is the hormone concentration, K_A represents the equilibrium dissociation constant for hormone binding that leads to activation, K_I represents the equilibrium dissociation constant for binding that leads to inhibition, A_{max} is the theoretical maximal adenylate cyclase activity, and A_{min} is basal adenylate cyclase activity in the absence of added hormone. This equation corresponds to the equilibrium

$$R \overset{K_A}{\rightleftharpoons} HR \overset{K_I}{\rightleftharpoons} H_2R \quad (3)$$

where R is the unoccupied hormone receptor, HR is the active hormone–receptor complex, and H_2R is an inactive complex formed at very high dose levels where two hormone molecules share in subsite occupancy of a single receptor site. This system represents the simplest possible case of the "multisubsite" receptor binding model described by De Lean et al. (1979) since there is only one (active) form of HR complex and thus no microscopic heterogeneity.

According to Table II and Figure 3, the inhibition becomes evident in most cases at concentrations greater than 5–10 μM with a K_I value of approximately 50–200 μM. Since not all of the derivatives were studied at sufficiently high concentrations, the K_I values for some of the derivatives were not equally well defined. Due to the limited solubility of glucagon and many derivatives at neutral pH, it was not possible to extend the data to sufficiently high concentration to determine whether activity returns to basal levels. In the case of N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon, however, activity falls to near basal levels, and for (oxindolylalanine^{25})glucagon, cyclase levels

are inhibited to about one-third of maximum activity.

Aggregation as a Possible Cause for Inhibition. Since glucagon readily self-associates into trimers (Wagman et al., 1980), aggregation effects were considered to be a possible explanation for the high-dose inhibition as has been considered for the apparent negative cooperativity of insulin–receptor binding (Cuatrecasas & Hollenberg, 1976). However, except for the unlikely possibility of some supersaturation effect (Swann & Hammes, 1969), the trimerization of glucagon at high concentrations would not actually reduce the maximum equilibrium concentration of the monomeric species, though its relative proportion with respect to the trimer would be reduced. Thus, trimer formation could conceivably cause inhibition at high concentrations, but only if the trimer were itself a competitive inhibitor of glucagon action. We are unaware of any studies on the effects of trimerization on glucagon action, but dimers of glucagon linked through the carboxyl-region tryptophan-25 provide activation equivalent to monomeric glucagon on a molar basis (Wright & Rodbell, 1980). Moreover, the approximate bell symmetry of the activation curves also suggests that inhibition, like activation, is first-order dependent on hormone concentration and therefore not caused by trimerization. Furthermore, the high-dose inhibition was apparent for both (*S*-methyl-Met[27])glucagon and (Met-sulfoxide[27])glucagon which do not exhibit the self-association properties of native glucagon (Rothgeb et al., 1978; Coolican et al., 1982). Since all peptide samples were kept in an ice bath prior to the assay, the possibility was considered that inhibition might be an experimental artifact resulting from precipitation of the most concentrated samples. However, there was no noticeable effect on the inhibition for native glucagon when samples were maintained at room temperature to ensure solubility.

Inhibition as a Cause for Partial Agonism. The equilibrium constants for activation (K_A values) determined by curve fitting to eq 2 are in most cases within 5% of the half-maximal activation concentrations ($K_{0.5}$ values) determined by curve fitting to eq 1. This close agreement reflects the fact that the midpoints of the activation and inhibition phases of the dose–response curves are sufficiently well separated (>3 log units) so that the midpoints closely approximate the true equilibrium constants of the curves (Dixon & Webb, 1979). However, for the two partial agonists, N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon and N^{α}-glycosylated glucagon, the K_A and K_I values are not well separated (<2.5 log units). Consequently, there is a significant difference (>10%) between $K_{0.5}$ and K_A values determined by the two procedures. The experimentally observed maximal activities are also somewhat lower (>10%) than the calculated values (A_{max}) for the partial agonists since the K_A and K_I values are not sufficiently separated for the activation curves to reach their theoretical maxima, raising the question of whether partial agonism might itself be explained in terms of a lessening of the separation between the stimulatory and inhibitory phases of the dose–response curve.

The latter possibility was further investigated by fitting the activation data for the two partial agonists to eq 2 with the maximal activity (A_{max}) set equal to 100%. The best-fit curves obtained by this procedure are shown in Figure 4. In both cases the theoretical curves have Hill coefficients greater than 1.2 (at half-maximal activation) and appear to provide a rather poor description of the experimental data, suggesting that the partial agonism is not related to the inhibition. However, partial agonism can be readily incorporated into the equilibrium of eq 3 by allowing for the formation of inactive hormone–receptor (HR) complexes which are an inherent feature

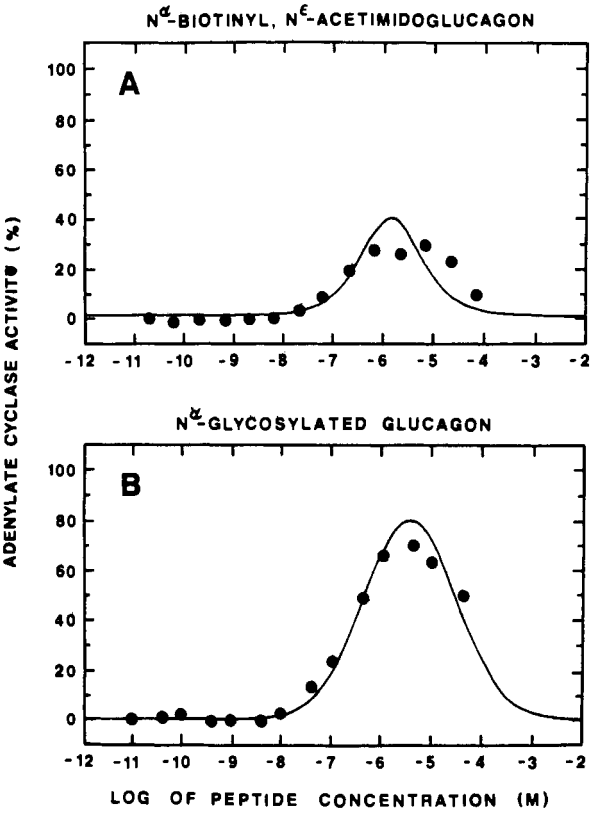
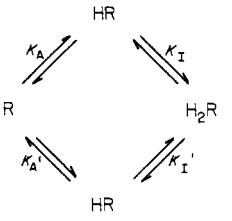


FIGURE 4: Partial agonism resulting from high-dose inhibition. The activation data for the two partial agonists, N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon and N^{α}-glycosylated glucagon, were fit to eq 2 of the text with the maximum activity set equal to 100%.

of the multisubsite model as originally proposed by De Lean et al. (1979). The equilibrium situation could be represented as



(4)

when the prime notation indicates nonproductive binding of the hormone to the receptor. The equilibrium equation for this system corresponding to eq 2 for the two equilibria system contains only three of the four dissociation constants since the fourth is redundant due to the thermodynamic constraints of the equilibrium. Thus, for the equilibrium of eq 4 this activity as a function of the hormone concentration would be

$$A = A_{min} + \frac{A_{max} - A_{min}}{1 + K_A/H + H/K_I + K_A/K_A'} \quad (5)$$

which can be rearranged to a form identical with that of eq 2:

$$A = A_{min} + \frac{(A_{max} - A_{min})r}{1 + K_A r/H + Hr/K_I} \quad (6)$$

where $r = K_A'/(K_A' + K_A)$. The ratio defined by r (between 0 and 1) is equivalent to the "intrinsic activity coefficient" as defined by De Lean et al. (1979). For a full agonist all binding is assumed to be productive so r is one ($K_A' = \infty$), and eq 6 becomes identical with eq 2 for a two-equilibria system. For a partial agonist r would be less than 1 so that the apparent

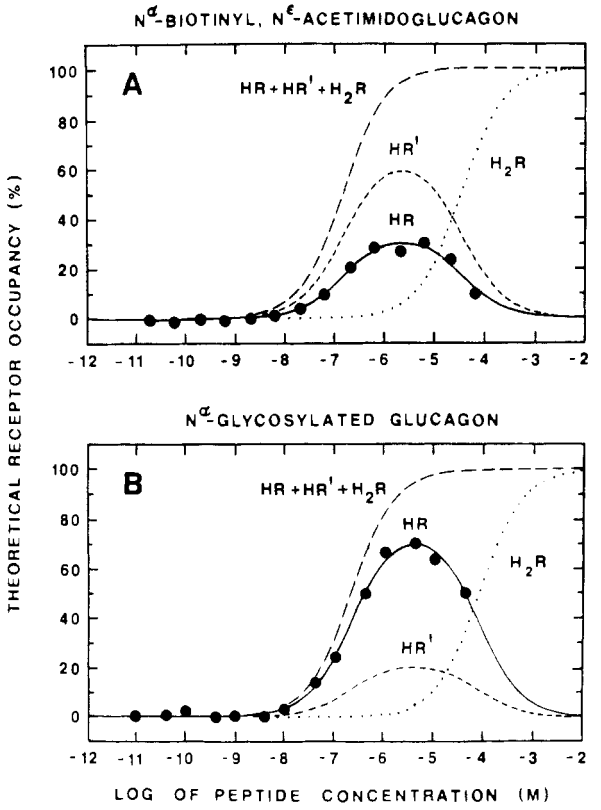


FIGURE 5: Partial agonism resulting from nonproductive binding. The activation data for the two partial agonist derivatives are superimposed on the theoretical curves representing the percentage of receptors in each of the hormone–receptor complexes of the equilibrium of eq 4 in the text. The solid curve represents the population of active HR complexes calculated from eq 6 and the values presented in the text. The other curves were calculated by using the appropriate permutations of eq 6.

maximal activity ($A_{max}r$) would be less than the true maximal activity (A_{max}) and the apparent K_A and K_I values would be respectively less than and greater than their true values.

If it is assumed that the true maximal activity of the partial agonist N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon is equal to that of native glucagon (100%), then from eq 6 and the data of Table II it is possible to calculate that the value of r is 0.34 and hence the true value of K_A is 400 nM and of K_I is 12 μM. From these can be calculated a value of K_A' of 210 nM and K_I' of 24 μM. Similar calculations for N$^{\alpha}$-glycosylated glucagon yield an r value of 0.77, a K_A value of 270 nM, K_I of 55 μM, K_A' of 910 nM, and K_I' of 16 μM. From these data it is possible to calculate the theoretical equilibrium populations of the various hormone–receptor complexes represented in the equilibria of eq 5, and these are plotted as a function of hormone concentration in Figure 5.

Figure 5 shows that for the glycosylated derivative about two-thirds of the hormone–receptor complexes represent productive binding and one-third represents nonproductive binding. This ratio is reversed for the biotinylated derivative which appears to be a weaker partial agonist, though as discussed elsewhere (Flanders et al., 1982) the same results would be obtained if the derivative only bound nonproductively and the productive binding represented trace agonist contamination. The experimental data are described equally well by both eq 2 and eq 6 since mathematically the equations are of exactly the same form. It is therefore not possible to determine by these methods whether the partial agonism results from a reduced number of fully active hormone–receptor complexes (eq 6) or a full complement of reduced activity binding complexes (eq 2). Neither the activation assay nor the binding assay allows for a separate quantitation of both the active and the inactive complexes which would be required in order to experimentally verify the dissociation constants of Table II. Unlike the case of true receptor heterogeneity, both the active and inactive hormone–receptor complexes are formed from the same receptor population and thus are not demonstrated in equilibrium binding studies. Finally, it should be noted in Figure 5 that despite the difference in the dissociation constants given above, the midpoints are identical for the curves corresponding to both the active and the inactive forms of the hormone–receptor complex. Therefore, no further information concerning the productive or nonproductive binding can be obtained from the apparent half-maximal activation (or binding) concentration of the equilibrium system of eq 4.

Discussion

Considerable uncertainty exists concerning the cooperative aspects of the glucagon–receptor interaction. Rodbell et al. (1974) and Lin et al. (1977) have reported that glucagon binding to rat liver plasma membranes demonstrates significant positive cooperativity (Hill coefficient = 1.5) at low concentrations while others have reported that this positive cooperativity is manifested only at high peptide concentrations (Demoliou-Mason & Epand, 1982). In contrast, negative cooperativity (Hill coefficient <1) or apparent negative cooperativity caused by receptor heterogeneity has been reported for glucagon binding to liver plasma membranes of rats (Sperling et al., 1980) and mice (Lafuse & Edidin, 1980) and for glucagon binding to isolated rat hepatocytes (Sonne et al., 1978, 1982). In contrast, Pingoud et al. (1982) have recently reported lack of cooperativity with binding to isolated hepatocytes.

In our studies with native glucagon both unweighted and statistically weighted analyses yield slope values from activation data (in the presence of GTP) not essentially different from 1. The Hill plot was linear and symmetrical over a concentration range, suggesting an absence of complex interactions and systematic error in the analyses. A similar lack of cooperativity was determined from the binding analyses. Hence, the data are inconsistent with either positive or negative cooperativity with respect to the glucagon–receptor interaction. The results thus confirm those of Pingoud et al. (1982) determined on isolated hepatocytes.

The binding data and adenylate cyclase activation data for the derivatives of glucagon were systematically examined. For all cases in which the data yielded slope values of less than 1 which might be interpreted to reflect cooperativity or receptor heterogeneity, the data were assessed to determine if a slope value different from 1 was likely to be significant. In no case was a reliable difference found.

The fact that a number of highly purified agonists containing modifications throughout the molecule and spanning a range of potency and agonism all have slope factors alike and equal to 1 indicates that these derivatives interact with a homogeneous glucagon–receptor system in a similar manner, at least with respect to the cooperativity of the interaction. The results should be compared, therefore, with the reported results in the β-adrenergic system in which subtle differences in cooperativity effects between full and partial agonists and antagonists are postulated to be directly related to the mechanism of adenylate cyclase activation (De Lean et al., 1980; Kent et al., 1980; Lefkowitz et al., 1982).

In the activation assay, GTP was present at 1 μM but it was not included in the binding assays of this study. Guanine nucleotides are known to affect the binding of glucagon and

its derivatives (Rodbell et al., 1971; Wright & Rodbell, 1980) and to modulate the slope factor for the receptor interaction of β-adrenergic compounds (Kent et al., 1980). Since the activation assays carried out in the presence of GTP and the binding assays carried out in the absence of GTP yielded slope factors which were essentially 1, it would seem unlikely that GTP has a significant effect upon the cooperativity of glucagon interactions. The physiological significance of this difference in the mechanism of glucagon and β-adrenergic interactions is unknown.

Our binding and activation data have been plotted with respect to total hormone concentration rather than the free hormone concentration which would be somewhat lower as a result of binding. Since there is no apparent correlation between the slope factors obtained and the binding affinities of the series of derivatives, the amount of receptor-bound hormone would seem to be insignificant for the purpose of defining the slope factor. This conclusion is consistent with an estimated receptor concentration of 0.1 nM obtained by fitting the native glucagon binding data to a generalized equilibrium binding equation (Hoffman et al., 1979) with the affinity of mono[^{125}I]iodoglucagon taken to be 3 times that of normal glucagon (Lin et al., 1976).

There are multiple technical problems associated with the determination of full binding and activation curves over the extended range of concentration required to define the slope factor accurately. Unless the extremes of the experimental curves are well-defined, the resulting slope factor may be misinterpreted to represent receptor heterogeneity or negative cooperativity.

Biphasic dose–response curves have been reported for a number of activators of adenylate cyclase including secretin (Moroder et al., 1980), β-adrenergic agonists, and prostaglandins (Sevilla et al., 1976; Ross et al., 1977), and GTP (Cooper et al., 1979). Less specific activators such as polylysine also produce a biphasic response in the cyclase system possibly through nonspecific electrostatic effects on membrane conformation (Wolff & Cook, 1975) brought about when the hormone adopts an α-helical conformation. The prominent inhibition seen in Figure 3 extends the observations of Wincek et al. (1975), England et al. (1982), and Demoliou-Mason & Epand (1982) for which the phenomenon was only suggested. Inhibition is not unique to full agonists and occurs with hormones modified throughout the molecule. Neither does it correlate with the ability to adopt a helical structure since the derivatives themselves have been examined for helical-forming potential (England et al., 1982; Coolican et al., 1982; Flanders et al., 1982) and found to differ in this regard. The K_I and K_A values differ sufficiently so that inhibition is not responsible for apparent partial agonism.

Our data have been fit to the multisubsite model of De Lean et al. (1979), but they can be fit to the antagonistic receptor model of Szabadi (1977) as well (unpublished), though we are unaware of evidence for the independent existence of inhibitory receptors for glucagon.

Nonspecific desensitization or exhaustion of the response system (Waud, 1968) is considered unlikely inasmuch as inhibition is clearly apparent for the partial agonists, the cyclase assay is linear with time up to 15 min in 1 μM glucagon, and the assays are unaltered by the inclusion of theophylline (unpublished). Birnbaumer & Pohl (1973) have found that the time course of glucagon action is inconsistent with the rate theory of drug action developed by Paton (1961). The rate theory furthermore predicts that partial agonists should have higher affinity than full agonists, which is contrary to the experimental data presented in Table I.

Although receptor cross-linking appears to be involved in other hormone systems (Mongar & Winne, 1966; Kahn et al., 1978; Schechter et al., 1979), we have considered this to be a less likely possibility for the glucagon system since the hexapeptide seems to activate adenylate cyclase and is unlikely to be of sufficient length to bridge two receptors though it might induce a conformation change in the receptor which would stabilize a receptor dimer (DeLisi, 1981). Furthermore, the data fit a cross-linking model somewhat less well in the region of maximum concentration (England, 1982). The theoretical binding isotherm which is necessary for any two-site receptor model should contain a second binding step (De Lean et al., 1979), but a lack of sufficiently high specific activity of available radiolabeled glucagon has precluded the differentiation of specific binding at the high concentration (μM) required.

Recently, two unifying models of β-adrenergic receptor interaction–adenylate cyclase activation have been presented (Swillens & Dumont, 1980; Stadel et al., 1982; Lefkowitz et al., 1982). In the β-adrenergic system, both high- and low-affinity receptors exist. GTP and the agonist properties of β-adrenergic ligands modulate the interconversion between the high- and the low-affinity state. These models explain the apparent differences in cooperativity for the binding of β-adrenergic agonists and antagonists as well as the higher affinity for binding than activation that is observed for β-adrenergic agonists but not for antagonists. In contrast, the studies reported here and elsewhere (Coolican, 1982; Coolican et al., 1982; England et al., 1982; Flanders et al., 1982) demonstrate that within experimental error, the half-maximum concentration for binding and activation for all these derivatives is equivalently reduced. Furthermore, there is no significant difference in cooperativity between full and partial agonists either in the activation of adenylate cyclase or in the binding to the receptor in the absence of GTP. Thus, it would appear that there is a significant difference between the interactions of glucagon and the interactions of β-adrenergic agonists with their respective receptors and that the two affinity state model developed for the β-adrenergic system may not be directly applicable to the glucagon system. Neither of the proposed models takes into consideration the inhibition of adenylate cyclase by high concentration of hormone or its agonists, although mechanisms of inhibition through auxiliary receptors (Lefkowitz et al., 1982) or by competition for the nucleotide binding protein (Swillens & Dumont, 1980) have been suggested. Since it would be unlikely that glucagon and 10 analogues would bind both receptor and transducer in the same manner, it seems unlikely that inhibition by competition for N could account for the inhibition noted.

Therefore, from the data and the analyses presented here, it can be seen that further studies on the receptor interaction of glucagon and its derivatives as well as various modulators of the adenylate cyclase system are needed in order to develop a comprehensive model for the glucagon effector system.

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Registry No. Glucagon, 16941-32-5; adenylate cyclase, 9012-42-4; (oxindolylalanine^{25})glucagon, 83874-24-2; (Met sulfoxide^{27})glucagon, 75217-63-9; [des-Thr^{29}]glucagon, 80722-24-3; N^{ϵ}-acetimidoglucagon, 74619-75-3; [des-Asn^{28},Thr^{29}](Hse^{27})glucagon, 42360-83-8; (*S*-methyl-Met^{27})glucagon, 68600-12-4; N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon, 84647-85-8; [des-Asn^{28},Thr^{29}](Hse lactone^{27})glucagon, 55758-07-1; [des-Asn^{28},Thr^{29}]glucagon, 25684-47-3; N^{α}-maltoglucagon, 84647-86-9; [des-Asn^{28},Thr^{29}](*S*-methyl-Met^{27})glucagon, 80722-25-4.

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