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Mechanism of action of des-His[1]-[Glu[9]]glucagon amide, a peptide antagonist of the glucagon receptor system

(saponin-permeabilized hepatocytes/glucagon–receptor interactions/adenylyl cyclase/GTP/Mg^{2+})

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ABSTRACT **We have investigated the mechanisms through which des-His[1]-[Glu[9]]glucagon amide functions as a peptide antagonist of the glucagon receptor/adenylyl cyclase system. Studies with radiolabeled peptides identified that (*i*) the antagonist bound to intact hepatocytes according to a single first-order process, whereas the rate of association of glucagon with the same preparation could be described only by the sum of two first-order processes; (*ii*) the interaction of the antagonist with saponin-permeabilized hepatocytes was not affected by the addition of GTP to the incubation medium or by the elimination of Mg^{2+}, whereas the interaction of glucagon with the same cell preparation was modified significantly by the presence of the nucleotide or by the absence of the divalent metal ion; (*iii*) the dissociation of antagonist from intact hepatocytes incubated in buffer was complete, whereas that of agonist was not; and (*iv*) the antagonist bound to intact hepatocytes at steady state according to a single binding isotherm (as did both agonist and antagonist in permeabilized hepatocytes), whereas glucagon bound to the intact cell system with two clearly defined apparent dissociation constants. A model is presented for the mechanism of action of the glucagon antagonist in which the analog binds to glucagon receptors in a Mg^{2+}- and GTP-independent fashion and in which resulting ligand–receptor complexes fail to undergo sequential adjustments necessary for the stimulation of adenylyl cyclase.**

Receptor antagonists are analogs of natural hormones that interact with receptor but that for one reason or another do not stimulate the production of second messengers and do not induce the usual complement of ensuing cellular and biological responses. The β-adrenergic receptor system has been particularly well-studied in this respect. Antagonist analogs of the natural hormone epinephrine have been shown to associate with the β-adrenergic receptor in competitive and specific ways and to inhibit the action of the hormone to stimulate the receptor-linked enzyme adenylyl cyclase (1–3). In contrast to ligand–receptor interactions characteristic of β-adrenergic agonists, those applicable to β-adrenergic antagonists are not sensitive to modulation by guanine nucleotides (4–6) and do not result in the slow dissociation of previously bound ligand that attends receptor desensitization (3, 6, 7). Although recent work has identified a detailed molecular framework on which to differentiate agonist and antagonist binding to the β-adrenergic receptor (8–10), relatively little is known about related mechanisms as they apply to the glucagon receptor.

Structure–function studies in the glucagon–receptor system have identified significant kinetic complexity in ligand–receptor interactions (11–13), important differences in these interactions that apply to intact cell and plasma membrane preparations (11–17), and difficulty in localizing structural elements that might play critical roles in directing the interaction of the hormone with its receptor (18–21). Further complexity in this system arises from membrane-associated glucagon–receptor complexes that exhibit high and low affinity for ligand and reversible and irreversible components of dissociation (11–13, 17). Although several glucagon analogs, including des-His[1]-glucagon and N^{α}-trinitrophenyl-His[1]-[Har[12]]glucagon (where Har is homoarginine), have been identified to represent partial agonists and antagonists of glucagon action (21–25), a high-affinity antagonistic glucagon analog with immeasureably low biological activity has only recently become available. Through a series of definitive studies, Merrifield and coworkers (26–28) have identified the peptide des-His[1]-[Glu[9]]glucagon amide as a full glucagon antagonist at receptors present on hepatic plasma membranes.

Because of the potential value of glucagon antagonists in discerning the mechanisms of glucagon action and in developing a drug that might alleviate the paradoxically elevated gluconeogenesis characteristic of diabetes (29, 30), we undertook to study in detail the interactions of des-His[1]-[Glu[9]]glucagon amide with intact and saponin-permeabilized canine hepatocytes. Our findings identify the analog as a glucagon antagonist in these systems and provide a framework on which to consider the mechanisms by which the analog exerts its antagonistic effects at the glucagon receptor/adenylyl cyclase couple.

MATERIALS AND METHODS

Peptides. Crystalline natural glucagon and lyophilized synthetic des-His[1]-[Glu[9]]glucagon amide were purchased from Elanco (Indianapolis) and Bachem, respectively. Both peptides were radioiodinated by the chloramine-T-based method that has previously been applied in this laboratory (31) and were purified on reverse-phase columns by use of high performance liquid chromatography (31) coupled with an isocratic solution phase consisting of a mixture of acetonitrile and triethylammonium phosphate buffer (31) in the ratio 27:73 (vol/vol). By use of a flow rate corresponding to 1 ml/min, radiolabeled glucagon was eluted after ≈42 min, and the radiolabeled analog was eluted after ≈35 min.

Hepatocyte Isolation and Incubation. Procedures for the isolation of intact canine hepatocytes through the use of collegenase digestion have been described (12, 31, 32), as have procedures for the permeabilization of isolated hepatocytes by use of saponin (final concentration, 30 μg/ml) (33). Intact hepatocytes exhibited >96% viability, as judged by exclusion of the dye trypan blue, and were incubated under an atmosphere of 95% O_2/5% CO_2 in standard Krebs–Ringer bicarbonate (KRB) buffer containing 115 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine, 1% bovine serum albumin (fraction V), and essential and nonessential amino acids formulated according to Eagle's minimal essential medium (all adjusted to pH 7.4). Saponin-permeabilized hepatocytes (prepared from intact cells exhibiting >96% viability) showed >99% of cells being

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stained by trypan blue and were incubated under an atmosphere of 95% O_2/5% CO_2 in reverse KRB buffer containing 20 mM NaCl, 100 mM KCl, 1 mM NaH_2PO_4, and 25 mM $NaHCO_3$ plus the supplements noted above. This reverse buffer was supplemented further with 1 mM EDTA or with 3 mM $MgSO_4$. Incubations of both intact and permeabilized cells occurred at cell densities of 2×10^6 cells per ml at 30°C (31, 33). The concentration of ^{125}I-labeled peptide was ≈12 pM (50,000 cpm/ml) in studies of ligand–receptor interactions. Each experiment consisted of at least duplicate determinations and was repeated on multiple occasions with similar results.

Assays for cAMP Formation. The accumulation of cAMP in intact hepatocytes (as altered by the presence of glucagon and des-His^1-[Glu^9]glucagon amide) followed the course as described (34). cAMP was determined by radioimmunoassay based on use of ^{125}I-labeled cAMP (from ICN) and an antibody directed against cAMP (from Sigma). The formation of cAMP in saponin-permeabilized hepatocytes followed from the incubation of permeabilized cells in reverse KRB buffer containing 1.5 mM ATP, 50 mM creatine phosphate, 2 mM theophylline, 3 mM Mg^{2+}, and creatine phosphokinase at 5 units/ml (all from Sigma). At the close of the incubation, cells were pelleted by centrifugation, aliquots of the supernatant were treated with calf intestine alkaline phosphatase, and the resulting mixture was subjected to radioimmunoassay for cAMP.

RESULTS

Initial studies compared the activities of des-His^1-[Glu^9]glucagon amide and glucagon in modulating the activity of adenylyl cyclase in isolated intact and saponin-permeabilized canine hepatocytes (11–13, 31–34). Fig. 1*a* identifies for intact hepatocytes (*i*) the ability of glucagon to stimulate the intracellular accumulation of cAMP at low concentrations, (*ii*) the inhibition of cAMP accumulation that applies when the concentration of hormone is raised above ≈3 nM (cf. ref. 34), (*iii*) the inability of the glucagon analog to affect basal intracellular cAMP levels at concentrations as high as 10 μM, and (*iv*) the action of the analog to inhibit by half the cAMP accumulation induced by 3 nM glucagon at a concentration of 30 nM during the coincubation of cells with both peptides. Fig. 1*b* presents related data obtained by use of permeabilized hepatocytes. Notably, (*i*) the action of high concentrations of glucagon to inhibit adenylyl cyclase is lost in this system whereas the stimulatory action of the hormone is clear, (*ii*) the analog is without effect on the basal activity of adenylyl cyclase, and (*iii*) the analog at ≈100 nM inhibits by half the stimulation of adenylyl cyclase that is induced by 10 nM hormone. Thus, these findings identify the analog des-His^1-[Glu^9]glucagon amide as a true antagonist of glucagon action in both experimental preparations.

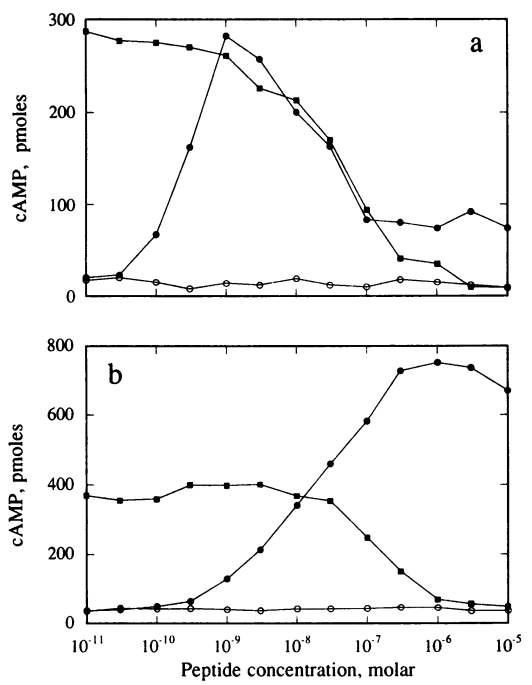

FIG. 1. Stimulation and inhibition of cAMP formation in intact and saponin-permeabilized canine hepatocytes by glucagon and des-His^1-[Glu^9]glucagon amide. (*a*) Intact hepatocytes: intracellular cAMP levels obtained by incubating cells with glucagon at the concentrations indicated (●), the analog at the concentrations indicated (○), or 3 nM glucagon plus the analog at the concentrations indicated (■). (*b*) Permeabilized hepatocytes: extracellular cAMP levels obtained by incubating cells with glucagon at the concentrations indicated (●), the analog at the concentrations indicated (○), or 20 nM glucagon plus the analog at the concentrations indicated (■). The ordinate plots the level of cAMP generated by 2×10^6 cells during incubations for 30 min at 30°C. The incubation medium for permeabilized cells contained 3 mM Mg^{2+}.

Further experiments were directed toward comparing des-His^1-[Glu^9]glucagon and glucagon in their interactions with the hepatic glucagon receptor. Fig. 2*a* presents findings identifying for ^{125}I-labeled glucagon (*i*) the enhanced binding of the radiolabeled hormone in permeabilized cells incubated in the presence of Mg^{2+} relative to that achieved in intact cells and (*ii*) the effect in permeabilized hepatocytes of deleting the divalent metal ion to decrease the steady-state level of radio-

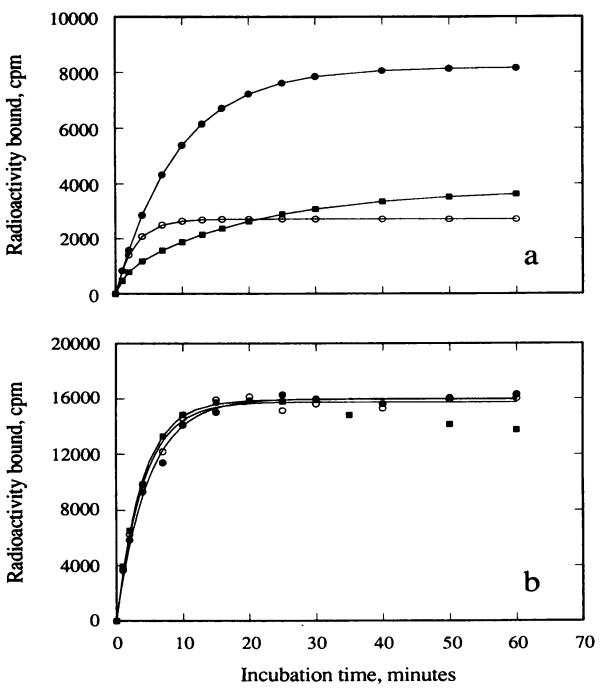

FIG. 2. Time courses for the association of ^{125}I-labeled glucagon and ^{125}I-labeled des-His^1-[Glu^9]glucagon amide with intact and saponin-permeabilized canine hepatocytes. Intact and permeabilized hepatocytes were incubated with ≈12 pM radiolabeled peptides for various periods at 30°C in standard KRB buffer or reverse KRB buffer, respectively. (*a*) Binding of ^{125}I-labeled glucagon to intact cells (■), to permeabilized cells incubated in the presence of 3 mM $MgSO_4$ (●), and to permeabilized cells incubated in the presence of 1 mM EDTA (○). (*b*) Binding of ^{125}I-labeled analog to intact cells (■), to permeabilized cells incubated in the presence of 3 mM $MgSO_4$ (●), and to permeabilized cells incubated in the presence of 1 mM EDTA (○). The curve representing glucagon binding to intact cells was mathematically modeled as the sum of two first-order processes. All other curves represent mathematically modeled functions describing single first-order processes. The data in *a* are adapted from ref. 33.

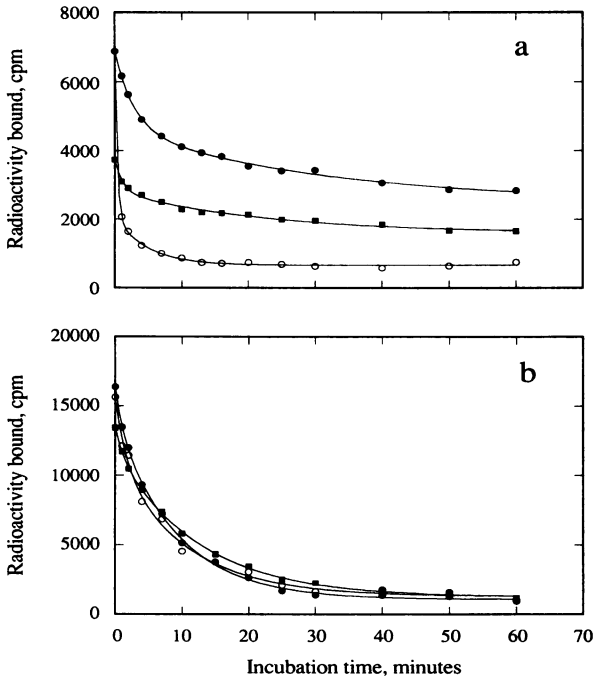

FIG. 3. Time courses for the dissociation of ^{125}I-labeled glucagon and ^{125}I-labeled des-His^1-[Glu^9]glucagon amide from intact and saponin-permeabilized canine hepatocytes. Cells were incubated with 12 pM radiolabeled ligand for 30 min at 30°C in standard KRB buffer or in reverse KRB buffer containing 3 mM $MgSO_4$, as appropriate, prior to removing a sample for the zero-time control. Radiolabeled ligand remaining bound to receptor subsequent to the addition of 1 μM glucagon or 1 μM analog (*a* or *b*, respectively, to prevent the measurable further binding of radiolabeled ligand) was assessed after various periods of further incubation at 30°C. (*a*) Dissociation of ^{125}I-labeled glucagon from intact cells (■), from permeabilized cells (●), and from permeabilized cells upon the addition of 30 μM GTP (○). (*b*) Dissociation of ^{125}I-labeled analog from intact cells (■), from permeabilized cells (●), and from permeabilized cells upon the addition of 30 μM GTP (○). All curves represent mathematically modeled functions describing the sum of two first-order processes. The data in *a* are adapted from ref. 33.

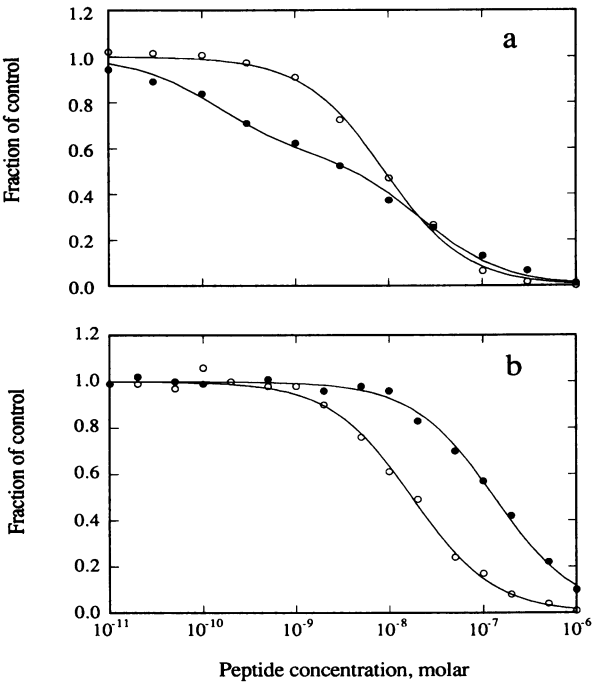

FIG. 4. Inhibition by glucagon and des-His^1-[Glu^9]glucagon amide of ^{125}I-labeled glucagon and ^{125}I-labeled des-His^1-[Glu^9]glucagon amide binding to intact canine hepatocytes at steady state. Hepatocytes were incubated in standard KRB buffer with 12 pM radiolabeled ligand in the presence of selected concentrations of either glucagon or the analog for 30 min at 30°C. (*a*) Inhibition of ^{125}I-labeled glucagon binding by glucagon (●) and by the analog (○). (*b*) Inhibition of ^{125}I-labeled analog binding by glucagon (●) and by the analog (○). The data have been corrected for so-called nonspecific binding (radioactivity remaining cell associated in the presence of 10 μM glucagon) and have been plotted for clarity as a fraction of control binding (that occurring in the absence of added competitor). The level of control binding was similar to that shown in Fig. 2. The curve representing the inhibition of ^{125}I-labeled glucagon binding by glucagon was mathematically modeled in terms of hormone association with two noninteracting populations of binding sites. All other curves represent mathematically modeled functions describing the inhibition of radiolabeled ligand binding to a single population of binding sites.

labeled hormone binding achieved (cf. ref. 33). In stark contrast, Fig. 2*b* shows that the rate and steady-state level of binding of the ^{125}I-labeled analog to hepatocyte receptors are the same in both intact and permeabilized cells and is independent of the presence of Mg^{2+} in the permeabilized cell preparation. The sum of two first-order processes required to model the association of ^{125}I-labeled glucagon with intact hepatocytes ($K_{observed}$ = 0.05 and 0.7 min^{-1}, cf. ref. 33) can be contrasted with the single first-order process that suffices for modeling the association of the ^{125}I-labeled analog ($K_{observed}$ = 0.1 min^{-1}) in the same preparation. Accordingly, it appears that complex aspects of ligand–receptor interactions applicable to glucagon do not apply to the analog. Related results that assess the dissociation of ligand from previously formed hormone–receptor complexes are presented in Fig. 3. Fig. 3*a* documents for previously formed complexes of receptor with ^{125}I-labeled glucagon (*i*) the incomplete dissociation of hormone from receptor in both intact and permeabilized cells and (*ii*) the effect of GTP to enhance the rate and extent of hormone dissociation from receptor in the permeabilized cell system (see ref. 33). Fig. 3*b* shows, again in stark contrast to results obtained with glucagon, that for complexes formed between receptor and ^{125}I-labeled analog, the dissociation of ligand from receptor is rapid and complete for both intact and permeabilized hepatocytes. Further, the dissociation of analog is independent of the presence of GTP in the permeabilized cell preparation.

Results presented above identify marked differences between the interactions of ^{125}I-labeled glucagon and ^{125}I-labeled des-His^1-[Glu^9]glucagon amide with hepatic glucagon receptors. Subsequent experiments assessed both the importance of these differences during steady-state binding and the relative affinities of ligand–receptor interactions that apply. On the one hand, Fig. 4*a* identifies for intact hepatocytes that the inhibition of radiolabeled glucagon association with receptor by glucagon follows a complex profile indicative of multiple ligand–receptor populations with different affinities for ligand (see refs. 11–13), whereas the inhibition of radiolabeled ligand association with receptor by the analog follows a much more simple course. Results obtained by mathematical modeling (12, 13, 33) identify two apparent dissociation constants for ligand–receptor interactions when glucagon is used as the competitor (K_d = 0.1 and 20 nM), but only one when the analog is used (K_d = 9 nM). On the other hand, Fig. 4*b* identifies for permeabilized hepatocytes that the inhibition of radiolabeled analog association with receptor by glucagon and by analog both exhibit simple binding profiles corresponding to single binding isotherms (K_d = 130 and 17 nM, respectively; see Table 1 for details). Thus, for intact hepatocytes, it appears that (*i*) ^{125}I-labeled glucagon has access to states of ligand–receptor interactions that are not available to the analog; (*ii*) all ligand–receptor interactions are inhibited

Table 1. Apparent dissociation constants for interactions of glucagon receptors on intact and permeabilized canine hepatocytes with glucagon and des-His[1]-[Glu[9]]glucagon amide

Cell preparation	Mg^{2+}, mM	Tracer	Inhibitor	K_d, nM
Intact	1.2	Glucagon	Glucagon	0.14, 23
	1.2	Glucagon	Analog	9.1
	1.2	Analog	Glucagon	130
	1.2	Analog	Analog	17
Permeable	3	Glucagon	Glucagon	7.9
	3	Glucagon	Analog	8.4
	3	Analog	Glucagon	30
	3	Analog	Analog	7.7
	0	Glucagon	Glucagon	34
	0	Glucagon	Analog	5.3
	0	Analog	Glucagon	36
	0	Analog	Analog	11

Intact and saponin-permeabilized isolated canine hepatocytes were incubated with radiolabeled glucagon or radiolabeled des-His[1]-[Glu[9]]glucagon amide and the corresponding unlabeled peptides in various combinations. Data obtained from resulting competition curves (all of which resulted in complete inhibition of radiolabeled ligand binding, see Fig. 4) were mathematically modeled by computer-assisted nonlinear methods according to equations developed to assess apparent dissociation constants in terms involving the inhibition of binding of radiolabeled tracer by unlabeled peptide (12, 13, 33). All inhibitions were successfully modeled in terms of single binding isotherms with the exception of the inhibition of radiolabeled glucagon to intact cells by glucagon. In this case, the two apparent dissociation constants required to describe the complex binding behavior are given.

completely, no matter which probe is used as tracer or as competitor; (*iii*) the binding of radiolabeled glucagon or radiolabeled analog to receptor does not allow the identification of sites that might be specific for one peptide or the other; and (*iv*) differences between the interactions of glucagon and analog with receptor must arise from sequential, rather than from parallel, processes.

Additional findings on steady-state ligand interactions with the glucagon receptor in saponin-permeabilized hepatocytes are presented in Table 1. It is notable that (*i*) no matter which ligand is used as tracer or competitor, all binding inhibitions are complete and can be modeled in terms of single binding equilibria; (*ii*) notwithstanding the decrease in affinity that attends the inhibition of radiolabeled glucagon interaction with receptors by glucagon in the absence of Mg^{2+} (cf. ref. 33), no interaction of the analog with receptor can be identified as being importantly affected by the presence of the divalent metal ion; and (*iii*) values for most relevant apparent dissociation constants fall into the range 5–36 nM, values quite different from those obtained for high-affinity glucagon–receptor interactions in intact hepatocytes (K_d = 0.14 nM) or in permeabilized hepatocytes incubated in the presence of GTP (K_d = 0.2 nM; ref. 33). In steady-state and in rate experiments (see Figs. 2 and 3), the interactions of the analog with glucagon receptors on permeabilized cells were not affected by the guanine nucleotide (data not shown). These results further identify that the complexity applicable to interactions of the glucagon receptor with glucagon does not apply to the interactions of the glucagon receptor with the antagonist analog. They also imply that the analog is capable of existing in combination with only a limited number of receptor states that apply to the natural hormone.

DISCUSSION

Results presented here confirm in two additional systems (intact and saponin-permeabilized isolated canine hepatocytes) the discovery of Merrifield and coworkers (26–28) that des-His[1]-[Glu[9]]glucagon amide is an antagonist of glucagon action in isolated hepatic plasma membranes. Since the analog competes fully for the interaction of glucagon with receptor (refs. 26–28 and this report), there seems little question that, overall, its antagonism of glucagon action depends upon its binding to the glucagon receptor and its ensuing action to prevent the binding of the natural hormone (thus to preclude the hormone from otherwise exerting an effect in stimulating the production of cAMP). Since the C^{α}-carboxamide group of the analog appears only to enhance somewhat the affinity of interaction between the glucagon receptor and the modified hormone and since the deletion of the NH_2-terminal histidinyl residue of the hormone results in only a partial agonist and antagonist in the system, the replacement of Asp^9 appears to be critical to the formation of an hepatic glucagon antagonist (26–28). Whereas the partial agonism exerted by des-His[1]-[Glu[9]]glucagon amide in stimulating glucose-potentiated insulin secretion from isolated pancreatic islets (35) remains incompletely understood, the behavior of the analog as full glucagon antagonist at the liver contributes to considering the beneficial role of related compounds in decreasing inappropriate hepatic glucose output (while minimally affecting insulin secretion) during a potential course of therapy for both type I and type II diabetes (25, 29, 30).

Notwithstanding the potential value of des-His[1]-[Glu[9]]glucagon amide or its congeners in diabetes therapy, an important set of questions concerns why the analog exhibits no ability to stimulate adenylyl cyclase in liver and how it exerts its actions as a glucagon antagonist. Results presented here provide important clues. That is, all in clear contrast to glucagon–receptor interactions, interactions of the analog with hepatic glucagon receptors (*i*) exhibit simple rather than complex binding isotherms at steady state in intact cells, (*ii*) show no dependence on the presence of Mg^{2+} or GTP in permeabilized cells (whether viewed in terms of steady-state levels of binding achieved, rate or extent of dissociation from preformed ligand–receptor complexes, or apparent affinities of receptor for ligand), and (*iii*) seem to define a subset of ligand–receptor states that might otherwise be achieved by the natural hormone. It is important to note, however, that this subset must represent a limitation to achieving sequential, rather than parallel, states of ligand–receptor interactions since the analog competes completely for glucagon–receptor interactions, and vice versa, under all conditions studied. For example, (*i*) notwithstanding the complexity with which glucagon competes for radiolabeled glucagon–receptor interactions in intact cells, des-His[1]-[Glu[9]]glucagon amide competes for glucagon–receptor interactions in a complete but simple way, and (*ii*) notwithstanding the high dissociation constant applicable to the inhibition of the binding of the analog to intact cells by glucagon, the inhibition is again complete and apparently homogenous.

The model based on studies of both intact and saponin-permeabilized hepatocytes presented previously to describe a glucagon receptor cycle (33) provides a framework on which to consider the interactions of des-His[1]-[Glu[9]]glucagon amide with the hepatic glucagon receptor. Most important, the model proposes a sequential process by which glucagon interacts with receptor in a Mg^{2+}-independent way, the resulting ligand–receptor complex is converted to a Mg^{2+}-sensitive state, and this state is converted to one that is capable of being affected by nucleotide exchange occurring on a heterotrimeric guanine nucleotide binding protein associated with the receptor. In agreement with data reported, for example, for antagonists of the β-adrenergic and vasopressin receptor systems (4–6, 36–39), the glucagon antagonist des-His[1]-[Glu[9]]glucagon amide appears to interact with hepatic glucagon receptors in a Mg^{2+}- and guanine nucleotide-independent fashion and fails to undergo modifications of initial ligand–receptor interactions that would otherwise re-

sult in the very slow dissociation of ligand from receptor and in consequent short-term receptor desensitization. On the one hand, results presented here suggest that limitations in the progress of antagonist–receptor complexes to achieve Mg^{2+}- and guanine nucleotide-sensitive states alter the kinetic characteristics observed for agonist interactions with the hepatic glucagon receptor and that these same limitations preclude the formation of ligand–receptor complexes that might reflect high-affinity binding, that might exhibit the incomplete dissociation typical of complexes containing glucagon, and that might stimulate adenylyl cyclase. On the other hand, our results identify more completely than before the critical roles of Mg^{2+} and guanine nucleotides in the sequential processing of ligand–receptor complexes to states effective in transmembrane signaling in the glucagon receptor system.

Thus, results presented here help to define the mechanism by which des-His^1-[Glu^9]glucagon amide behaves as a glucagon antagonist and to augment our understanding of the interactions of ligand with the glucagon receptor system. Many questions remain to be addressed, including how the structure of the analog precludes attainment of the full complement of ligand–receptor interactions achieveable by glucagon, how the structures of initial ligand–receptor complexes in the glucagon–receptor system determine their potential for assuming Mg^{2+}- and guanine nucleotide-dependent states, and how glucagon receptor antagonists might be applied to the treatment of both insulin-dependent and insulin-independent diabetes mellitus.

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