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HETEROGENEITY OF GLUCAGON RECEPTORS OF RAT HEPATOCYTES: A SYNTHETIC PEPTIDE PROBE FOR THE HIGH AFFINITY SITE

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Summary: A glucagon analog with the following sequence has been synthesized: His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Leu-Gln-Glu-Phe-Leu-Gln-Trp-Ala-Leu-Gln-Thr. When interacting with rat hepatocytes, the analog mimics, in part, the activities of glucagon in receptor binding and inhibition of carbohydrate incorporation into glycogen. Comparison of the binding of the analog with that of glucagon demonstrates the existence of two distinct homogeneous populations of glucagon receptors. The synthetic analog acts as a specific probe for those receptors that have a high affinity for glucagon.

The binding of glucagon to plasma membrane receptors represents the initial event in an extensively studied pathway of hormone action (1-5). It is thought that association of the occupied receptor with adenylyl cyclase results in the elevation of cytoplasmic cAMP levels and in the concomitant modulation of cell metabolism (2,3,5-9). Other membrane components, such as nucleotide binding proteins, have been shown to play important roles in the regulation of this functional complex (9-12). Receptor activation occurs over a wide range of hormone concentrations indicating either multiple receptor populations, or cooperative glucagon-receptor interactions and subsequent actions. Previous results concerning both isolated hepatocytes and hepatic plasma membranes have suggested the existence of two glucagon receptor populations (1,13-18). More recently, the glucagon binding to hepatocytes and plasma membrane vesicles has been analyzed successfully in terms of two non-interacting receptor populations (5).

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To demonstrate the existence of distinct receptor populations and to establish their several roles in mediating hormone action, a hormone analog which interacts selectively with one of the two receptor types is required. As a result of a recent investigation involving the synthesis of glucagon analogs with defined secondary structure, we had available to us a series of analogs with sequence changes restricted to their COOH-terminal moieties. All analogs contained the NH_2-terminal hexapeptide of the natural hormone because previous studies had shown that this fragment of glucagon has the ability to bind to glucagon receptors and to stimulate adenylyl cyclase (19). One of these peptides (peptide A) not only showed glucagon activity, but also behaved as a partial inhibitor of glucagon binding. For this reason, we undertook a characterization of the interactions of this peptide with hepatocytes.

MATERIALS AND METHODS

Glucagon Analog - The glucagon analog, peptide A, was synthesized by the solid-phase method as previously described (20) and removed from the resin by cleavage with HF. The formyl protecting group was removed by treatment with 0.5 M piperidine. Purification of the peptide utilized gel filtration on Sephadex G-25F and partition chromatography. Amino acid analysis gave results consistent with the expected composition: Ala(1)1.0, Asx(2)2.0, Arg(2)2.2, Gly(1)1.0, Glx(5)4.9, His(1)0.9, Leu(4)4.1, Lys(1)1.0, Phe(2)2.0, Ser(4)3.9, Thr(3)2.6, Trp(1)0.8, Tyr(2)2.0. The purified peptide behaved as a homogeneous species when analyzed by reverse-phase HPLC.

Cell Incubations - Rat hepatocytes were isolated and incubated as described (21,22) for measurement of both ^{125}I-glucagon binding at 30° and glucagon inhibition of ^{14}C-fructose incorporation into glycogen at 37° (4).

RESULTS AND DISCUSSION

The glucagon analog peptide A is identical to glucagon in its amino-terminal octadecapeptide, whereas in the remaining portion of the peptide six substitutions have been effected: Ala^{19} is replaced by Leu, Asp^{21} is replaced by Glu, Val^{23} is replaced by Leu, Leu^{26} is replaced by Ala, Met^{27} is replaced by Leu, and Asn^{28} is replaced by Gln. The complete sequence of peptide A is given in the summary at the beginning of this article. The binding of glucagon and of peptide A to hepatocyte receptors was assessed by measuring the abilities of these peptides inhibit the binding of ^{125}I-glucagon to intact hepatocytes. Fig. 1 (curve A) shows the results of the binding assay when glucagon was used as the unlabeled ligand. The curve displays the

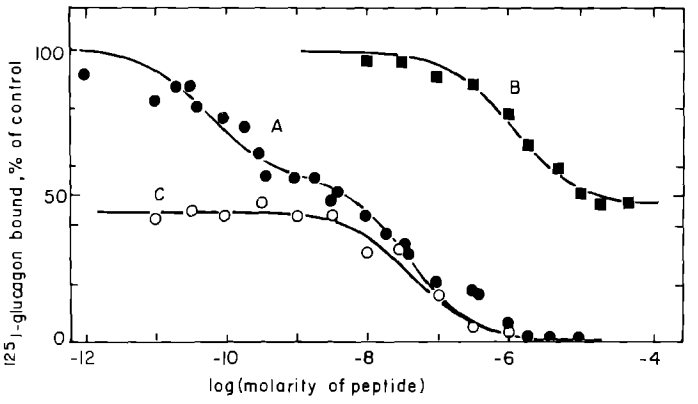

Fig. 1 Competition for ^{125}I-glucagon binding to rat hepatocytes by glucagon and peptide A. Competition by glucagon alone (●), by peptide A alone (■), and by glucagon in the presence of 10 μM peptide A (○). Curve A is theoretical and was generated by using equation 1 and the parameters of glucagon binding given in Table I. Curves B and C were generated by using equation 2 and the appropriate parameters in Table I. The data are corrected for the nonspecific binding of ^{125}I-glucagon which occurred in the presence of 10 μM unlabeled glucagon. Control binding is defined as binding of ^{125}I-glucagon occurring in the absence of any competitor. Constants derived from theoretical treatment of the experimental data are presented in Table I.

double sigmoidal behavior reminiscent of that observed using canine hepatocytes (5).

The data were analyzed according to the equation proposed earlier for the case of two non-interacting receptor populations. In this analysis, the two experimental variables are F, defined as the fraction of ^{125}I-glucagon bound in the absence of competitor which remains bound when competitor is added, and G, the ligand concentration. For the case of two non-interacting receptor populations, the experimental variables should obey the following relationship:

$$FG = P_1 K_{D1} + P_2 K_{D2} - (K_{D1} + K_{D2})F + K_{D1} K_{D2} ((1-F)/G) \qquad (1)$$

where P_1 and P_2 are the fractions of total bound labeled hormone associated, respectively, with the high and low affinity receptors in the absence of competitor, and K_{D1} and K_{D2} are the corresponding dissociation constants of the ligand (5). Treating FG, F, and (1-F)/G as independent variables,

Table 1

Parameters for Receptor Binding

Parameter	Ligand		
	Glucagon[a]	Glucagon[b]	Peptide A[c]
K_{D1}	57 ± 14 pM		1.2 μM
P_1	0.45[d]		0.54[e]
K_{D2}	41 ± 10 nM	46 nM	
P_2	0.55[d]	0.44[f]	

a Experiment where glucagon alone competed for ^{125}I-glucagon binding; constants were derived from equation 1.

b Experiment where glucagon competed for the binding of ^{125}I-glucagon in the presence of 10 μM peptide A; constants were derived from equation 2.

c Experiment where peptide A alone competed for ^{125}I-glucagon binding; constants were derived from equation 2.

d P_1 and P_2 are terms identified in equation 1 and represent the fractions of total bound radiolabeled ligand associated with the high and low affinity receptor populations in the absence of competitor, respectively.

e P_1, the fraction of total bound radiolabeled ligand associated with the high affinity receptor population, is the value of the lower asymptote of curve B shown in Fig. 1.

f P_2, the fraction of total bound radiolabeled ligand associated with the low affinity receptor population in the absence of competitor, was estimated from the upper asymptote of curve C shown in Fig. 1.

equation 1 permits the analysis of the experimental F vs. G curve of Figure 1A by multiple linear regression. The parameters obtained from this analysis are presented in Table I and document the similarity of glucagon binding to canine and rat hepatocytes. Indeed, both cell types show high and low affinity glucagon receptor populations (with respective dissociation constants differing by about a factor of 10^3), and both cell types show an almost equal distribution of binding of labeled ligand to the two receptors in the absence of competitor.

In contrast, the binding of peptide A shows a single sigmoidal binding curve which extends over a concentration range of only two orders of magnitude and which leaves 45% of the labeled ligand bound even at 50 μM unlabeled

ligand (Fig. 1, curve B). These data are readily amenable to analysis according to a scheme where peptide A competes for ^{125}I-glucagon binding at only one of the two receptor populations present on rat hepatocytes. Data for peptide A binding were analyzed graphically and by linear regression according to equation 2, yielding a single dissociation constant, K_D, equal to 1.2 μM.

$$(1-F)/(F-P_2) = (1/K_D) \times G \quad (2)$$

Note that equation 2 is the simplified form of equation 1 when $K_{D2} = \infty$ and P_2 is a constant, namely the value of the asymptote of F at high values of G. In other words, equation 2 describes binding of the inhibitor to only one of the two receptor populations.

As the inhibition of ^{125}I-glucagon binding by peptide A is sharply circumscribed, experiments were designed to investigate whether the low affinity or the high affinity glucagon receptor accounts for interaction of peptide A with isolated hepatocytes. We reasoned that in the presence of 10 μM peptide A (a saturating concentration of the ligand) glucagon itself should compete for residual ^{125}I-glucagon binding according to a single equilibrium. As shown in Fig. 1 (curve C), under these conditions, the binding curve for glucagon becomes a simple sigmoid which is extended over only two orders of magnitude of competitor concentration. These data for glucagon binding could then be analyzed using equation 2, yielding a simple dissociation constant equal to 46 nM. The close agreement of this dissociation constant with one of the two values obtained when glucagon alone was the competitor (K_{D2} = 41 nM, see Table I) establishes that in the presence of 10μM peptide A only low affinity receptors are available for glucagon binding. Thus, peptide A interacts solely with the high affinity glucagon receptor population and does not affect the binding of glucagon to low affinity receptors.

Further studies involved a comparison of the activities of peptide A and glucagon in stimulating glycogenolysis. Fig. 2 compares the actions of glucagon and peptide A in inhibiting the incorporation of ^{14}C-fructose into hepatocyte glycogen. It can be seen that the analog indeed possesses glucagon-like biological activity. Peptide A inhibits ^{14}C-fructose incorpo-

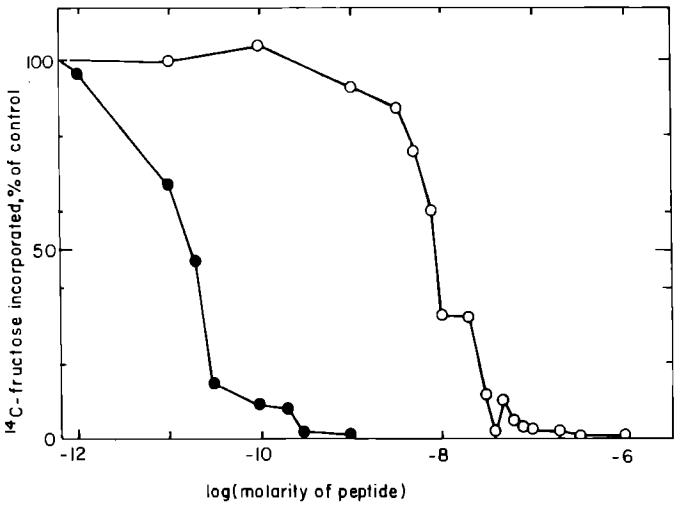

Fig. 2 Biological activities of glucagon and peptide A. Inhibition of ^{14}C-fructose incorporation into hepatocyte glycogen by glucagon (●) and peptide A (○). Control incorporation is defined as incorporation occurring in the absence of any added peptide. Data have been corrected for residual incorporation of ^{14}C-fructose which occurred in the presence of 1 μM glucagon; residual incorporation corresponded to 10-12% of control incorporation in all cases.

ration into glycogen to the same maximal extent as that shown by glucagon itself. Importantly, dose response curves for the two peptides are parallel and half-maximal effects for both peptides occur at concentrations below their respective dissociation constants for interaction with the high-affinity glucagon receptor (Fig. 2 and Table I). Further experiments will be required to dissect the multiple binding and catalytic events involved in cellular response to glucagon and its analogs.

Our use of a synthetic glucagon analog greatly facilitated investigation of the roles of different glucagon receptors in regulating cell metabolism. In particular, the guaranteed absence of native hormone in our synthetic samples makes the use of hormone analogs of low potency an excellent tool for the study of hormone action. The association of peptide A with high affinity receptors, in fact, demonstrates the existence of two separate populations of hepatocyte glucagon receptors, a hypothesis which has previously been inferred only from mathematical modeling. It is likely that

peptide A and related analogs will find additional use as specific probes of the high affinity glucagon receptor.

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