

Synthetic peptide antagonists of glucagon

(des-His¹-[Glu⁹]glucagon amide/hepatic membrane binding/adenylate cyclase activation/solid-phase peptide synthesis)

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ABSTRACT Several glucagon analogs were synthesized in an effort to find derivatives that would bind with high affinity to the glucagon receptor of rat liver membranes but would not activate membrane-bound adenylylase and, therefore, would serve as antagonists of the hormone. Measurements on a series of glucagon/secretin hybrids indicated that replacement of Asp⁹ in glucagon by Glu⁹, found in secretin, was the important sequence difference in the N terminus of the two hormones. Further deletion of His¹ and introduction of a C-terminal amide resulted in des-His¹-[Glu⁹]glucagon amide, which had a 40% binding affinity relative to that of native glucagon but caused no detectable adenylylase activation in the rat liver membrane. This antagonist completely inhibited the effect of a concentration of glucagon that alone gave a full agonist response. It had an inhibition index of 12. The pA₂ was 7.2. An attempt was made to relate conformation with receptor binding. The peptides were synthesized by solid-phase methods and purified to homogeneity by reverse-phase high-performance liquid chromatography on C₁₈-silica columns.

Due to the renewed interest in the role of glucagon in diabetes and the maintenance of normal blood glucose levels (1), the development of antagonists of glucagon has become increasingly important. The recent discoveries of dual cell membrane binding sites for glucagon and secretin, which lead not only to the activation of adenylylase but also to stimulation of the hydrolysis of phosphatidylinositol bisphosphate and the events that follow, have already demonstrated the value of selective analogs of these hormones (2, 3). Our initial efforts to design potential antagonists of glucagon, based on the synthesis of replacement analogs with altered peptide chain conformation, led to some derivatives that were completely inert toward activation of adenylylase in the liver plasma membrane but retained weak binding affinity and could be demonstrated to reversibly inhibit the action of glucagon (4). Our next approach to the search for antagonists was based on the idea that secretin, which has significant homology with glucagon but does not bind to the glucagon receptors of the hepatocyte (5), may have evolved from glucagon or a common precursor by a series of steps such that the intermediates retained significant affinity for the glucagon receptor but lost the ability to transduce the signal to activate adenylylase (6). If that were true, hybrids of secretin and glucagon might have the desired properties. A comparison of the structure of secretin relative to that of glucagon shows only three sequence changes in the first 12 N-terminal residues: Asp³ for Gln³, Glu⁹ for Asp⁹, and Arg¹² for Lys¹². Initial studies showed that the synthetic hybrids [Asp³,Glu⁹,Arg¹²]glucagon and [Asp³,Glu⁹]glucagon were totally inactive (<0.001%) in terms of cAMP production but retained approximately 2% of glucagon binding affinity and could completely inhibit a concentration of glucagon that alone gave a maximal rise in cAMP (6). From this and

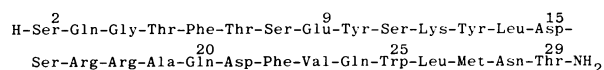


FIG. 1. Sequence of des-His¹-[Glu⁹]glucagon amide.

subsequent work, it was concluded that neither Arg¹² nor Asp³ were primarily responsible for the effect (7). We report here that replacement of Asp⁹ by Glu⁹ was the important change. We also show that further changes, including deletion of residue 1 and introduction of a C-terminal amide, lead to a fully inactive peptide, des-His¹-[Glu⁹]glucagon amide (Fig. 1), which has high receptor affinity and a relatively good inhibition index.

MATERIALS AND METHODS

Materials. *tert*-Butyloxycarbonyl (Boc)-protected amino acids were from Peninsula Laboratories (San Carlos, CA), *p*-methylbenzhydramine-resin (0.45 mmol/g) was from United States Biochemical (Cleveland, OH), and Boc-*O*-benzylthreonine-4-oxymethyl-phenylacetamidomethylcopoly-(styrene/1% divinylbenzene) was prepared as described by Mitchell *et al.* (8). All other reagents used for syntheses on the Applied Biosystems 430 A peptide synthesizer were obtained from the manufacturer. Natural porcine glucagon (Sigma) was purified on a dedicated reverse-phase C₁₈-silica column as described (7) to avoid cross-contamination with the synthetic analogs. ¹²⁵I-labeled glucagon from New England Nuclear was used without further purification for periods up to 1 month after its preparation. Creatine phosphate, creatine kinase, bovine serum albumin, dithiothreitol, GTP, and ATP were from Sigma. A cAMP assay kit containing [8-³H]cAMP was from Amersham. Nuflow membrane filters (0.45 μm) were from Oxoid (Basingstoke, England).

Peptide Synthesis. All peptides were synthesized by solid-phase methods (9) on the ABI instrument using procedures developed for the synthesis of natural glucagon (10). Analogs with a free C-terminal carboxyl were made on phenylacetamidomethyl-resin supports, and those with C-terminal amides were made on a methylbenzhydramine-resin. Side chain protection was Arg(Tos), Asp(OcHx), Glu(OcHx), His(Tos), Lys[Z(Cl)], Ser(Bzl), Thr(Bzl), Trp(For), and Tyr[Z(Br)]. [Tos, tosyl; cHx, cyclohexyl; Z(Cl), 2-chlorobenzoyloxycarbonyl; Z(Br), 2-bromobenzoyloxycarbonyl; Bzl, benzyl; For, formyl.] Double couplings with preformed symmetric anhydrides in dimethylformamide were used routinely for all *tert*-butyloxycarbonyl-protected amino acids except for tosyl arginine, glutamine, and asparagine, where *N*¹-hydroxybenzotriazole esters in dimethylformamide were required (11). The assembled protected peptide-resins were cleaved by the "low/high HF" technique (12), which had been developed to avoid a number of potential side reactions. After evaporation of HF and washing with ether, the crude free peptide was extracted with 10% acetic acid and lyophilized.

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Table 1. Synthetic antagonists of glucagon: The roles of His¹, Glu⁹, and C-terminal amide

No.	Analog	Membrane binding, %	Adenylate cyclase activation		
			Maximum activity, %	Relative potency, %	(I/A) ₅₀ * pA ₂ †
1	Glucagon	100	100	100	
2	Glucagon amide	100	100	15	
3	[Glu ⁹]Glucagon	7	24	0.13	
4	[Glu ⁹]Glucagon amide	14	62	0.05	
5	des-His ¹ -glucagon	8	36	0.10	
6	des-His ¹ -glucagon amide	63	44	0.16	
7	des-His ¹ -[Glu ⁹]glucagon	11	0	<0.0008	36 7.6
8	des-His ¹ -[Glu ⁹]glucagon amide	41	0	<0.0001	12 7.2

* (I/A)₅₀, the inhibition index—i.e., the ratio of inhibitor concentration to agonist concentration when the response is reduced to 50% of the response to the agonist in the absence of inhibitor.

† pA₂, the negative logarithm of the concentration of inhibitor that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist.

lized. Purification of the synthetic peptides was performed by preparative low-pressure reverse-phase liquid chromatography on C₁₈-silica as described (6). The overall yields were between 35 and 40%. Homogeneity was demonstrated by analytical HPLC in a different solvent system, and identity was confirmed by amino acid analysis.

Circular Dichroism. Circular dichroism spectra were recorded on an Aviv 60 DS CD spectropolarimeter, using the Prosec program. The spectra were measured in a 1-mm cell from 300 to 185 nm at 1-nm intervals, and three scans were averaged. The Prosec program was used to calculate the fractional amounts of α -helix, β -sheet, β -turn, and random coil.

Membrane Binding Assay. Liver plasma membranes were prepared from male Sprague-Dawley rats (Charles River Breeding Laboratories) by the Neville procedure as described by Pohl (13). The receptor binding assay was as described by Wright and Rodbell (14) in which competition for glucagon receptors between ¹²⁵I-labeled natural glucagon (1.6 nM) and the unlabeled synthetic analog was measured. After correction for the blank, the percentage of displacement of label was compared with that of a purified glucagon standard, and the relative binding affinity was calculated.

Adenylate Cyclase Assay. The assay on liver membranes was performed according to Salomon *et al.* (15). The released cAMP was mixed with [8-³H]cAMP and was measured with a high-affinity cAMP binding protein.

RESULTS

Results reported previously (6, 7) showed that [Asp³,Glu⁹,Arg¹²]glucagon and [Asp³,Glu⁹]glucagon bound to liver membranes approximately 2% as tightly as glucagon but were without detectable cAMP activity (<0.001% of glucagon). Their inhibition indices were 75 and 35 and their pA₂ values were 5.5 and 5.6, respectively (see below). The single-replacement analog [Asp³]glucagon bound slightly, but it was a weak partial agonist with a relative potency of 2.2% and a maximum adenylate cyclase activity of 17%. Thus, neither Asp³ nor Arg¹² appeared to be primarily responsible for the observed effects of the secretin/glucagon hybrids, and we turned to the Glu⁹ position. Table 1 shows the results of our progression from the glucagon structure through the single changes involving replacement of Asp⁹ by Glu⁹, the deletion of His¹, and the introduction of a C-terminal amide, to the three double changes, and finally to an analog with all three changes. The new synthetic analogs were examined for membrane binding by competition with ¹²⁵I-labeled glucagon, for adenylate cyclase activation in liver membranes by measurement of released cAMP, and for competitive inhibition of cAMP production by natural glucagon. The inhibition

index is defined as the ratio of inhibitor to agonist when the response is reduced to 50% of the response to the agonist in the absence of inhibitor. The antagonism was also expressed as the pA₂ value of Schild (16), which represents the negative logarithm of the concentration of inhibitor that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist. A relatively precise calculation could be obtained by the graphic method of Arunlakshana and Schild (17).

Either the replacement by Glu⁹ (Table 1, analog 3) or the deletion of His¹ (analog 5) from glucagon reduced binding to 6 or 8% and gave weak partial agonists with 0.1% relative potency and 24 to 36% maximum responses, respectively, at high concentrations. However, the two changes together (analog 7) gave a peptide with 11% binding but no detectable (<0.0008%) activity. The inhibition index of 36 was significant, as was the pA₂ value of 7.6. The third change, the introduction of a terminal primary amide to replace the normal carboxyl group of the other derivatives, consistently increased the binding affinity. This was especially evident when the amide was in combination with the His¹ deletions (analog 6 and 8), where increases in binding affinity from 8 to 63 and from 11 to 41, respectively, were found. Des-His¹ glucagon amide was a partial agonist, but it still had low potency (0.16%). On the other hand, des-His¹-[Glu⁹]glucagon amide, in spite of its high binding affinity, was completely inactive in the cAMP assay and was a pure antagonist. The inhibition index dropped to 12, and the pA₂ remained relatively high at 7.2. The sequence is shown in Fig. 1.

The competitive binding assays for the two antagonists, des-His¹-[Glu⁹]glucagon and des-His¹-[Glu⁹]glucagon amide relative to purified natural glucagon are shown in Fig. 2. This assay is quite reproducible, but because of the logarithmic relationship, differences of less than 20% are not easily detected.

The assay involving stimulation of adenylate cyclase and the release of cAMP is shown in Fig. 3. This shows standard response curves for natural glucagon and for des-His¹-[Glu⁹]glucagon amide plotted as percent of maximum cAMP rise above the membrane blank vs. the logarithm of the peptide concentration. The analog showed no evidence of a stimulation of cAMP release at concentrations up to 2×10^{-6} M, and at higher concentrations an inhibition of baseline became evident. Finally, in Fig. 3 the competitive inhibition of a constant amount (3.5×10^{-8} M) of glucagon (a level of hormone giving approximately 90% of the maximum response) by increasing concentrations of the analog is shown. From this curve, the inhibition index (I/A)₅₀ was estimated to be 4.2×10^{-7} M/ 3.5×10^{-8} M = 12. The 50% response was measured from the normal baseline since an analog concentration of 4.2×10^{-7} M was not high enough to reduce the

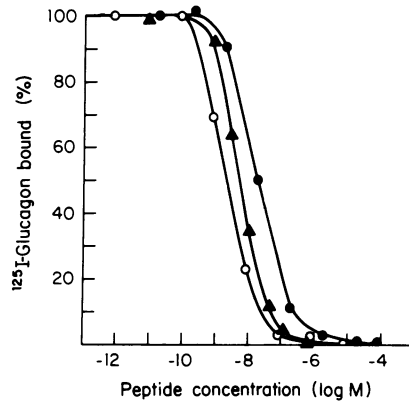


FIG. 2. Displacement of ¹²⁵I-labeled glucagon from rat liver plasma membranes by natural (unlabeled) glucagon (○) and the synthetic analogs des-His¹-[Glu⁹]glucagon amide (▲) and des-His¹-[Glu⁹]glucagon (●).

blank below baseline. Values of the inhibition index were quite reproducible in assays performed on different days with different membrane preparations.

The inhibition index was also estimated from data using a different protocol (Fig. 4). Here, increasing amounts of glucagon were assayed in the presence of a constant amount of inhibitor, and the I/A ratio giving a 50% decrease in response was calculated. Such curves were prepared at constant inhibitor concentrations of 1.55, 8.5, and 67 μM; (I/A)₅₀ values of 19, 15, and 17, respectively, were obtained. These are comparable to, but somewhat higher than, the value found by the alternative protocol where the 50% concentration of inhibitor was lower.

A general principle for enhancing the potency of an inhibitor was proposed and demonstrated by Rosenblatt *et al.* (18)—structural changes leading to mild inhibition are combined with other changes leading to superactivity. [Lys^{17,18}, Glu²¹]glucagon was reported (19) to give a 5-fold increase in binding affinity and a 7-fold increase in adenylate cyclase activation in liver membranes, and a sample prepared in our laboratory gave approximately a 3-fold increase in cyclase assay. In addition, [D-Phe⁴]glucagon was reported (20) to be seven times more active than glucagon in elevating *in vivo*

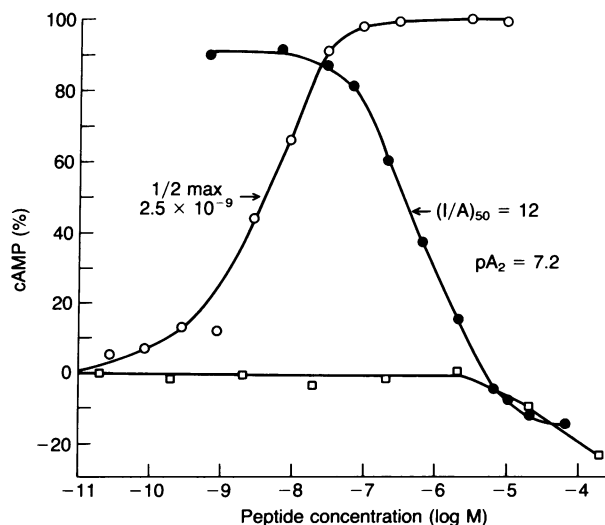


FIG. 3. Activation of adenylate cyclase in rat liver membranes by natural glucagon (○) and des-His¹-[Glu⁹]glucagon amide (□). Inhibition of 3.5×10^{-8} M glucagon by increasing amounts of the analog des-His¹-[Glu⁹]glucagon amide is also shown (●).

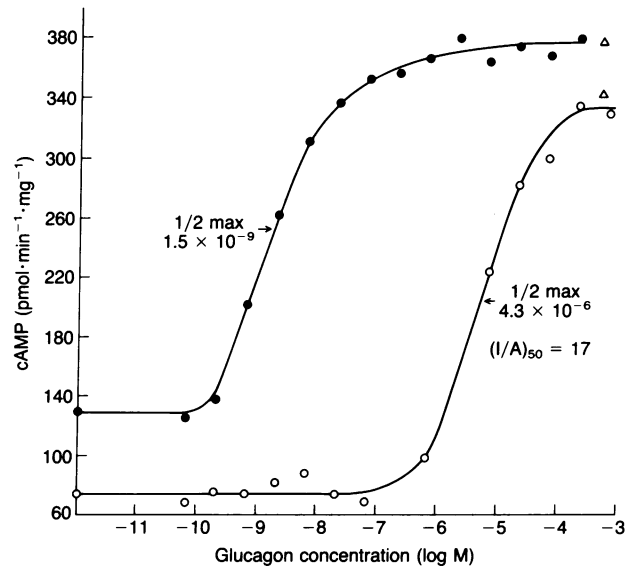


FIG. 4. Activation of adenylate cyclase by glucagon in the absence (●) and presence (○) of 6.7×10^{-5} M des-His¹-[Glu⁹]glucagon amide.

blood glucose. The results of combining these changes with some of those leading to antagonists are shown in Table 2. The combination of [Glu⁹] with [Lys^{17,18}, Glu²¹] did not change the binding affinity or potency, and the analog remained a partial agonist. Combination of des-His¹ with [Lys^{17,18}, Glu²¹] or with [D-Phe⁴] increased the binding slightly, but the analogs remained partial agonists of low potency. Combination of des-His¹-[Glu⁹] with [Lys^{17,18}, Glu²¹] did not change the binding, and the analog remained totally inactive. However, the inhibition index was reduced from 36 to 13 even though the pA₂ value was unchanged. This result remains to be confirmed. Finally, the combination of [Asp³, Glu⁹] with [D-Phe⁴] did not change the binding, but the analog became a weak agonist of very low potency and had a slightly elevated inhibition index. [D-Phe⁴]glucagon was then synthesized and was found to bind only 63% as well as glucagon to liver membranes and, in the adenylate cyclase assay, to be a full agonist with 100% relative potency. Therefore, significant changes in properties of the analogs by introduction of D-Phe⁴ probably should not have been expected in this *in vitro* system.

It was of interest to estimate the effects that these various changes in composition might have on the overall conforma-

Table 2. Membrane binding and adenylate cyclase activation by analogs containing changes leading to superactivity

Changes in glucagon	Membrane binding, %	Adenylate cyclase activation		(I/A) ₅₀
		Maximum activity, %	Relative potency, %	
[Glu ⁹]	7	24	0.1	
[Glu ⁹ , Lys ^{17,18} , Glu ²¹]	5	64	0.5	
des-His ¹	8	36	0.1	
des-His ¹ -[Lys ^{17,18} , Glu ²¹]	15	28	0.6	
des-His ¹ -[D-Phe ⁴]	13	48	0.1	
des-His ¹ -[Glu ⁹]	11	0	<0.0008	36
des-His ¹ -[Glu ⁹ , Lys ^{17,18} , Glu ²¹]	10	0	<0.0001	13
[Asp ³ , Glu ⁹]	2	0	<0.001	35
[Asp ³ , Glu ⁹ , D-Phe ⁴]	2	14	0.005	100

Table 3. Conformation by circular dichroism of glucagon analogs

TFE %	Glucagon		Glucagon amide		[Glu ⁹]		[Glu ⁹] amide		des-His ¹		des-His ¹ amide		des-His ¹ - [Glu ⁹]		des-His ¹ - [Glu ⁹] amide	
	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β
10	7.5	60.2	17.0	49.7			13.5	55.7	10.7	51.6	19.5	43.3	6.2	62.5	7.1	66.4
20	32.7	30.3	35.9	25.9	32.9	31.0	35.5	29.8	33.5	25.1	42.4	17.9	28.7	36.4	39.9	24.3
50	48.0	18.2	55.8	2.6	53.8	7.2			57.4	2.5	60.9	0	54.7	6.2	57.8	4.4
70	54.7	8.6	62.5	0	62.5	0	62.5	0	61.0	0.2	64.1	0	60.3	0.7	61.3	0.9
86	61.4	0.1	64.7	0	64.9	0	63.5	0	65.6	0	68.8	0	67.7	0	68.8	0

Analogues were in TFE/0.01 M sodium phosphate (pH 6.9) at 22°C. TFE, trifluoroethanol. α and β refer to the percentage of α -helix and β -sheet conformations, respectively.

tion of glucagon. Therefore, circular dichroism was measured on the series of analogs in 0.01 M sodium phosphate (pH 6.9) at 22°C, containing from 10 to 86% trifluoroethanol, and percentages of α - and β -conformers were calculated using the Prosec program (Table 3). The following general conclusions seem warranted: (i) for all peptides, increasing the trifluoroethanol concentration enhanced the α -helix and destabilized the β -sheet; (ii) in all cases the amide increased α -helix and decreased β -sheet content; (iii) [Glu⁹] caused some increase in α -helix and decrease in β -sheet content; (iv) des-His¹ caused some increase in α -helix and decrease in β -sheet content; (v) effects of [Glu⁹] and des-His¹ were not additive; and (vi) [des-His¹]glucagon amide was the most helical and contained the least β -sheet. In data not shown, the helical structure was increasingly stabilized by lowering the temperature.

DISCUSSION

It is generally true that if a pure antagonist of a peptide hormone can be made it will prove to be a valuable tool for investigating the mechanism of hormone action. In several instances, antagonists have also been of therapeutic value in the treatment of disease states where the patient could benefit from a reduction in the level of activity of the endogenous natural hormone. It was recently shown (2) that *N*^α-trinitrophenyl[12-homoarginine]glucagon, prepared by chemical modification of natural glucagon (21), will bind to the glucagon receptor but not transduce the signal to form cAMP. However, this analog does activate another binding system in the hepatocyte membrane leading to production of inositol trisphosphate (InsP₃) and Ca²⁺, and it was crucial for demonstrating the existence of the second glucagon receptor (2). At about the same time, it was shown (3) that the acinar cells of the pancreas have two independent receptors for secretin, one leading to formation of cAMP and the other to release of InsP₃ and Ca²⁺. This conclusion was made possible because the totally synthetic analogs [Tyr¹⁰,Tyr¹³]secretin and [Tyr¹⁰,Tyr¹³,Phe²²,Trp²⁵]secretin (7) were able to stimulate production of cAMP without affecting InsP₃ or Ca²⁺ levels.

These results have further encouraged our continuing search for glucagon analogs that have no intrinsic activity toward either one or both of the known receptor pathways of the hepatocyte but will bind to the receptor with high affinity and result in pure antagonists of potential benefit.

We have concentrated on totally synthetic analogs because they allow a much wider range of structures to be made and because they now can be synthesized readily in high yield and purity by solid-phase synthesis procedures that were developed for this hormone (10). The analogs reported here were synthesized in the 0.5–1 g range of crude product, and approximately 20 mg of each was purified to homogeneity.

The design of potent hormone inhibitors has a large empirical component, but several guiding generalities have emerged in recent years, and there is hope that a more

theoretical approach will eventually evolve. For the development of our antagonists, we followed several leads: (i) the suggestion (22) that predictable changes in conformation of the 19–27 region will be correlated with receptor binding and activity; (ii) the idea that hybrids of secretin and glucagon may result in molecules that bind to the receptor but do not transduce the signal; (iii) the fact that deletion of the N-terminal histidine from glucagon reduces binding and transduction to different extents (23); (iv) the finding that addition of a C-terminal amide to peptides enhances the helical dipole[†], which may be important for receptor binding; (v) the demonstration (18) that a combination of structural changes leading to moderate antagonism with those known to result in superagonists can give improved antagonists. Leads (i) and (v) have, so far, been of only limited value, but the successive combination of (ii), (iii), and (iv) has produced a good antagonist. Although each of the three structural changes contributed to the properties of the analogs, they were not strictly additive.

The removal of His¹ from glucagon was known to give a weak partial agonist and to reduce adenylate cyclase activation. In addition, we had shown that its removal from [Tyr²²]glucagon (24) led to a molecule that retained some membrane binding capacity but no observable cyclase activation. Therefore, we decided to synthesize the series of des-His¹ derivatives of the secretin/glucagon analogs described here.

The introduction of a C-terminal amide into these analogs was originally selected because many hormones (secretin in particular) and other biologically active peptides terminate in amides, which in some cases are more active than those with a free carboxyl. The choice was later reinforced by work coming from the laboratories of Hruby and co-workers (25) and also of Stewart, Baldwin and co-workers.[†] The former found that [Phe¹⁰,Phe¹³]glucagon amide was more active than [Phe¹⁰,Phe¹³]glucagon. The latter group has shown that removal of the C-terminal negative charge of peptides by replacement with an amide improves the helix dipole and stabilizes an enhanced proportion of helix. Since it is generally believed that an amphipathic helix in the 19–29 half of glucagon is important for receptor binding, a change that enhances such a structure may increase the binding and lead to an improved antagonist. The present data are in agreement with this idea.

According to the circular dichroism results, the introduction of a C-terminal amide did increase the proportion of α -helix and decrease the β -sheet structure for all the analogs over the whole range of trifluoroethanol concentrations studied, although the effect was not always dramatic. Because of the conformational flexibility of a peptide like glucagon, one abrupt change to a single fixed structure would not be expected in the solutions being measured, whereas the

[†]Stewart, J. M., York, E. J., Baldwin, R. L. & Shoemaker, K. R., 19th European Peptide Symposium, Aug. 31–Sept. 5, 1986, Porto Carras, Greece, p. 22 (abstr.).

parts of the molecule in contact with the receptor may be conformationally more rigid. A small preference for such a structure may make a large change in the binding constant, and that appears to be the case with these amide derivatives. Thus, going from des-His¹-glucagon to des-His¹-glucagon amide increased the binding from 8 to 63% relative to natural glucagon, and the change from des-His¹-[Glu⁹]glucagon to des-His¹-[Glu⁹]glucagon amide increased binding from 11 to 41%. Changes in transduction of hormone signal are probably not related to the amide but to the removal of histidine and the presence of glutamic acid at position 9.

The best of the antagonists was des-His¹-[Glu⁹]glucagon amide. Its binding to the receptor, as measured by competitive displacement of ¹²⁵I-labeled glucagon from liver membranes, was approximately 40% as good as glucagon itself. Moreover, this analog did not have the ability to activate adenylate cyclase or to generate cAMP at any concentration tested. The response remained at baseline up to a peptide concentration of 2 μM, but at higher concentrations a pronounced decrease below baseline was observed (Fig. 3). In the presence of a high level of analog, the maximum response to excess glucagon was also decreased (Fig. 4). However, the total increase in cAMP was nearly the same with or without antagonist, suggesting that the same number of glucagon receptors were still available in the presence of the inhibitor. Since this is a complex system, several explanations are possible, but the correct one is not known.

des-His¹-[Glu⁹]glucagon amide is a relatively potent competitive antagonist of glucagon in the *in vitro* activation of adenylate cyclase in hepatocyte membranes and has an inhibition index of 12. Since the normal circulating level of glucagon is thought to be about 0.1 nM, an analog with such an index *in vivo* might need to be present at a concentration of only about 100 nM (0.4 μg/ml) to completely inhibit the stimulation of cAMP and would be expected to be useful in further studies on the mode of action of this hormone.

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