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Characterization of Glucagon Receptors in Golgi Fractions of Rat Liver: Evidence for Receptors That Are Uncoupled from Adenylyl Cyclase

Kenneth E. Lipson,‡ Angeli A. Kolhatkar, Bruce D. Cherksey, and David B. Donner*
Memorial Sloan-Kettering Cancer Center and The Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, New York, New York 10021

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ABSTRACT: Glucagon receptors have been identified and characterized in intermediate (G_i) and heavy (G_h) Golgi fractions from rat liver. At saturation, plasma membranes bound 3500 fmol of hormone/mg of membrane protein, while G_i and G_h bound 24 and 60 fmol of ^{125}I-glucagon/mg of protein, respectively. Half-maximal saturation of binding to plasma membranes, G_i, and G_h occurred at approximately 4, 10, and 20 nM ^{125}I-glucagon, respectively. Trichloroacetic acid precipitation of intact, but not degraded, glucagon was used to correct binding isotherms for hormone degradation. After such correction, half-maximal saturation of binding to plasma membranes, G_i, and G_h was observed in the presence of approximately 2, 7, and 14 nM hormone, respectively. After 90 min of dissociation in the absence of guanosine 5′-triphosphate (GTP), 86% of ^{125}I-glucagon remained bound to plasma membranes, whereas only 42% remained bound to Golgi membranes. GTP significantly increased the fraction of ^{125}I-glucagon released from plasma membranes but only slightly augmented the dissociation of hormone from Golgi fractions. ^{125}I-Glucagon/receptor complexes solubilized from plasma membranes fractionated by gel filtration as high molecular weight (K_{av} = 0.16), GTP-sensitive complexes and lower molecular weight (K_{av} = 0.46), GTP-insensitive complexes. ^{125}I-Glucagon complexes solubilized from Golgi membranes fractionated almost exclusively as the lower molecular weight species. The lower affinity of Golgi than plasma membrane receptors for hormone, the ability of glucagon to stimulate plasma membrane, but not Golgi membrane, adenylyl cyclase, and the near absence of high molecular weight, GTP-sensitive complexes in solubilized Golgi membranes demonstrate that plasma membrane contamination of Golgi fractions cannot account for the ^{125}I-glucagon binding. These observations are novel and significant in demonstrating that (1) Golgi fractions contain specific binding sites for glucagon, (2) such sites bind hormone with lower affinity than plasma membrane receptors, and (3) these sites are uncoupled from other components of adenylyl cyclase.

Receptor/adenylyl cyclase systems are composed of at least three distinct membrane components: a specific receptor such as that for glucagon, guanine nucleotide regulatory proteins (N proteins), and the catalytic subunit of the enzyme adenylyl cyclase (Rodbell, 1980). In such systems, hormone action is initiated by binding to a specific receptor in the plasma membrane. Interactions between occupied receptors and N proteins ("coupling") are obligatory for transmembrane signaling. Guanosine 5′-triphosphate (GTP)[1] binding to the N protein promotes dissociation of the occupied receptor from the N protein and thereby diminishes the affinity of receptor for hormone. The activated N protein that results from this process then stimulates cAMP production by the enzyme until the bound GTP is hydrolyzed to GDP (Rodbell, 1980; Gilman, 1984).

Hormone and neurotransmitter receptors have been characterized in cell fractions other than the plasma membrane. For example, receptors for insulin and prolactin have been identified in Golgi fractions from rat liver (Bergeron et al., 1973b, 1978; Posner et al., 1979). β-Adrenergic receptors that were uncoupled from other components of the adenylyl cyclase system have been isolated in "light" membranes that could not

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GDP, guanosine 5′-diphosphate; Gpp(NH)p, guanylyl imidodiphosphate; GTP, guanosine 5′-triphosphate; G_i, intermediate Golgi fraction; G_h, heavy Golgi fraction; HBSS, Hank's balanced salt solution; K_D, dissociation constant, Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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be resolved from the Golgi enzyme marker galactosyl transferase by sucrose gradient centrifugation (Waldo et al., 1983). Such uncoupled receptors bind agonists with low affinity (Fishman et al., 1981) and are insensitive to GTP (Harden et al., 1979).

The glucagon receptor on the surface of rat hepatocytes (Sonne et al., 1978) and on liver plasma membranes (Rodbell et al., 1971; Rodbell, 1980) has been extensively investigated. In contrast to the other receptors alluded to above, intracellular glucagon binding sites have not been previously characterized. The goals of this study were (1) to determine if Golgi fractions from rat livers contain specific receptors for glucagon, (2) to characterize the properties (affinity, GTP sensitivity) of such receptors, and (3) to determine if glucagon binding to these receptors could activate adenylyl cyclase which is also present in Golgi fractions (Cheng & Farquhar, 1976).

Experimental Procedures

Materials. Crystalline glucagon (lot 258-25J-120) was the generous gift of Eli Lilly Inc. Kits to measure cAMP by radioimmunoassay and protein binding were purchased from New England Nuclear and Amersham Inc., respectively. The sources of other materials were previously described (Corin et al., 1982).

Methods. Glucagon was iodinated according to Greenwood et al. (1963) as modified by Lesniak et al. (1973). ^{125}I-Glucagon was separated from $^{125}I^-$ by selective desorption from a microfine silicate QUSO 32 (Corin et al., 1982). Partially purified liver plasma membranes were prepared by the Neville procedure (1968) as described by Pohl et al. (1971). Liver Golgi fractions were prepared from fasted, non-ethanol-intoxicated, 150–200-g male Sprague-Dawley rats (Bergeron, 1979). After discontinuous sucrose gradient centrifugation, light, intermediate (G_i), and heavy (G_h) Golgi vesicles were harvested from the interfaces of the 0.25/0.6, 0.6/0.86, and 0.86/1.0 M sucrose layers, respectively. Since very little or no light Golgi vesicles are obtained from unintoxicated rats (Ehrenreich et al., 1973), it was not possible to obtain sufficient material to characterize glucagon binding in this fraction. Unless stated otherwise, Golgi fractions were frozen and thawed 4 times in order to achieve maximal vesicle disruption (Bergeron et al., 1978).

The concentration of membrane protein (reported in the figure legends) was measured by the method of Lowry et al. (1951) or by the fluorescamine method (Böhlen et al., 1973) using bovine serum albumin (fraction V) as a standard. 5′-Nucleotidase was assayed according to Evans (1978), and the released phosphate was measured by a modification (Peterson, 1978) of the Fiske–Subbarow method (Fiske & Subbarow, 1925). β-*N*-Acetylglucosaminidase was assayed as described by Hubbard et al. (1983), and galactosyl transferase was assayed according to Bretz and Stäubli (1977) using ovomucoid as an acceptor.

For assay of adenylyl cyclase, Golgi fractions were prepared from livers homogenized in the presence of 1 mM EGTA to prevent loss of enzyme activity (Cheng & Farquhar, 1976). Adenylyl cyclase was assayed by a modification of the procedure described by Pohl et al. (1971). The assay mixture consisted of 40 mM Tris, pH 7.6 (30 °C), 0.1% bovine serum albumin, 5 mM $MgCl_2$, 1 mM EDTA, 0.8 mg/mL bacitracin, 2 mM isobutylmethylxanthine, 3 mM ATP, 20 mM phosphocreatine, 0.25 mg/mL (40–50 units/mL) creatine phosphokinase, and glucagon (0–100 nM), fluoride (15 mM), or Gpp(NH)p (10 μM) as indicated; 20–30 μg of plasma membranes or 75–330 μg of Golgi membranes was assayed at 30 °C for 5–20 min and then boiled for 3 min. The concentration of cAMP was measured by radioimmunoassay (Steiner et al., 1972) after acetylation of cAMP (Harper & Brooker, 1975) or by protein binding (Gilman, 1970; Brown et al., 1971). The amount of cAMP formed during assay of adenylyl cyclase was determined by subtracting the endogenous levels present in boiled membrane fractions (Cheng & Farquhar, 1976).

Binding of ^{125}I-glucagon to plasma membranes was performed at 23 °C in the presence of 0.5% bovine serum albumin in HBSS, pH 7.4. Triplicate 100-μL aliquots were assayed for hormone binding by microcentrifugation (1 min, 10000*g*) through 200 μL of 5% sucrose (0 °C) (Corin et al., 1982). The membrane pellet was washed once with 200 μL of ice-cold 5% sucrose and centrifuged for 30 s. The tip of each microcentrifuge tube was cut off and placed in a tube for measurement of ^{125}I. To ensure complete pelleting of Golgi membranes, centrifugation times were increased to 2 and 1 min for the initial centrifugation and wash step, respectively. Radioactive uptake by membranes in the presence of ^{125}I-glucagon alone is defined as total binding. Nonspecific binding is defined as uptake of ^{125}I-glucagon in the presence of unlabeled glucagon (7–29 μM). Specific binding is the difference between total and nonspecific binding.

The integrity of unbound ^{125}I-glucagon was assessed by the ability of Cl_3CCOOH to precipitate intact but not degraded glucagon or free ^{125}I. This procedure is as sensitive as radioimmunoassay for measurement of glucagon degradation (Eisentraut et al., 1968). An aliquot (20 μL) of supernatant recovered from a binding incubate was added to 0.5 mL of 1% bovine serum albumin. Approximately 1.5 mL of 10% Cl_3CCOOH was added, and the resulting precipitate was pelleted by centrifugation (1100*g*, 3 min). The integrity of ^{125}I-glucagon was defined as the ratio of counts per minute in the precipitate to the total counts per minute in the precipitate and supernatant.

Dissociation of membrane-bound ^{125}I-glucagon was initiated by dilution of incubates into a 200-fold excess of hormone-free medium (0.1% bovine serum albumin in HBSS, pH 7.4, 23 °C). Dissociation was measured by vacuum filtration through cellulose acetate membrane filters (Millipore, type EG, 0.2 μm). The filters were soaked in 5% bovine serum albumin for 1 h at 23 °C, mounted on a manifold, and washed with 10 mL of medium immediately prior to use. Binding assayed by filtration of six 10-mL aliquots immediately after dilution was defined as binding at zero time. At various times thereafter, triplicate 10-mL aliquots were removed for filtration. To assess the effect of GTP (11 μM) on the dissociation of bound ^{125}I-glucagon, a freshly prepared stock solution of this reagent was added to the dissociation medium immediately after assay of binding at zero time. The data are presented as the fraction of ^{125}I-glucagon remaining bound at any time relative to binding at zero time.

The properties of solubilized hormone/receptor complexes were characterized by gel filtration chromatography. G_h or plasma membranes were incubated with ^{125}I-glucagon (30 min, 23 °C), centrifuged (80000*g*, 10 min), and resuspended in solubilization buffer [25 mM Tris (pH 7.4), 1 mM EDTA, 1% Lubrol PX, 4 °C, and 4.7 mg of protein/mL]. After 30 min of incubation, insoluble material was removed by centrifugation (185000*g*, 30 min). An aliquot of the supernatant (0.8 mL) was applied to an Ultrogel AcA 34 column (0.9 × 100 cm) equilibrated with elution buffer [25 mM Tris (pH 7.4), 1 mM EDTA, and 0.01% Lubrol PX, 4 °C] and eluted in 1-mL fractions at 12 mL/h. The void (fraction 31) and salt (fraction 81) volumes of the column were determined by elution of blue dextran and reduced DTNB, respectively.

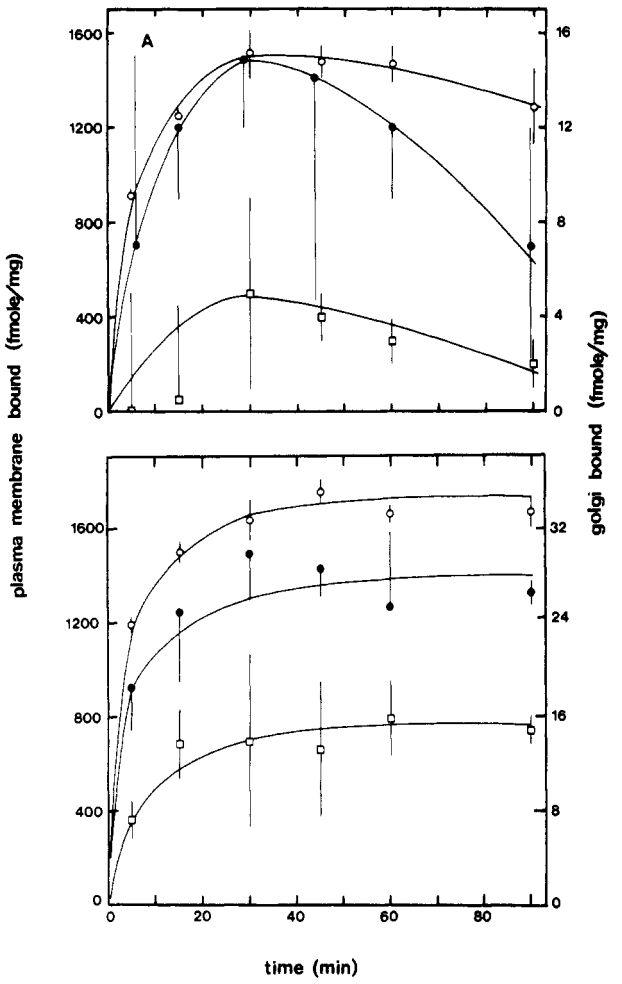

FIGURE 1: Association of ^{125}I-glucagon with plasma membranes or Golgi membranes. (A, top) Specific binding of ^{125}I-glucagon (2.6 nM) to plasma membranes (0.4 mg of protein/mL) (○), intermediate (□), or heavy (●) Golgi membranes (0.5 mg of protein/mL) was assayed at 23 °C. Representative experiments are shown. (B, bottom) Plasma membranes (0.8 mg of protein/mL) (○) were incubated with 3.5 nM ^{125}I-glucagon, and intermediate (□) or heavy (●) Golgi membranes (1 mg of protein/mL) were incubated with 5.4 nM ^{125}I-glucagon at 23 °C in the presence of bacitracin (0.8 mg/mL), and the specific binding was assayed. Data points represent the mean ± SEM of triplicate experiments.

Degradation products of ^{125}I-glucagon (assayed as Cl_3CCOOH-soluble radioactive label) eluted near the salt volume (K_{av} = 0.87–1.06), and intact hormone eluted slightly after the salt volume of the column (K_{av} = 1.09) (not shown).

RESULTS

To establish if Golgi fractions contain glucagon receptors, the binding of ^{125}I-glucagon to intermediate and heavy Golgi and plasma membranes was examined and compared. During an association reaction, binding of ^{125}I-glucagon to plasma membranes reached a maximal value of 1500 fmol/mg of protein after 30 min and then slowly diminished (Figure 1, panel A). ^{125}I-Glucagon bound to Golgi membranes specifically, although much less binding per milligram of protein was observed than in plasma membranes. Because of the low uptake, there was considerable uncertainty in the magnitude of specific ^{125}I-glucagon binding to Golgi membranes. Maximal binding to Golgi membranes also occurred after 30 min (about 5 fmol/mg of G_i, 15 fmol/mg of G_h) and thereafter decreased (Figure 1, panel A).

To test if the decrease of ^{125}I-glucagon binding to Golgi membranes after 30 min resulted from hormone degradation,

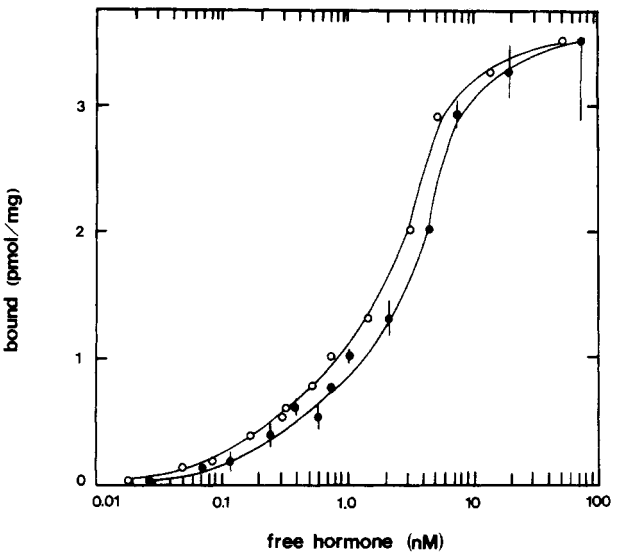

FIGURE 2: Incubation of plasma membranes with various concentrations of ^{125}I-glucagon. Specific binding of various concentrations of ^{125}I-glucagon to plasma membranes (0.33 mg of protein/mL) was assayed after 60 min at 23 °C. Data are shown without (●) or with (○) correction for hormone degradation. Error bars have been omitted from the corrected data for clarity. Each data point represents the mean of duplicate or triplicate measurements.

binding to Golgi or plasma membranes was examined in the presence of bacitracin (Figure 1, panel B). With the enzyme inhibitor present, maximal uptake of ^{125}I-glucagon by plasma membranes occurred within 60 min (1700 fmol/mg) and remained constant for 30 min thereafter. Specific ^{125}I-glucagon binding to Golgi fractions also remained constant after 60 min but was still considerably less (15 fmol/mg of G_i, 27 fmol/mg of G_h) than to plasma membranes. Bacitracin was included in all subsequent experiments since it stabilized hormone binding to membranes.

The capacities of plasma and Golgi membranes to bind ^{125}I-glucagon were compared. At saturation, plasma membranes bound approximately 3500 fmol of ^{125}I-glucagon/mg of protein (Figure 2). In typical experiments, G_i and G_h membranes bound 24 and 60 fmol of ^{125}I-glucagon/mg of protein, respectively, in the presence of sufficient hormone to saturate receptor binding capacity (Figure 3). The binding capacity of Golgi fractions varied somewhat from one preparation to another without alteration in the apparent affinity of the receptor for hormone. Factors that could have contributed to such variability include hormonal differences in the rats used for preparation of Golgi fractions as well as undefined seasonal differences that may alter the Golgi apparatus (Morré, 1973). Although ^{125}I-glucagon binding to Golgi membranes was low compared to plasma membranes, the Golgi apparatus contains more of a liver cell's mass (about 3%) (Bergeron et al., 1973a) than the plasma membrane (about 0.4%) (Ray, 1970). This suggests that approximately 10% of total glucagon receptors could reside within the cell under basal conditions.

Half-maximal saturation of binding to plasma membranes occurred in the presence of approximately 4 nM ^{125}I-glucagon (Figure 2), whereas that of Golgi membranes occurred at a higher concentration (10–20 nM ^{125}I-glucagon) (Figure 3). Since Golgi membranes are more effective at degrading ^{125}I-labeled human growth hormone than plasma membranes (Posner et al., 1979), it is possible that degradation could reduce the concentration of ^{125}I-glucagon sufficiently to diminish the apparent affinity of Golgi membranes for hormone. To determine if this was so, the integrity of ^{125}I-glucagon was

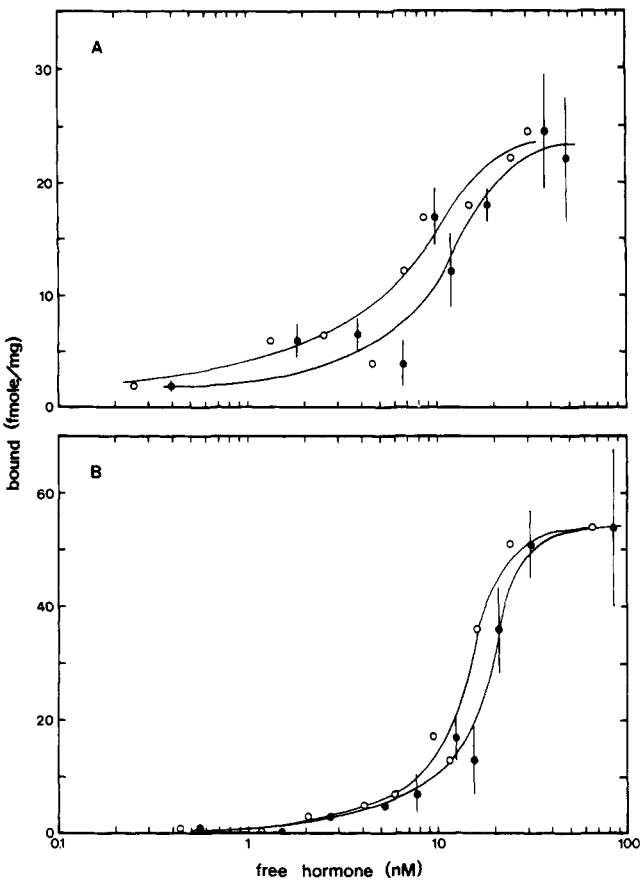

FIGURE 3: Incubation of Golgi membranes with various concentrations of ^{125}I-glucagon. Specific binding of various concentrations of ^{125}I-glucagon to intermediate (panel A) or heavy (panel B) Golgi membranes (1 mg of protein/mL) was assayed after 60 min at 23 °C. Data are shown without (●) or with (○) correction for hormone degradation. Error bars have been omitted from the corrected data for clarity. Representative experiments are shown.

assayed by Cl_3CCOOH precipitation (Methods), and saturation curves were corrected to reflect only intact hormone. In the presence of bacitracin, 50–80% of ^{125}I-glucagon remained undegraded after 60 min of association with plasma or Golgi membranes. Correction for hormone degradation lowered the concentration at which half-maximal saturation of ^{125}I-glucagon binding to plasma membrane receptors occurred from 4 to approximately 2 nM hormone (Figure 2). Half-maximal saturation of binding to intermediate and heavy Golgi membranes occurred at 7 and 14 nM ^{125}I-glucagon, respectively, after correction for hormone degradation (Figure 3).

Binding sites of high (K_D = 0.2 nM) and low affinity (K_D = 5 nM) were evident when the data for ^{125}I-glucagon binding to plasma membranes were plotted according to the method of Scatchard (1949) (not shown). Computer analysis of binding isotherms that had been corrected for hormone degradation determined that 540 and 4070 fmol of ^{125}I-glucagon/mg of membrane protein were bound to high- and low-affinity sites, respectively. Scatchard analysis of hormone binding to G_i and G_h membranes indicated the presence of a single class of low-affinity sites in these fractions (not shown). Computer analysis estimated the dissociation constants for ^{125}I-glucagon binding to G_i and G_h membranes to be 7 and 10 nM, respectively.

The dissociation of ^{125}I-glucagon from Golgi membranes was compared to dissociation from plasma membranes in the presence and absence of GTP. After 90 min of dissociation, 86% of ^{125}I-glucagon remained bound to plasma membranes

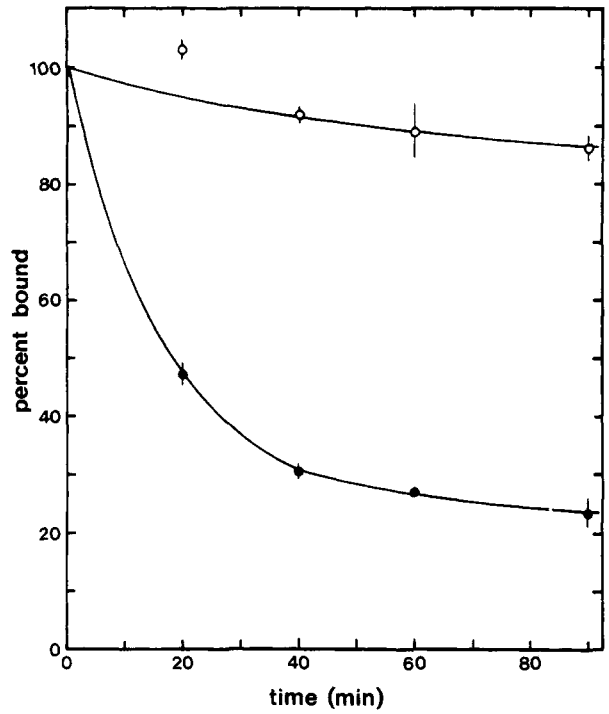

FIGURE 4: Dissociation of ^{125}I-glucagon from plasma membranes. Plasma membranes (1.7 mg of protein/mL) were incubated at 23 °C with ^{125}I-glucagon (4.1 nM) for 60 min prior to 200-fold dilution into hormone-free medium. The fraction of ^{125}I-glucagon remaining bound relative to binding immediately after dilution was determined in the absence (○) or presence (●) of GTP at various times. A representative experiment is shown.

in the absence of GTP, whereas only 23% remained bound in the presence of GTP (Figure 4). ^{125}I-Glucagon dissociated more rapidly from Golgi than from plasma membranes in the absence of GTP (Figure 5). For example, after 90 min of dissociation, 42% of ^{125}I-glucagon remained bound to heavy Golgi membranes. GTP augmented the rate of ^{125}I-glucagon release from Golgi membranes somewhat; however, this may not be statistically significant.

Plasma and Golgi membranes were treated differently prior to binding experiments. Golgi fractions were stored at −20 °C and then subjected to four freeze/thaw cycles to disrupt vesicles (Bergeron et al., 1978). Plasma membranes were stored in liquid nitrogen (Pohl et al., 1971) and were used without further treatment. To determine if freeze/thaw cycles accounted for the different properties of Golgi and plasma membrane receptors, ^{125}I-glucagon dissociation from plasma membranes that were frozen and thawed prior to use was examined (Figure 6). After 90 min of dissociation in the absence of GTP, approximately 95% of ^{125}I-glucagon remained bound to plasma membranes subjected to four freeze/thaw cycles. Golgi membranes subjected to only two freeze/thaw cycles released bound ^{125}I-glucagon rapidly (Figure 6).

A possible, although unlikely, explanation for the presence of glucagon receptors in Golgi fractions with the properties described above is that they are contaminating plasma membrane receptors that are affected by factors in the Golgi. To test for this, plasma membranes (0.06 mg of protein/mL) were incubated with ^{125}I-glucagon (0.7 and 5 nM) for 60 min at 23 °C in the absence or presence of G_h membranes (0.45 mg of protein/mL). In the presence of 0.7 nM hormone, 5.9 ± 0.1, 0.9 ± 0.2, and 8 ± 1 fmol of ^{125}I-glucagon were bound per 0.1-mL aliquot of plasma membranes, Golgi membranes, or mixed plasma and Golgi membranes, respectively. Similarly, plasma membranes and Golgi membranes incubated with 5 nM ^{125}I-glucagon bound 13.5 ± 1 and 3.9 ± 0.5 fmol of

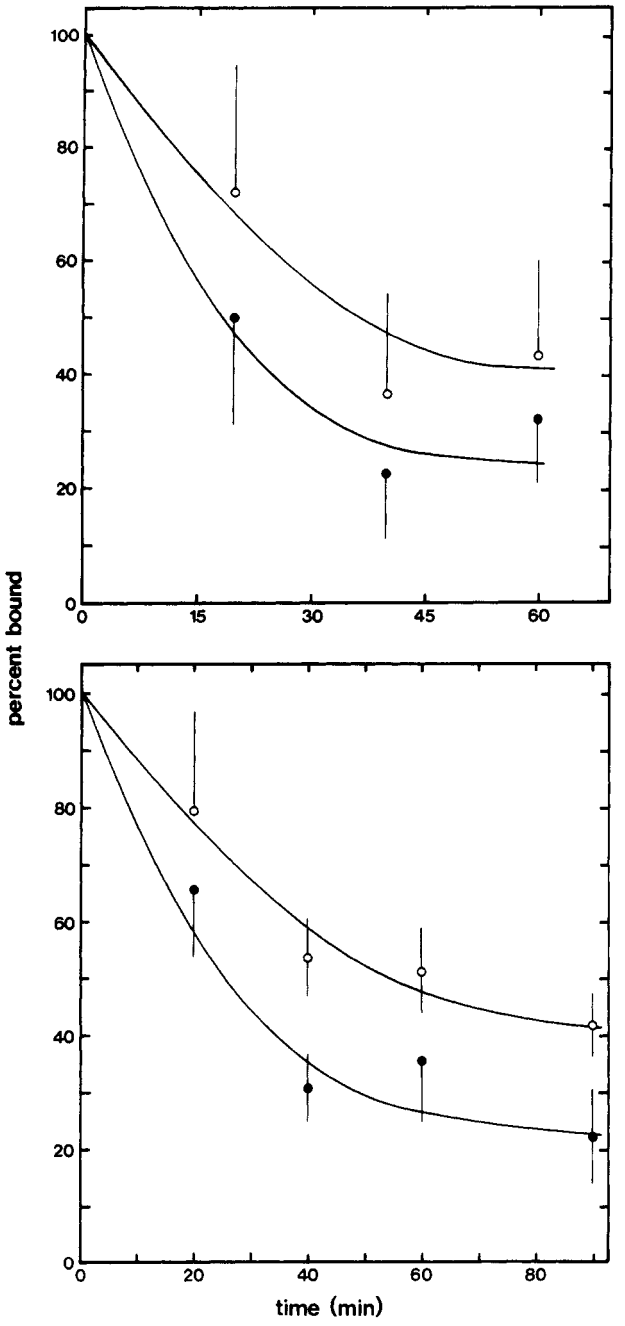

FIGURE 5: Dissociation of ^{125}I-glucagon from Golgi membranes. Intermediate (1 mg of protein/mL) (panel A, top) or heavy (2 mg of protein/mL) (panel B, bottom) Golgi membranes were incubated at 23 °C with ^{125}I-glucagon (5.3 nM) for 60 min prior to 200-fold dilution into hormone-free medium. The fraction of hormone remaining bound relative to binding immediately after dilution was determined in the absence (○) or presence (●) of GTP at various times. Representative experiments are shown.

hormone, respectively, and the mixture of membranes bound 18.4 ± 0.8 fmol of ^{125}I-glucagon.

It was considered possible that incubation with Golgi membranes produced a subtle alteration of ^{125}I-glucagon that would diminish the apparent affinity of the hormone for receptors. To test for this, ^{125}I-glucagon (61.4 nM) was incubated with G_h membranes (1 mg of protein/mL) for 60 min at 23 °C, and unbound hormone was recovered by centrifugation (80000*g*, 10 min). Plasma membranes (1.1 mg of protein/mL) incubated with 6 nM control or preincubated ^{125}I-glucagon (equal amounts of Cl_3CCOOH-precipitable hormone) bound 960 ± 58 and 923 ± 20 fmol of hormone/mg of protein, respectively. In a related experiment, G_h membranes (1 mg of protein/mL) were incubated with ^{125}I-glucagon (6.8 nM) for 60 min, and the unbound hormone was removed by centrifugation. Of the ^{125}I-glucagon bound to G_h membranes, 98.7% was precipitated by Cl_3CCOOH.

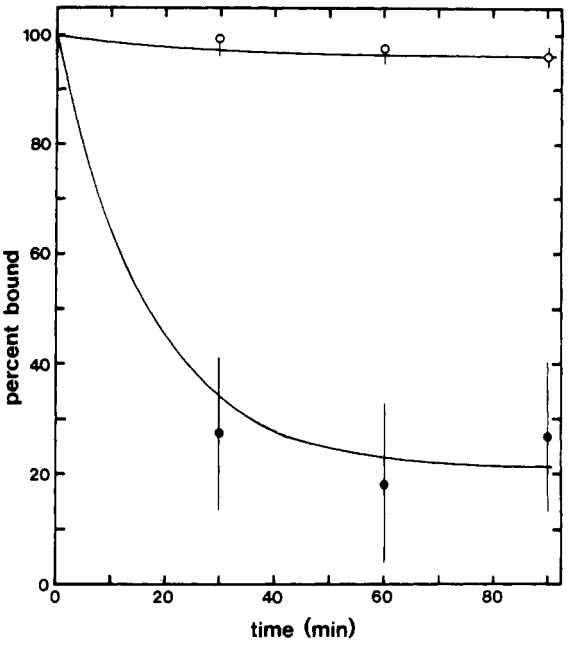

FIGURE 6: Effect of freeze/thaw treatment on ^{125}I-glucagon dissociation. Plasma and heavy Golgi membranes were frozen and thawed 4 and 2 times, respectively. Plasma membranes (1 mg of protein/mL) (○) or G_h membranes (2.1 mg of protein/mL) (●) were incubated with ^{125}I-glucagon (0.4 and 5.4 nM, respectively) for 60 min prior to 200-fold dilution into hormone-free medium. The fraction of hormone remaining bound relative to binding immediately after dilution was determined in the absence of GTP at various times. A representative experiment is shown.

Since the binding properties of Golgi receptors did not result from subtle alteration of glucagon or from contaminating plasma membrane receptors altered by factors from Golgi fractions, the hydrodynamic properties of hormone/receptor complexes solubilized from G_h or plasma membranes were compared by gel filtration chromatography (Figure 7). ^{125}I-Glucagon complexes from plasma membranes (panel A) eluted from an Ultrogel AcA 34 column in two peaks of K_{av} 0.16 and 0.46. Incubation of plasma membranes with ^{125}I-glucagon and unlabeled glucagon prior to solubilization and fractionation eliminated 96% of the ^{125}I-glucagon in the high molecular weight peak (K_{av} = 0.16) and diminished the amount of hormone in the lower molecular weight complex (K_{av} = 0.46) by 80% (not shown). GTP diminished the fraction of ^{125}I-glucagon in high molecular weight complexes (panel B). ^{125}I-Glucagon complexes from Golgi membranes (panel C) eluted almost exclusively in a single, low molecular weight peak (K_{av} = 0.46), 46% of which was eliminated by the presence of excess unlabeled glucagon in the incubation prior to solubilization and fractionation. Less than 0.6% and 0% of specifically bound ^{125}I-glucagon eluted as high molecular weight complexes (K_{av} = 0.16) in solubilized G_h and G_i membranes, respectively (not shown).

The specific activities of marker enzymes in Golgi and plasma membranes were measured (Table I). Golgi membranes were purified 20–30-fold with respect to the Golgi enzyme marker galactosyl transferase, whereas plasma membranes had a relative specific activity of less than 7 for this enzyme. In contrast, plasma membranes were more highly enriched in 5′-nucleotidase than Golgi membranes. The lysosomal enzyme marker *N*-acetylglucosaminidase was depleted

Table I: Assay of Marker Enzymes in Various Cell Fractions

	GT[a]		5′-N		NAG	
	SA[b]	RSA	SA	RSA	SA	RSA
homogenate	0.15	1	7.24	1	5.9	1
microsomes	0.15	1	28.4	3.9	3.4	0.58
G_i	4.9	33	103	14	21	3.6
G_h	3.5	23	192	27	16	2.7
plasma membranes	1.0	6.7	388	54	2.0	0.34

[a] Abbreviations: GT, galactosyl transferase; 5′-N, 5′-nucleotidase; NAG, β-*N*-acetylglucosaminidase; SA, specific activity; RSA, relative specific activity (*x*-fold purification). [b] The specific activities are presented in picomoles per minute per milligram for galactosyl transferase and in nanomoles per minute per milligram for 5′-nucleotidase and *N*-acetylglucosaminidase.

Table II: Adenylyl Cyclase Specific Activity[a] in Golgi Fractions Exposed to Glucagon

		glucagon concentration (nM)				
fraction	expt	0	0.1	1	10	100
G_i						
fresh	1	4.4 ± 0.5	6.4 ± 0.8	4.7 ± 0.3	5.9 ± 1.1	4.5 ± 0.6
frozen	1	6.3 ± 1.7	3.4 ± 2.3	5.2 ± 2.3	6.7 ± 1.8	6.5 ± 0.9
frozen	2	7.7 ± 0.6	9.1 ± 1.3	7.6 ± 0.7	7.8 ± 0.5	7.0 ± 1.8
G_h						
fresh	1	3.6 ± 1.1	2.6 ± 0.7	2.6 ± 0.2	3.0 ± 0.3	2.2 ± 0.4
frozen	1	3.8 ± 1.2	3.6 ± 1.2	2.8 ± 0.5	2.8 ± 0.2	3.7 ± 1.0
frozen	2	8.7 ± 1.7	10.2 ± 2.3	11.3 ± 2.4	10.7 ± 1.2	11.6 ± 1.5
plasma membranes		33.4 ± 3.5	31.0 ± 7.5	52.5 ± 5.5	290 ± 70	339 ± 34

[a] The concentration of cAMP was measured by radioimmunoassay, and the specific activity of adenylyl cyclase is presented in picomoles per minute per milligram.

Table III: Adenylyl Cyclase Specific Activity[a] in Golgi Fractions Exposed to Fluoride or Gpp(NH)p

	basal	fluoride	Gpp(NH)p
G_i membranes	10.4 ± 1.4	8.8 ± 0.2	8.5 ± 1.7
G_h membranes	1.2 ± 0.4	2.3 ± 0.6	5.5 ± 0.6
plasma membranes	10 ± 4	119 ± 13	74 ± 8

[a] The concentration of cAMP was measured by protein binding, and the specific activity of adenylyl cyclase is presented in picomoles per minute per milligram.

in plasma membranes and slightly enriched in Golgi membranes relative to a crude liver homogenate. Adenylyl cyclase is stimulated approximately 4–10-fold by 10^{-7} M glucagon in plasma membranes (Pohl et al., 1971; Table II) but not in freshly isolated Golgi fractions (Cheng & Farquhar, 1976; Table II). We also did not observe glucagon stimulation of adenylyl cyclase in Golgi fractions after two freeze/thaw cycles (Table II). Fluoride ion and Gpp(NH)p enhanced the specific activity of adenylyl cyclase in plasma membranes and consistently produced a small stimulation of the enzyme in G_h membranes but did not significantly alter cAMP formation in G_i membranes. This result was observed whether cAMP was measured by protein binding (Table III) or by the method of Salomon et al. (1974) (not shown).

Four freeze/thaw cycles enhanced ^{125}I-insulin binding to intermediate Golgi vesicles 1.8–3.2-fold without appreciably affecting hormone binding to plasma membranes (Bergeron et al., 1978). In this study, binding of ^{125}I-insulin (1 nM) or ^{125}I-glucagon (5 nM) to freshly isolated Golgi or plasma membranes (0.5 mg/mL) was assayed after 60 min at 4 °C in the presence of 0.1% bovine serum albumin in HBSS. ^{125}I-Insulin binding to intermediate and heavy Golgi membranes was enhanced 3- and 2-fold, respectively, after four freeze/thaw cycles (not shown). ^{125}I-Glucagon binding to each Golgi fraction was enhanced up to 1.7-fold by freeze/thaw cycles. The binding of both hormones to plasma membranes was unaffected by similar treatment.

Discussion

Golgi fractions in which glucagon receptors were observed have enzymatic characteristics similar to those in which insulin and prolactin receptors were identified (Bergeron et al., 1973b, 1978; Posner et al., 1979). The enrichment of galactosyl transferase specific activity in intermediate (33-fold) and heavy (23-fold) Golgi fractions relative to a crude liver homogenate (Table I) was nearly identical with those of intermediate (33.4-fold) and heavy (17.7-fold) fractions reported by Khan et al. (1981). A small enrichment of *N*-acetylglucosaminidase in intermediate (3.6-fold) and heavy (2.7-fold) Golgi fractions is also consistent with previously reported values (2.0- and 1.7-fold, respectively) (Khan et al., 1982). In addition, the specific activities of 5′-nucleotidase in intermediate and heavy Golgi membranes (103 and 192 nmol min^{-1} mg^{-1}, respectively) are slightly lower than reported values (170 and 249 nmol min^{-1} mg^{-1}, respectively) (Bergeron et al., 1978). Enrichment in 5′-nucleotidase or the lysosomal marker *N*-acetylglucosaminidase is not indicative of contamination of Golgi fractions, as Farquhar et al. (1974) identified 5′-nucleotidase and acid phosphatase, another lysosome marker, as enzymes indigenous to Golgi fractions.

Enhancement of ^{125}I-insulin and ^{125}I-glucagon binding to Golgi fractions was observed after freeze/thaw cycles. This property results from the isolation of Golgi membranes as sealed vesicles in which receptors are sequestered. The observed enhancement was somewhat more variable (0–3-fold) than previously reported for ^{125}I-insulin binding to intermediate Golgi vesicles (1.8–3.2-fold enhancement) (Bergeron et al., 1978). We speculate that this may result from different amounts of vesicle disruption during the isolation of Golgi fractions. As previously reported for insulin binding (Bergeron et al., 1978), glucagon and insulin binding to plasma membranes was not enhanced by freeze/thaw cycles. Thus, enhancement of hormone binding after freeze/thaw cycles as well as marker enzyme activities indicates that Golgi fractions containing glucagon receptors are very similar to those previously demonstrated to contain insulin and prolactin receptors (Bergeron et al., 1978).

The receptors of Golgi fractions, containing only low-affinity binding sites, have a 3.5–5-fold lower apparent affinity for ^{125}I-glucagon than receptors of plasma membranes which

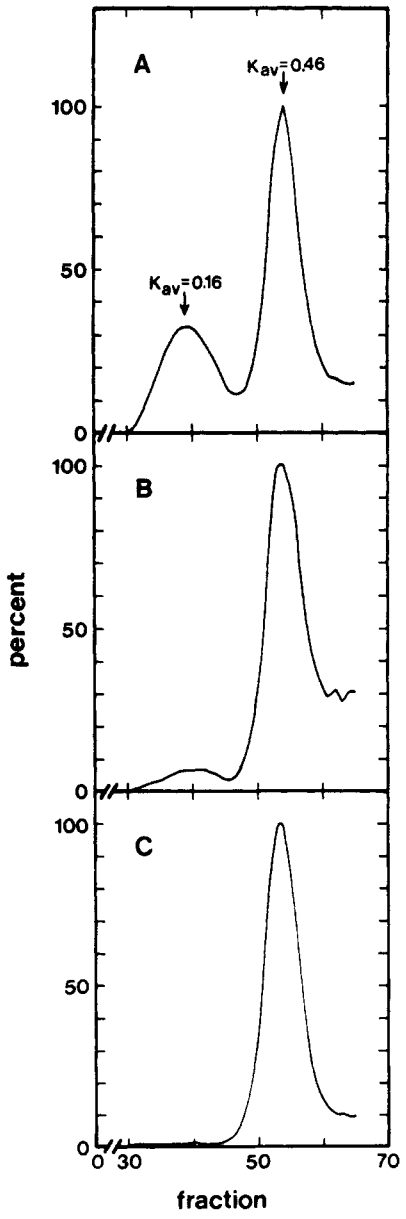

FIGURE 7: Fractionation of solubilized glucagon receptors on Ultrogel AcA 34. Heavy Golgi (panel C) or plasma membranes (panels A and B) (2.3 mg of protein/mL) were incubated for 30 min at 23 °C with ^{125}I-glucagon (11 nM) in the absence (panels A and C) or presence (panel B) of GTP (1.7 μM). The membranes were collected by centrifugation, solubilized, and fractionated on Ultrogel AcA 34 as described under Methods. To test the effect of GTP on the hydrodynamic properties of solubilized glucagon receptors (panel B), nucleotide was also added to the supernatant prior to fractionation (100 μM) and to the elution buffer (10 μM). The eluted radioactivity is expressed as a fraction of the maximal radioactivity of the second peak (K_{av} = 0.46).

contain high- as well as low-affinity sites (Figures 2 and 3). In the experiment illustrated in Figure 4, high-affinity binding sites were preferentially occupied when plasma membranes were incubated with 4 nM ^{125}I-glucagon, a concentration below the K_D of the lower affinity sites. Under these conditions, a greater proportion of bound ^{125}I-glucagon was released from Golgi membranes than from plasma membranes during dissociation (Figures 4 and 5). The diminished affinity of Golgi fractions does not result from hormone degradation, from a subtle alteration of the hormone by Golgi membranes, from factors present in Golgi fractions that can alter the affinity of contaminating plasma membrane receptors, or from the freeze/thaw treatment used to disrupt the isolated vesicles. Freshly isolated Golgi fractions (Cheng & Farquhar, 1976) also differ from plasma membranes (Pohl et al., 1971) in the inability of glucagon to stimulate cAMP production (Table II). Two freeze/thaw cycles enhanced hormone binding to Golgi membranes (not shown) without loss of basal adenylyl cyclase activity (Table II). Since glucagon did not stimulate Golgi membrane adenylyl cyclase after two freeze/thaw cycles (Table II), receptor sequestration within Golgi vesicles cannot explain the inability of hormone to stimulate the enzyme. These properties provide additional evidence that contamination of Golgi fractions with plasma membrane receptors does not account for our observations and suggest that glucagon receptors in Golgi fractions are uncoupled from adenylyl cyclase.

The affinity of glucagon receptors for hormone, as well as the ability of the receptor to stimulate activation of adenylyl cyclase, is determined by interaction with a regulatory protein (N or G protein) (Rodbell, 1980; Gilman, 1984). In plasma membranes, glucagon receptors that are coupled to N proteins have a high affinity for ^{125}I-glucagon. GTP diminishes the receptor affinity for hormone (Rodbell et al., 1971) and augments hormone dissociation (Figure 4) by promoting dissociation of receptor/N protein complexes (Rodbell, 1980). GTP significantly increased the rate of dissociation of glucagon from plasma membranes (Figure 4) but only produced a slight effect on the release of hormone from Golgi membranes (Figure 5). These observations also suggest that glucagon receptors in Golgi fractions are not coupled to N proteins.

Fluoride and Gpp(NH)p stimulate adenylyl cyclase by activating N proteins (Rodbell, 1980; Gilman, 1984). The lack of significant stimulation of adenylyl cyclase in G_i membranes suggests that N proteins are absent in this fraction. In contrast, a small stimulation of G_h membrane adenylyl cyclase by fluoride and Gpp(NH)p was observed. Cheng and Farquhar (1976) reported a greater fluoride stimulation of Golgi fraction adenylyl cyclase (25- and 10-fold for G_i and G_h, respectively) than is shown in Table III. The reason for this difference is not clear. A small amount of fluoride stimulation of adenylyl cyclase in "light" membranes containing β-adrenergic receptors has also been observed, although N proteins and catalytic subunits were not translocated into such fractions along with receptors (Stadel et al., 1983; Waldo et al., 1983).

Welton et al. (1977) demonstrated that active adenylyl cyclase and ^{125}I-glucagon/receptor complexes could be solubilized from rat liver plasma membranes. When solubilized membranes were fractionated by gel filtration chromatography, ^{125}I-glucagon eluted in high molecular weight, GTP-sensitive complexes that partially cofractionated with adenylyl cyclase activity. This suggests that high molecular weight complexes contain ^{125}I-glucagon bound to receptors coupled to the other components of the adenylyl cyclase system. ^{125}I-Glucagon also eluted in a lower molecular weight, GTP-insensitive peak that was not unambiguously demonstrated to contain hormone/receptor complexes since it cofractionated with lipid/detergent micelles containing ^{125}I-glucagon (Welton et al., 1977). We observed similar fractionation of ^{125}I-glucagon complexes solubilized from plasma membranes (Figure 7). High molecular weight complexes (K_{av} = 0.16) were GTP sensitive and therefore presumably coupled to N proteins. Formation of the lower molecular weight species (K_{av} = 0.46) was inhibited by unlabeled glucagon, suggesting that this peak also contains specific ^{125}I-glucagon/receptor complexes. The lower molecular weight complexes are presumed to be uncoupled from N proteins since they were insensitive to GTP. Golgi membranes contain almost exclusively lower molecular weight

species (K_{av} = 0.46), consistent with the suggestion that glucagon receptors in such fractions are not coupled to the other components of adenylyl cyclase. This probably accounts for the inability of glucagon to stimulate adenylyl cyclase in Golgi fractions. The absence of high molecular weight complexes in solubilized G_i membranes also confirms by yet another criterion that this fraction is not contaminated by plasma membrane. The small amount of specific, high molecular weight complexes in G_h membrane suggests that less than half of the ^{125}I-glucagon binding to this fraction could result from plasma membrane contamination.

Observation of low-affinity, intracellular glucagon receptors, although unique for a peptide hormone receptor, is not without precedent. β-Adrenergic receptors of astrocytoma cells (Su et al., 1980), C_6 glioma cells (Homburger et al., 1980; Fishman et al., 1981), S49 lymphoma cells (Insel et al., 1983), and frog erythrocytes (Kent et al., 1980) rapidly uncoupled from adenylyl cyclase upon exposure to catecholamines. Uncoupled receptors have a low affinity for agonists (Fishman et al., 1981), are insensitive to guanine nucleotides (Harden et al., 1979), and may be isolated in plasma membrane fractions of cells treated with concanavalin A (Toews et al., 1984) or subjected to especially gentle disruption (Strader et al., 1984). In the absence of such special treatment and after uncoupling, some β-adrenergic receptors are translocated from plasma membranes to intracellular, light membranes (Waldo et al., 1983; Stadel et al., 1983), where only low-affinity receptors are found (Stadel et al., 1983; Toews et al., 1984). Such light membranes cannot be completely resolved from Golgi elements (galactosyl transferase activity) by centrifugation through sucrose density gradients (Waldo et al., 1983).

Hepatic glucagon receptors appear to be regulated by mechanisms similar to those summarized above (Heyworth & Houslay, 1983). For example, glucagon receptors of rat liver membranes become insensitive to the dissociating effects of GTP after extended association with ^{125}I-glucagon (Corin et al., 1982). This may be analogous to uncoupling of β-adrenergic receptors from N proteins which is an early event in desensitization that occurs at the plasma membrane. Subsequent to binding, ^{125}I-glucagon is internalized by rat hepatocytes (Barazzone et al., 1980), and the glucagon receptor is down-regulated (Noda et al., 1984). Thus, a likely explanation for the observation of low-affinity, GTP-insensitive glucagon receptors in Golgi fractions of rat liver is that during down-regulation induced by endogenous glucagon in the serum of fasted rats, uncoupled glucagon receptors may be translocated from plasma membranes to membranes that copurify with Golgi fractions, as has been shown for insulin receptors (Kay et al., 1984). Cultured hepatocytes also appear to have a reserve pool of intracellular receptors.[2] We have observed that glucagon receptors on the surface of cultured hepatocytes can be degraded by digestion with chymotrypsin and that the synthesis of new receptors can be halted by incubation of such cells with cycloheximide. Under these conditions, hormone binding at the cell surface is recovered with time. This may suggest a function for intracellular receptors in the maintenance of cellular sensitivity to hormone.

Glycoproteins, such as the glucagon receptor (Iyengar & Herberg, 1984), are glycosylated in the Golgi apparatus prior to transport to the cell surface (Farquhar, 1983). Therefore, another explanation for glucagon receptors in Golgi fractions is that they are newly synthesized. β-Adrenergic receptors and N proteins are synthesized independently. For example, the number of β-adrenergic receptors on HeLa cells increases upon exposure to butyrate, without a concomitant increase of hormone-stimulatable cAMP production (Henneberry et al., 1977). Conversely, during postnatal development of rat parotid gland secretory function, expression of N proteins increases without an increase of β-adrenergic receptors (Ludford & Talamo, 1983). Since both receptors and N proteins can be expressed independently, newly synthesized receptors would not be expected to be coupled to N proteins in the Golgi.

Several of the observations described in this report are both novel and significant. This is the first characterization of intracellular glucagon receptors. These receptors have a lower affinity for hormone than glucagon receptors in plasma membranes. Golgi membrane receptors are relatively insensitive to GTP, are unable to activate adenylyl cyclase, and fractionate by gel filtration chromatography with a K_{av} that is identical with that of uncoupled glucagon receptors from plasma membranes. These facts demonstrate that the glucagon receptors in Golgi fractions are predominantly uncoupled from N proteins. Thus, this is also the first demonstration of an intracellular *peptide* hormone receptor that is uncoupled from an effector system.

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Registry No. GTP, 86-01-1; Gpp(NH)p, 34273-04-6; glucagon, 9007-92-5; adenylyl cyclase, 9012-42-4; fluoride, 16984-48-8.

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