Identification of distinct receptor complexes that account for high- and low-affinity glucagon binding to hepatic plasma membranes

(receptor heterogeneity/solubilization/wheat germ lectin/GTP)

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ABSTRACT We have analyzed ligand–receptor complexes resulting from (i) the incubation of canine hepatic plasma membranes with [125I]iodoglucagon and (ii) subsequent gentle solubilization of receptor-bound ligand with digitonin. The complexes (molecular weight = 500,000) retain the radiolabeled ligand during gel filtration and subsequent manipulation at 4°C in the absence of covalent crosslinking. Affinity chromatography of the glucagon–receptor complexes on columns of wheat germ lectin linked to agarose resulted in two fractions, one of which was not bound by the column and the other of which was specifically eluted by N-acetylgalactosamine. The presence of GTP during the incubation of plasma membranes with [125I]iodoglucagon caused about a 50% decrease in total ligand binding but affected only the ligand–receptor complexes that bound to wheat germ lectin. Moreover, it was found that the proportion of the two forms of ligand–receptor complexes identified by chromatography on wheat germ lectin depended on the degree of saturation of the membrane receptor. Thus, both the inhibition by glucagon of radiolabeled glucagon binding to membranes and the concomitantly decreased extent of association of the radiolabeled ligand with solubilized receptor complexes could be modeled in terms of two noninteracting receptor populations (having dissociation constants of about 0.35 and 4.94 × 10−9 M). We conclude that (i) glucagon–receptor complexes formed on canine hepatic plasma membranes exist in two forms that differ after solubilization by digitonin in their avidities for wheat germ lectin, (ii) the high- and low-affinity binding of glucagon characteristic of hepatic plasma membranes arises from distinct receptor populations that probably differ in glycosylation, and (iii) the effect of GTP to decrease binding of glucagon to membranes arises from interactions of the nucleotide with the receptor complex that binds to wheat germ lectin.

Although the binding of ligand to plasma membrane receptors is known to initiate the mechanism of peptide hormone action, many details of ligand–receptor interactions remain to be clarified. Such interactions often fail to conform to those expected for single binding equilibria, and it has been noted that such deviations from the simple case is particularly acute in the binding of glucagon to hepatic plasma membranes and to surface receptors of isolated hepatocytes (1–6); as much as a 50,000-fold increase in the concentration of hormone is required to cause a reduction from 90% to 10% of maximal binding of [125I]iodoglucagon, whereas theory predicts that a 100-fold increase should be sufficient for a single binding equilibrium. In fact, the complex binding of glucagon both to rat hepatocytes and to canine hepatocytes has been successfully modeled in terms of two noninteracting populations of receptors having different affinities for the ligand (1, 3, 5). In the case of canine hepatocytes, cells that appear to bind glucagon with a notable degree of complexity, high- and low-affinity receptor populations were calculated to have dissociation constants of about 0.5 and 100 × 10−9 M for the hormone and to represent about 1% and 99% of total glucagon receptors, respectively (3).

Since the complex binding of glucagon to plasma membrane receptors has not been uniformly observed (7, 8) and since both negative and positive homotropic interactions have been suggested to play roles in the binding of glucagon to receptor (4, 9, 10), the validity of mathematical models involving two populations of glucagon receptors has yet to be placed within a biochemical framework. Nevertheless, the hepatic glucagon receptor has been identified as a protein with an apparent molecular weight of 60,000 (11–14), and other studies have demonstrated (i) the interaction of the receptor with additional membrane components during hormonal activation of adenyl cyclase and (ii) the importance of GTP in both increasing the activity of that enzyme and decreasing the affinity of the receptor for glucagon (9, 15–19).

Indeed, findings placing the glucagon receptor within multimeric complexes of membrane-associated proteins (20) permit consideration of the receptor as existing in two or more states of both physical complexity and affinity.

The aim of the present study was to examine, by biochemical techniques, glucagon receptors of canine hepatic membranes and to reconcile the kinetics of glucagon–receptor interactions with the nature of membrane receptors and associated proteins. As detergent-solubilized glucagon receptors appear to bind the radiolabeled ligand rather poorly (refs. 11, 15, cf. ref. 14), and as the affinities of glucagon receptors appear to be modulated by their environment (3), we specifically examined ligand-receptor complexes resulting from the prior association of glucagon to hepatic plasma membranes. Complexes were gently solubilized with digitonin and were analyzed without disruption of their macromolecular components. Affinity chromatography on lectin columns separated the gel-filtered hormone–receptor complexes into two forms. Further analysis showed that (i) the effect of GTP to decrease hormone binding to plasma membranes arises from its interaction with the receptor complex that shows highest avidity for the N-acetylgalactosamine-directed lectin, (ii) the high- and low-affinity binding sites for glucagon that are characteristic of the hepatic plasma membrane result from distinct receptor complexes that differ in their binding to wheat germ lectin, and (iii) the complexity of ligand interaction with hepatic glucagon receptors most probably arises from the summed interaction of glucagon with each receptor population.

MATERIALS AND METHODS

Membrane Isolation and Incubation. Hepatic plasma membranes were isolated from canine liver as described by Neville (21) and Ray (22). Membranes were suspended in 35% (vol/vol) glycerol prepared in 1 mM NaHCO3/0.5 mM CaCl2 (0.5 ml/100 mg wet weight of membranes) and were

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stored at −80°C. Prior to use, membranes were washed with Tris buffer (0.05 M Tris adjusted to pH 7.4 with HCl) containing 0.1 M NaCl, 0.01 M MgCl₂, 0.2% bovine serum albumin (RIA grade, Sigma), and 100 units of Trasylol (FBA Pharmaceuticals, New York) per ml. Binding assays were performed in the same buffer. The incubation volume (3 ml) and the amount of membrane (≈4 mg wet weight) were chosen to result in the binding of ≤10% of added [125I]iodo-
glucagon (100,000 cpm; ≈10 pmol); assay tubes contained unlabeled glucagon and GTP as appropriate for each experiment. Incubation proceeded for 45 min at 20°C, conditions which resulted in steady-state binding. At the close of the incubation period, the tubes were cooled to 4°C, the membranes were recovered by centrifugation (2000 × g for 30 min at 4°C), and the pelleted membranes were washed with ice-cold incubation buffer (1 ml). The final pellet was then assayed for radioactivity at ≤10°C by use of a refrigerated γ counter (Packard, model 5130).

Solubilization and Analysis of Glucagon–Receptor Complexes. Replicate plasma membrane samples resulting from binding assays were pooled and treated by vortex mixing (30 s) with a solution (1 ml/24 mg of membranes, wet weight) containing 1% (wt/vol) digitonin, 0.01 M MgCl₂, 100 units of Trasylol per ml, and 0.1 M Tris adjusted to pH 7.4 with HCl; all procedures took place at 4°C. After the mixtures had stood on ice for 15 min, insoluble material was removed by centrifugation (40,000 × g for 1 hr at 4°C), and the supernatant was assayed for radioactivity. This solution containing soluble membrane components was gel-filtered at 4°C on columns (0.9 × 100 cm) of Sepharose CL-6B (Pharmacia) equilibrated with a buffer similar to that used for solubilization, except that the concentration of digitonin was decreased to 0.05%; 1-ml fractions were collected and assayed for radioactivity. Protein concentrations were determined by using the Bradford reagent (23). Those fractions containing 125I-labeled glucagon bound to high molecular weight proteins were pooled and applied to columns (0.9 × 12 cm) of wheat germ lectin bound to agarose (P-L Biochemicals), which had been equilibrated with the buffer used for gel filtration. After the application of sample, the columns were washed first with 25 ml of equilibration buffer to remove material not bound to the lectin and then with 25 ml of equilibrium buffer (solution containing 0.2 M N-acetylglucosamine) to elute specifically bound material. Fractions containing 1.5 ml were collected and assayed for radioactivity.

Preparation of 125I-Labeled Glucagon. Glucagon for use both as competitor and as substrate for radiolabeling was obtained from Elanco (Indianapolis, IN). For radioiodination (24), glucagon (20 μg) was dissolved in 25 μl of 0.01 N NaOH and was added to 1 mCi (1 Ci = 37 GBq) of carrier-free Na125I (Industrial Nuclear, St. Louis, MO). The reaction was initiated by the addition of chloramine T (16 μg dissolved in 20 μl of 0.2 M sodium phosphate buffer, pH 7.4) and was terminated after 20 s by the addition of 48 μg of Na2S2O5, dissolved in 20 μl of 0.04 M sodium phosphate buffer (pH 7.4). The mixture was then applied to a column (1 × 50 cm) of QAE-Sephadex A-25 (Pharmacia) equilibrated with a buffer containing 0.08 M Tris adjusted to pH 8.9 with HCl, 0.08 M NaCl, 0.2% bovine serum albumin, and 200 units of Trasylol per ml (25). 125I-labeled glucagon was eluted under isocratic conditions with 90–120 ml of the equilibration buffer. Fractions containing 1.5 ml were collected; material corresponding to the peak of radioactivity was pooled, divided into 0.5-ml aliquots, and stored at −80°C. By use of a reverse-phase HPLC method developed in these laboratories (26), the iodinated product was found to contain a mixture of three radioiodinated glucagons, [125I][I3-iodotyrosine]glucagon, [125I][I3-iodotyrosine][I10-iodotyrosine]glucagon, and [125I][I10,13-iodotyrosine]glucagon, in the relative propor-
tions 24:29:47. For some experiments these radiolabeled hormones were isolated (26) and examined separately.

RESULTS

Initial studies on the solubilization of glucagon receptors from canine hepatic membranes used a variety of detergents (including Lubrol, Triton X-100, CHAPS, CHAPSO, and digitonin at concentrations ranging from 0.01% to 10%). Although the use of none of these agents resulted in soluble proteins that would bind glucagon efficiently (see also refs. 11, 14, and 15), soluble glucagon–receptor complexes that were precipitated by polyethylene glycol 6000 [12.5% (wt/vol) final concentration] were often observed when membranes previously incubated with 125I-iodoglucagon were treated with the detergent solutions (data not shown). Treatment with digitonin at 1% (wt/vol) reproducibly led to apparently uniform, high molecular weight complexes, which were unlikely to have undergone dissociation of receptor components. Fig. 1 shows the profile obtained from gel filtration of digitonin-solubilized 125I-labeled glucagon–receptor complexes on Sepharose CL-6B. Most of the radioactivity (80%) eluted was associated with proteins having an apparent molecular weight of about 500,000; the elution of this material preceded that of the major fraction of digitonin-solubilized membrane proteins. Importantly, when the addition of 125I-labeled glucagon was delayed until just before the solubilization of membranes, radioactivity was eluted from gel filtration columns at the position taken by 125I-labeled glucagon (Fig. 1). Additional control studies showed that (i) radioactivity appearing in the low molecular weight fractions was further decreased by washing the pellet a second time after incubation of plasma membranes with 125I-labeled glucagon, (ii) the high molecular weight material maintained its elution position during rechromatography on Sepharose CL-6B, and (iii) identical profiles were obtained for membranes treated with 0.25, 0.5, 1.0, and 2.0% solutions of digitonin. These findings demonstrate the inability of the ligand to associate nonspecifically with either high molecular weight proteins or detergent micelles during solubilization and the stability of the ligand–receptor complexes subsequent to solubilization. Further, the high molecular weight of the soluble material suggested that it contained, in addition to the glucagon receptor itself (11, 12, 14), other receptor-associated proteins in multimeric soluble complexes (11, 12, 16, 20).

![Fig. 1. Gel filtration on Sepharose CL-6B of solubilized proteins resulting from treatment of plasma membranes with digitonin sub-
sequent to incubation with 125I-labeled glucagon. Radioactivity recovered in fractions (1 ml) from samples incubated with 125I-
labeled glucagon for 5 sec (∆) and 45 min (○) and the protein concentration of the fractions (●) are shown. The column was calibrated with the molecular weight markers M1, blue dextran; M2, thyroglobulin; M3, ferritin; M4, catalase; M5, aldolase; and M6, 125I-labeled glucagon.](image-url)
The high molecular weight complexes identified above were applied to a variety of lectin columns in order to assess heterogeneity in soluble components to which \(^{125}\)I-labeled glucagon had been bound. Whereas columns of concanavalin A apparently bound the material irreversibly, Fig. 2A shows that columns of wheat germ lectin separated the radiolabeled material into two fractions, one of which did not bind to lectin (45% of the total) and the other of which was biospecifically eluted by buffers containing N-acetylglucosamine (55% of the total). Importantly, the fraction of soluble ligand–receptor complexes retained by the lectin did not depend on the choice of radiolabeled probe: \(^{57, 51}\), and 55% of soluble complexes were retained by and were specifically eluted from columns of wheat germ lectin subsequent to incubation of membranes with \(^{125}\)I[10-iodotyrosine]glucagon, \(^{125}\)I[10,13-iodotyrosine]glucagon, and \(^{125}\)I[13-iodotyrosine]glucagon, respectively. A similar analysis of soluble ligand–receptor complexes formed during incubation of plasma membranes with \(^{125}\)I-labeled glucagon in the presence of 0.03 mM GTP (an effector known both to decrease the binding of glucagon to plasma membranes and to induce a lower average affinity state of the receptor (9, 15–19)) showed that the nucleotide specifically decreased association of ligand with only the fraction that bound to wheat germ lectin (Fig. 2A). Control studies illustrated in Fig. 2B further showed that both receptor fractions retained their properties during both gel-filtration and reapplication to the affinity column. Therefore, we could exclude the possibility that the heterogeneity of the receptor–ligand complexes identified by affinity chromatography could be due to nonspecific binding of material to the lectin columns, dissociation of the ligand–receptor complex, degradation of soluble complexes, or overloading of the affinity matrix.

Fig. 3 presents a more complete analysis of the effect of guanyl nucleotides on \(^{125}\)I-labeled glucagon binding to plasma membranes and on association of the ligand with the two populations of ligand–receptor complexes identified by affinity chromatography. Plasma membranes incubated with radiolabeled glucagon and either GTP or its analog guanyl-5'-yl imidophosphate bound progressively less ligand as the concentration of nucleotide was increased from 3 nM to 0.3 mM (Fig. 3A). The concentration of nucleotide causing half maximal effect was about 6.5 \(\mu M\) in both cases, and in both cases the inhibition of radiolabeled glucagon binding to membrane receptors reached a plateau at higher concentrations of the effector. Fig. 3B and C compare the effect of the concentration of GTP on identification of soluble receptor–ligand complexes that bind or do not bind to wheat germ lectin. In parallel with the effect of GTP to decrease ligand binding to plasma membranes, increasing concentrations of GTP markedly decreased the association of radiolabeled glucagon with those receptor complexes that bind to and are specifically eluted from the lectin. The results of Figs. 2 and 3 demonstrate that the circumscribed inhibition of glucagon binding to plasma membranes by GTP can be accounted for by the interaction of the nucleotide with only one of the two populations of glucagon–receptor complexes identified by affinity chromatography.

Further experiments examined the saturation of glucagon receptors by ligand both in the membrane environment and after solubilization of preformed receptor complexes by digitonin. Fig. 4A compares the ability of glucagon to compete for the binding of the \(^{125}\)I-labeled hormone to hepatic membranes with the retention of radiolabeled glucagon by solubilized receptors, both as functions of glucagon concentration. For intact membranes and for soluble receptors analyzed before or after gel filtration, inhibition of the binding of \(^{125}\)I-labeled glucagon from 90% to 10% of the maximal value required about a 1,000-fold increment in the concentration of glucagon, rather than the 300-fold increment predicted for a single binding equilibrium. As expected, the character of curves reflecting inhibition of radiolabeled ligand binding to plasma membranes by glucagon was unaffected by use of \(^{125}\)I[10-iodotyrosine]glucagon, \(^{125}\)I[10,13-iodotyrosine]glucagon, or \(^{125}\)I[13-iodotyrosine]glucagon as the receptor probe (Fig. 4B). Therefore, as has been proposed.

![Fig. 2](image)

![Fig. 3](image)

![Fig. 4](image)
matches the experimental data points (▲ in Fig. 4A). Although the value for the dissociation constant of the high-affinity receptor is similar to that for the binding of glucagon to the high-affinity receptor population of isolated hepatocytes (0.5 × 10⁻⁹ M), the binding of glucagon to the low-affinity receptor population, a parameter notably dependent on receptor environment (3), is markedly decreased.

We finally examined the importance of glucagon concentration and, therefore, receptor saturation on the association of ligand with the two forms of hormone–receptor complexes identified by use of gel filtration and affinity chromatography. Analysis of material obtained from membranes incubated with ¹²⁵I-labeled glucagon and with unlabeled glucagon showed that low concentrations of the competitor caused radioactivity in the fraction specifically bound to the lectin column to decrease markedly, whereas higher concentrations of competitor were required to decrease radioactivity in the unbound fraction. A quantitative analysis of the effect of ligand concentration on association of ¹²⁵I-labeled glucagon with glucagon–receptor complexes separated by affinity chromatography is presented in Fig. 5. For complexes bound to the lectin column, 0.3 nM glucagon was sufficient for half-maximal inhibition of ¹²⁵I-labeled hormone association with receptor, whereas for complexes not bound to the lectin, 3 nM glucagon was required. In fact, these values are little different from those derived earlier by mathematical modeling for the dissociation constants corresponding to the high- and low-affinity components of total glucagon binding to soluble receptor complexes (Kₐ₁ = 0.35 × 10⁻⁸ M, Kₐ₂ = 4.94 × 10⁻⁹ M). The close fit of the experimental data to the theoretical curves for these mathematically modeled, single equilibria (Fig. 5) identifies (i) high-affinity glucagon binding as resulting from hormone association with the receptor complex that binds to wheat germ lectin and (ii) low-affinity glucagon binding as resulting from hormone association with the complex that does not bind to the lectin.

**DISCUSSION**

Our findings provide a framework for understanding the kinetic complexity observed for glucagon binding to hepatic plasma membranes. First, gel filtration and affinity chromatography of glucagon–receptor complexes has resulted in the identification of two states of the receptor, one of which

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*Dissoication constants were calculated from equations 10 and 13 in ref. 3 by using Gauss–Newton and multiple linear-regression data analysis, respectively. Six data points (including replicates) were included in the calculations.
under our conditions binds to wheat germ lectin and the other of which does not. Second, the two types of hormone–receptor complexes exhibit differential sensitivity to competing glucagon when they are in the membrane environment, with the fraction binding to the lectin approaching saturation at concentrations of the hormone lower than those required for the fraction not binding to the lectin. Third, concentrations of hormone causing half-maximal inhibition of radiolabeled glucagon association with the two populations of receptor complexes do not differ from the respective dissociation constants derived mathematically from the overall interaction of glucagon with soluble membrane receptors. Fourth, the sum of the two single equilibria proposed for the association of hormone with glucagon receptor complexes (equilibrium having dissociation constants differing by about 10-fold) yields a binding curve that spans a 1000-fold increment in ligand concentration and that mimics the curves for both inhibition of $[^{125}I]_4$iodoglucagon binding to plasma membranes and retention of $[^{125}I]_4$iodoglucagon by solubilized receptor complexes. Thus, our experiments provide evidence that the low- and high-affinity binding previously assigned to glucagon–receptor interactions by mathematical modeling (1, 3, 5) is due to association of hormone with distinct receptor complexes that differ in their binding to wheat germ lectin and probably in their content of N-acetylglucosamine.

It should be noted that our experimental approach (one involving gentle methods for receptor solubilization and investigation of ligand–receptor complexes arising from direct interaction of $[^{125}I]_4$-labeled glucagon with isolated plasma membranes) was designed to ensure the examination of the broadest possible range of potential glucagon–receptor interactions. Evidence presented in this report for the existence of soluble glucagon–receptor complexes of about 500,000 molecular weight is compatible with the known molecular weight of the receptor itself (60,000, as determined by photoaffinity labeling and other techniques) (11–14), with the occurrence of the receptor as part of a multimeric complex of proteins (9, 15–20), and with the possible existence of receptor dimers in the plasma membrane (14). Experiments assessing the effects of GTP on ligand–receptor interactions provide additional information on the kinetics of glucagon association with plasma membrane receptors and on the nature of the high molecular weight complexes identified by use of our methods. Thus, analysis of soluble glucagon–receptor complexes has shown that the well-documented effect of GTP to decrease glucagon binding to plasma membranes (9, 15–19) arises from a defined loss of hormone association with receptor complexes that are specifically retained by columns of wheat germ lectin—that is, receptor complexes that have been identified as giving rise to the high-affinity interactions of the hormone with plasma membrane receptors.

Although chemical dissection of the high molecular weight glucagon–receptor complexes identified here will require considerable further investigation, our findings form a base for determining the identity of receptor components that (i) provide for ligand recognition in the two populations of glucagon–receptor complexes, (ii) determine the affinity of the receptor for glucagon in each case, (iii) confer specific binding of the high-affinity complex to wheat germ lectin, (iv) result in sensitivity of the receptor to the nucleotide GTP, and (v) modulate both binding and biological activities. In fact, the participation of multiple forms of plasma membrane receptors in the binding of ligand to a variety of cells appears not to be unique for the interactions of glucagon with canine hepatic membranes. Thus, very recent reports have documented, by the use of both chemical and physical methods, heterogeneity in receptor populations serving for the interaction of catecholamines with 1321N1 human astrocytoma cells (30), insulin with Fao/Reuber H35 rat hepatoma cells (31), dopamine with bovine anterior pituitary (32), and angiotensin with rat liver plasma membranes (33). It will be important to assess further the extent to which receptor heterogeneity (perhaps in conjunction with homotropic interactions and potential conformational conversion of receptor forms) contributes to the kinetic complexity of hormone interactions with target cells and to the mechanisms by which target cells respond to receptor-bound ligand.

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