Divalent Cations Regulate Glucagon Binding. Evidence for Actions on Receptor–N_s Complexes and on Receptors Uncoupled from N_s

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ABSTRACT: The effects of Mg^{2+} or ethylenediaminetetraacetic acid (EDTA) on ^{125}I-glucagon binding to rat liver plasma membranes have been characterized. In the absence of guanosine 5′-triphosphate (GTP), maximal binding of ^{125}I-glucagon occurs in the absence of added Mg^{2+}. Addition of EDTA or Mg^{2+} diminishes binding in a dose-dependent manner. In the presence of GTP, maximal binding occurs in the presence of 2.5 mM Mg^{2+} (EC_{50} = 0.3 mM) while EDTA or higher concentrations of Mg^{2+} diminish binding. Response to exogenous Mg^{2+} or EDTA depends on the concentration of Mg^{2+} in the membranes and may vary with the method used for membrane isolation. Solubilized ^{125}I-glucagon–receptor complexes fractionate on gel filtration columns as high molecular weight, GTP-sensitive complexes in which receptors are coupled to regulatory proteins and lower molecular weight, GTP-insensitive complexes in which receptors are not coupled to other components of the adenylyl cyclase system. In the absence of GTP, 40 mM Mg^{2+} or 5 mM EDTA diminishes receptor affinity for hormone (from K_D = 1.2 ± 0.1 nM to K_D = 2.6 ± 0.3 nM) and the fraction of ^{125}I-glucagon in high molecular weight receptor–N_s complexes without affecting site number (B_{max} = 1.8 ± 0.1 pmol/mg of protein). Thus, while GTP promotes disaggregation of receptor–N_s complexes, Mg^{2+} or EDTA diminishes the affinity with which these species bind hormone. In the presence of GTP, hormone binds to lower affinity (K_D = 9.0 ± 3.0 nM), low molecular weight receptors uncoupled from N_s. Binding site affinity is diminished by 40 mM Mg^{2+} or 5 mM EDTA (K_D = 20 ± 5 nM) and increased by 0.5 mM Mg^{2+} (K_D = 3.0 ± 0.4 nM) without significantly changing the amount of ^{125}I-glucagon in high molecular weight receptor–N_s complexes. The ability of Mg^{2+} to alter binding to receptors uncoupled from N_s suggests the presence of a cation binding site in the glucagon receptor. GTP enhances hormone dissociation from control membranes or membranes incubated with 0.5 mM Mg^{2+} (EC_{50} = 270 nM). EDTA (5 mM) diminishes (EC_{50} = 500 nM GTP) while Mg^{2+} (40 mM) enhances (EC_{50} = 30 nM GTP) the sensitivity of glucagon–receptor complexes to GTP-induced dissociation. Thus, divalent cations also affect the sensitivity of hormone–receptor–regulatory protein complexes to GTP-induced disaggregation.

Transmembrane signaling through receptors that stimulate adenylyl cyclase activity is affected by interaction of ligand–receptor complexes with regulatory proteins (N_s or G_s) (Rodbell, 1980; Gilman, 1984; Birnbaumer et al., 1985). Regulatory proteins contain binding sites for low molecular weight cofactors, such as guanosine 5′-triphosphate (GTP)[1] and Mg^{2+}, which affect their conformation and activity. The hormone-stimulated activation of N_s promotes the conversion of ATP to cAMP by adenylyl cyclase. Subsequent hydrolysis of bound GTP deactivates the regulatory protein, thereby diminishing the rate of production of cAMP.

Glucagon and β-adrenergic receptors coupled to N_s bind ligand with high affinity. Uncoupled receptors bind ligand with lower affinity (Rodbell, 1980; Gilman, 1984; Birnbaumer et al., 1985). Factors such as guanine nucleotides and Mg^{2+} that affect the conformation of N_s (Sternweis et al., 1981; Codina et al., 1984) affect receptor–regulatory protein interactions and ligand–receptor binding. For example, Mg^{2+} increases β-adrenergic agonist binding by increasing receptor affinity for ligand (Williams et al., 1978; Bird & Maguire, 1978). GTP promotes disaggregation of receptor–N protein complexes (Rodbell, 1980) and thereby increases the dissociation of ^{125}I-glucagon from rat liver plasma membranes (Rodbell et al., 1971b). EDTA also affects the binding and dissociation of glucagon from rat liver plasma membranes (Rodbell et al., 1971a,b), suggesting that divalent cations stabilize glucagon–receptor as well as β-adrenergic agonist–receptor interactions. In contrast to the observations summarized above, Rojas and Birnbaumer (1985) reported that Mg^{2+} does not affect glucagon binding.

The present study characterizes the effects of Mg^{2+} and EDTA on ^{125}I-glucagon binding to rat liver plasma membranes in the absence or presence of GTP. Our observations indicate that divalent cations affect both glucagon–receptor binding and the sensitivity of receptor–N protein complexes to dissociation by GTP.

Experimental Procedures

Materials

[^{125}I-Tyr^{10}]Glucagon was from Amersham Corp. The sources of other materials were previously described (Corin

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; cAMP, adenosine 3′,5′-cyclic phosphate; EC_{50}, effective concentration that produces 50% of the maximal effect; GTP, guanosine 5′-triphosphate; HBSS, Hank's balanced salt solution; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; [^{125}I-Tyr^{10}]glucagon, monoiodoglucagon labeled on Tyr-10; N_s, stimulatory, guanine nucleotide binding, regulatory protein of adenylyl cyclase; EDTA, ethylenediaminetetraacetic acid; TEA, triethylamine; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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et al., 1982; Lipson et al., 1986b).

Methods

Partially purified liver plasma membranes were prepared from 150–250-g male Sprague-Dawley rats by the Neville (1968) procedure as modified by Pohl et al. (1971). The concentration of protein in membranes was measured by the fluorescamine method (Böhlen et al., 1973) using bovine serum albumin (fraction V) as a standard. 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 column with bovine serum albumin (Corin et al., 1982).

Analysis of ^{125}I-Glucagon by HPLC. A C_{18} Sep Pak cartridge (Waters Associates), prewashed sequentially with acetonitrile (5 mL) and 75 mM formic acid adjusted to pH 3 with triethylamine (TEA–formate), was used to remove bovine serum albumin from ^{125}I-glucagon recovered from QUSO-32. ^{125}I-Glucagon (500 μL) diluted 1:1 with 0.15 M TEA–formate was applied to the Sep Pak cartridge which was then washed with 2 mL of TEA–formate (75 mM) followed by 2 mL of 1:9 acetonitrile/75 mM TEA–formate. ^{125}I-Glucagon was eluted from the cartridge with 1:1 acetonitrile/75 mM TEA–formate (three sequential 1-mL washes). Acetonitrile in fractions containing ^{125}I-glucagon was partially evaporated by blowing a stream of compressed air over the liquid.

^{125}I-Glucagon from the Sep Pak cartridge was analyzed by modification of the procedure described by Hagopian and Tager (1984). ^{125}I-Glucagon was injected onto a C_{18} reversed-phase HPLC column (Bio-Rad Hipore 318, 0.46 × 25 cm) equilibrated with acetonitrile/75 mM TEA–formate (1:9) in a Varian Model 5020 high-performance liquid chromatography system. The column was washed with acetonitrile/TEA–formate buffer (5 min, 1 mL/min) before the proportion of acetonitrile was increased to 25% over 15 min. Isomers of ^{125}I-glucagon were eluted with a linear gradient from 25% acetonitrile and 75% TEA–formate to 35% acetonitrile and 65% TEA–formate over 2 h. [^{125}I-Tyr^{13}]Glucagon, [^{125}I-Tyr^{10,13}]glucagon, and [^{125}I-Tyr^{10}]glucagon eluted at 28.3%, 29.5%, and 30.6% acetonitrile, respectively. The fraction of radioactive label eluted as [^{125}I-Tyr^{10}]glucagon, [^{125}I-Tyr^{13}]glucagon, and [^{125}I-Tyr^{10,13}]glucagon was 11.3%, 63.2%, and 7.2%, respectively.

^{125}I-Glucagon–Receptor Binding. Binding to plasma membranes was assayed at 23 °C in Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} but containing bovine serum albumin (0.5%), 25 mM HEPES, pH 7.4, and various concentrations of Mg^{2+} or EDTA. Unless otherwise specified, the isomeric mixture of ^{125}I-glucagon was employed in binding measurements. Substitution of Tris buffer for HBSS did not alter the effects of divalent cations on glucagon binding (not shown). When binding was measured in the presence of GTP, the incubation mixture also contained 20 mM phosphocreatine and 0.25 mg/mL (40–50 units/mL) creatine phosphokinase. Addition of phosphocreatine and creatine phosphokinase did not significantly alter hormone binding (not shown). Triplicate aliquots (100 μL) were assayed for 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 for measurement of ^{125}I. Radioactive uptake by membranes in the presence of ^{125}I-glucagon is defined as total hormone binding. Nonspecific binding is defined as uptake of ^{125}I-glucagon in the presence of unlabeled glucagon (3–6 μM). Specific binding is the difference between total and nonspecific binding. Binding isotherms were analyzed by nonlinear regression (Donner et al., 1980). Each point is the mean of triplicate determinations. The data shown represent the average of two to five experiments ± the SEM.

Dissociation of bound ^{125}I-glucagon was initiated by 100-fold dilution of incubation mixtures into hormone-free medium. The extent of dissociation was measured by vacuum filtration through Durapore filters [Millipore poly(vinylidene difluoride) GVWP, 0.2 μm]. The filters were soaked in 5% bovine serum albumin for 1 h at 23 °C, mounted on a filtration manifold, and washed with 10 mL of dissociation medium (0.1% bovine serum albumin in HBSS, pH 7.4, 23 °C) immediately prior to use. Binding assayed by filtration of three 10-mL aliquots immediately after dilution into dissociation medium containing phosphocreatine and creatine phosphokinase was defined as the zero time point. After 60 min of dissociation, triplicate aliquots were removed from the incubates for assay of binding. To characterize the effect of GTP 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.

Characterization of Solubilized Receptors. Solubilized hormone–receptor complexes were characterized by gel filtration chromatography (Welton et al., 1977; Lipson et al., 1986a,b). Membranes were incubated with ^{125}I-glucagon (30 min, 23 °C), centrifuged (80000*g*, 10 min), resuspended in a solubilization buffer (5 mg of protein/mL in 25 mM Tris, pH 7.4, 1% Lubrol PX, and the indicated concentration of Mg^{2+} or EDTA at 4 °C), and incubated on ice for 30 min. Insoluble material was then removed by centrifugation (185000*g*, 30 min). An aliquot of the supernatant (0.8 mL, about 40000 cpm) was applied to an Ultrogel AcA 34 column (0.9 × 100 cm) equilibrated with 25 mM Tris, pH 7.4, 0.01% Lubrol PX, and the indicated concentration of Mg^{2+} or EDTA at 4 °C and eluted in 1-mL fractions at 12 mL/h. The void (fraction 29) and salt (fraction 80) volumes of the column were determined by elution of blue dextran and reduced DTNB, respectively. Degradation products of ^{125}I-glucagon (assayed as trichloroacetic acid soluble radioactive label) eluted near the salt volume (K_{av} = 0.87–1.06), and intact hormone eluted slightly after the salt volume (K_{av} = 1.09) (not shown), suggesting that ^{125}I-glucagon interacts with the column. However, all applied radioactivity was recovered from each column, and the fraction of ^{125}I-glucagon in each peak was highly reproducible between experiments (≤±2% for each eluted peak).

To test if physiological salt concentrations altered elution profiles, hypotonic Tris buffer was replaced with HBSS without Ca^{2+} or Mg^{2+}. This did not significantly change the elution pattern. When the Lubrol concentration was increased from 0.01% to 0.1% or 1.0% during gel filtration, ^{125}I-glucagon fractionated exclusively with detergent micelles or as free hormone. This indicates that the conditions used in this study, which were previously defined by Welton et al. (1977), are optimal for fractionation of solubilized glucagon receptors.

Measurement of the Mg^{2+} Content of Control and Mg^{2+}-Washed Membranes. Plasma membranes (3.2 mg of protein/mL) were incubated with 50 mM Mg^{2+} (15 min, 4 °C), centrifuged (10 min, 100000*g*), washed with Ca^{2+}- and Mg^{2+}-free HBSS (4 °C), and resuspended in this medium. An equal volume of extraction buffer (20% trichloroacetic acid/5% lanthanum oxide) was added to control or Mg^{2+}-washed membranes which were incubated for 30 min and then diluted to one-fifth of their original volume. Particulate matter was removed by centrifugation, and the concentration of Mg^{2+}

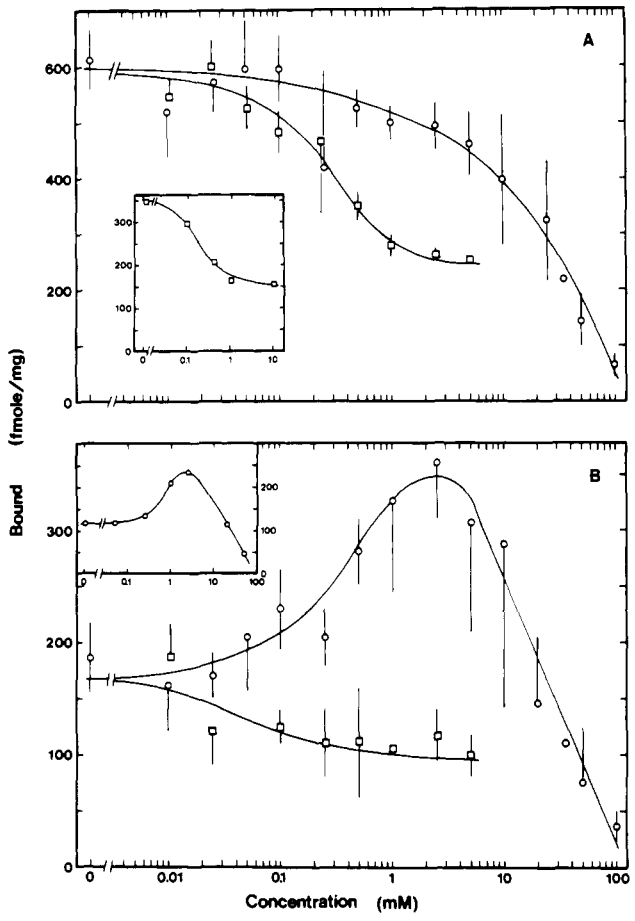

FIGURE 1: Effect of Mg^{2+} or EDTA concentration on ^{125}I-glucagon binding. (Panel A) Plasma membranes (0.36 mg of protein/mL) in HBSS without Ca^{2+} or Mg^{2+} were incubated with ^{125}I-glucagon (1 nM) and various concentrations of Mg^{2+} (○) or EDTA (□) at 23 °C for 60 min before specific hormone binding was measured. (Inset) Membranes (0.36 mg of protein/mL) were incubated with [^{125}I-Tyr^{10}]glucagon (0.6 nM) and various concentrations of EDTA for 60 min at 23 °C before assay of binding. (Panel B) Membranes (0.3 mg of protein/mL) were incubated with ^{125}I-glucagon (1 nM) at 23 °C for 60 min in the presence of GTP (10 μM) and various concentrations of Mg^{2+} (○) or EDTA (□) before assay of binding. (Inset) Membranes (0.36 mg of protein/mL) were incubated with [^{125}I-Tyr^{10}]glucagon in the presence of GTP (10 μM) and various concentrations of Mg^{2+} for 60 min at 23 °C before assay of binding.

in the supernatant from control or washed membranes was measured by using a Perkin-Elmer Model 372 flame atomic absorption spectrometer.

RESULTS

To determine if hormone binding is affected by Mg^{2+}, the amount of ^{125}I-glucagon specifically bound to plasma membranes was assayed in the presence of various concentrations of Mg^{2+} or EDTA (Figure 1). In the absence of added Ca^{2+} or Mg^{2+}, approximately 600 fmol of ^{125}I-glucagon bound per milligram of membrane protein. Binding remained constant until the Mg^{2+} concentration exceeded about 1 mM Mg^{2+} and then diminished (panel A). The decreased binding observed in the presence of high concentrations of Mg^{2+} could not be entirely reproduced by increasing the ionic strength of the medium with NaCl (not shown). EDTA decreased binding of the mixture of ^{125}I-glucagon isomers (panel A) and of [^{125}I-Tyr^{10}]glucagon (inset) with a half-maximal effect (EC_{50}) in the presence of 0.27 mM EDTA.

The effects of Mg^{2+} or EDTA on ^{125}I-glucagon binding were determined in the presence of GTP (Figure 1, panel B). Binding increased from approximately 175 fmol/mg to a maximum of about 350 fmol/mg of protein in the presence of 2.5 mM Mg^{2+} (EC_{50} = 0.3 mM) and was diminished by higher concentrations of Mg^{2+}. Mg^{2+} affected [^{125}I-Tyr^{10}]-glucagon binding similarly (inset). In the presence of GTP, EDTA decreased binding from 175 to approximately 100 fmol/mg of protein (EC_{50} = 50 μM).

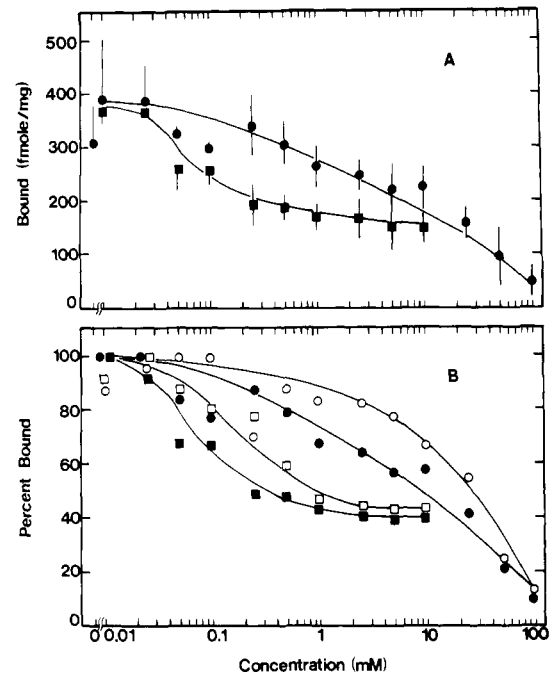

FIGURE 2: Effect of Mg^{2+} or EDTA concentration on ^{125}I-glucagon binding to Mg^{2+}-washed membranes. (Panel A) Membranes (3.6 mg of protein/mL) were washed with 50 mM Mg^{2+} for 15 min at 4 °C (Experimental Procedures). The Mg^{2+}-washed membranes (0.33 mg of protein/mL) were incubated with ^{125}I-glucagon (0.7 nM) at 23 °C for 60 min in the presence of various concentrations of Mg^{2+} (●) or EDTA (■), and specific hormone binding was measured. (Panel B) Specific binding to control (○, □) and Mg^{2+}-washed (●, ■) membranes in the presence of various concentrations of Mg^{2+} (○, ●) or EDTA (□, ■) expressed as a percentage of binding in the absence of added reagent.

Rojas and Birnbaumer (1985) reported that the affinity of glucagon receptors was similar in the presence of 5 mM Mg^{2+} or 5 mM EDTA and that hormone binding is therefore not dependent on Mg^{2+}. It is possible that the apparent discrepancy between this report and that of Rojas and Birnbaumer (1985) may result from differences in the amount of Mg^{2+} retained by membranes prepared in the two laboratories. To test this, plasma membranes were washed with Mg^{2+} (see Methods), and ^{125}I-glucagon binding was measured in the presence of Mg^{2+} or EDTA (Figure 2, panel A). The wash procedure increased the amount of Mg^{2+} retained by membranes from 15.4 ± 2.0 (n = 2) to 147.5 nmol/mg of protein, as determined by atomic absorption spectroscopy. The effect of added Mg^{2+} or EDTA on binding to Mg^{2+}-washed or control (Figure 1, panel A) membranes was qualitatively similar. However, less exogenous Mg^{2+} was required to decrease glucagon binding to washed membranes which now was not statistically different in the presence of 5 mM Mg^{2+} or 5 mM EDTA (Figure 2, panel A). Comparison of binding to washed and control membranes (Figure 2, panel B) shows that pretreatment with Mg^{2+} shifted the Mg^{2+} and EDTA titration curves to lower concentrations of added reagent (i.e., left-shifted). Thus, the sensitivity of glucagon binding to added Mg^{2+} or EDTA depends on the concentration of Mg^{2+} in membranes before any additions.

To determine if Mg^{2+} or EDTA acts by altering receptor affinity for hormone, ^{125}I-glucagon binding was measured in

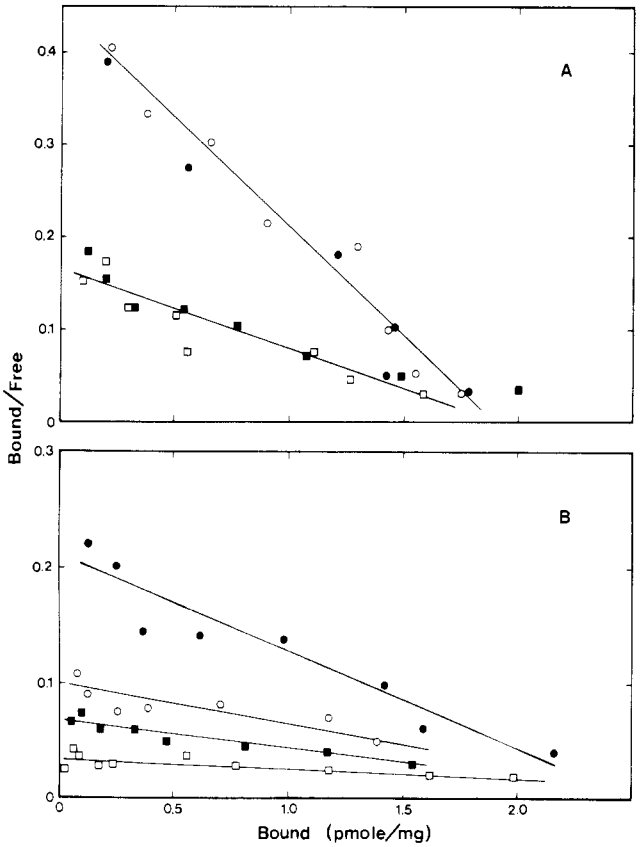

FIGURE 3: Effect of Mg^{2+} or EDTA concentration on receptor affinity. (Panel A) Membranes (0.3 mg of protein/mL) were incubated with ^{125}I-glucagon (0.15–18 nM) in HBSS without Ca^{2+} or Mg^{2+} and no additions (○), 0.5 mM Mg^{2+} (●), 40 mM Mg^{2+} (□), or 5 mM EDTA (■). After 60 min, specific binding was assayed. Error bars have been omitted from the figure for clarity. (Panel B) Membranes (0.3 mg of protein/mL) were incubated at 23 °C with ^{125}I-glucagon (0.18–32 nM) and GTP (10 μM) in HBSS without Ca^{2+} or Mg^{2+} and no additions (○), 0.5 mM Mg^{2+} (●), 40 mM Mg^{2+} (□), or 5 mM EDTA (■). After 60 min, specific binding was assayed.

the presence of these reagents and plotted according to the method of Scatchard (1949) (Figure 3, panel A). Computer analysis estimated that 1.8 ± 0.1 pmol of ^{125}I-glucagon bound to higher affinity receptors with a K_D of 1.2 ± 0.1 nM in the absence of other reagents. Binding to lower affinity sites could only be measured with inordinately high hormone concentrations and was not characterized in this experiment. Mg^{2+} (0.5 mM) did not affect binding to higher affinity sites whereas EDTA (5 mM) or 40 mM Mg^{2+} diminished the apparent affinity of these sites for glucagon (K_D = 2.6 ± 0.3 nM) without changing the number of sites.

Extrapolation of data obtained in the presence of GTP made it possible to examine binding to lower affinity receptors uncoupled from N_s (Figure 3, panel B). In the absence of added Mg^{2+} or EDTA, membranes bound 2.5 pmol of ^{125}I-glucagon/mg of protein (K_D = 9.0 ± 3.0 nM). The apparent affinity of uncoupled receptors for hormone was diminished (K_D = 20 ± 5 nM) by 40 mM Mg^{2+} or 5 mM EDTA but increased (K_D = 3.0 ± 0.4 nM) in the presence of 0.5 mM Mg^{2+}.

Solubilized ^{125}I-glucagon–receptor complexes eluted from an Ultrogel AcA 34 gel filtration column as high (K_{av} = 0.16) and low molecular weight (K_{av} = 0.46) species (Figure 4, panel A). Almost all hormone in high molecular weight complexes (21% of the eluted radioactivity) was specifically bound, but only 80% of the hormone in lower molecular weight complexes (50% of the eluted radioactivity) was specifically bound (Lipson et al., 1986a) because lipid–detergent micelles also

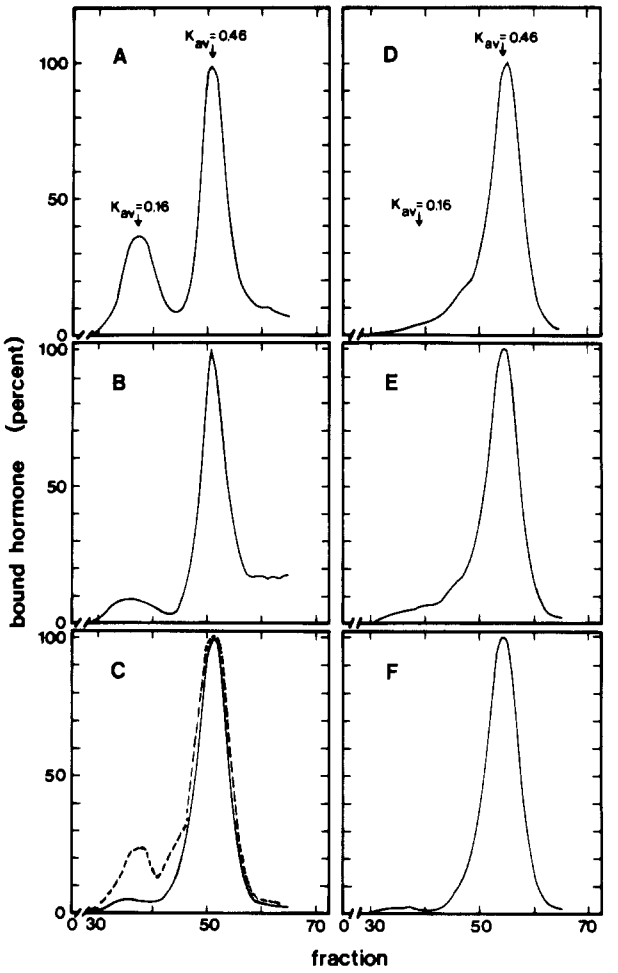

FIGURE 4: Fractionation of solubilized glucagon receptors on Ultrogel AcA 34. Membranes (5 mg of protein/mL) were incubated for 30 min at 23 °C with ^{125}I-glucagon (12 nM) in HBSS without Ca^{2+} or Mg^{2+} and no additions (panel A), 5 mM EDTA (panel B), 40 mM Mg^{2+} (panel C, solid line), 0.5 mM Mg^{2+} (panel C, dashed line), 11 μM GTP (panel D), 11 μM GTP and 0.5 mM Mg^{2+} (panel E), or 11 μM GTP and 40 mM Mg^{2+} (panel F). The membranes were collected by centrifugation, solubilized, and fractionated on Ultrogel AcA 34. Samples incubated with GTP were supplemented with fresh nucleotide (100 μM) after solubilization and fractionated in the presence of 11 μM GTP (panels D–F). Eluted radioactivity is expressed as a fraction of the maximal radioactivity of the second peak (K_{av} = 0.46).

elute in this volume (Welton et al., 1977). Only the higher molecular weight complexes were sensitive to GTP (panel D), indicating that they were coupled to N_s. Inclusion of 5 mM EDTA (panel B) or 40 mM Mg^{2+} (panel C, solid line) decreased the proportion of hormone in higher molecular weight complexes from 21% (panel A) to 7% or 4% of the total radioactivity, respectively. The effect of 40 mM Mg^{2+} was specific as HBSS without Ca^{2+} or Mg^{2+}, which has a greater ionic strength, did not produce a similar result (not shown). A greater fraction of the hormone (15%) was recovered in the high molecular weight peak after incubation with 0.5 than 40 mM Mg^{2+} (panel C, dashed line), which was consistent with increased receptor affinity for glucagon in the presence of the lower concentration of divalent cation. A two-sample t test (Freund, 1984) indicated that the fraction of hormone eluting in higher molecular weight complexes in the presence of 40 mM Mg^{2+} or 5 mM EDTA was significantly different ($p < 0.005$) from that of control membranes or membranes incubated with 0.5 mM Mg^{2+} (which were also significantly different from one another, $p < 0.005$). In the absence or presence of Mg^{2+} or EDTA, the ability of GTP to diminish

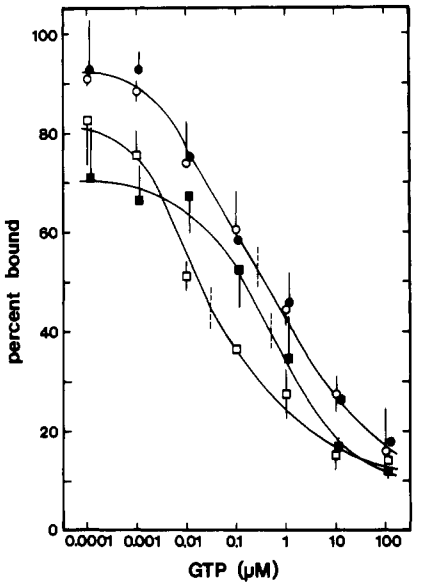

FIGURE 5: Effect of Mg^{2+} or EDTA concentration on GTP-promoted dissociation of ^{125}I-glucagon. Membranes (1.2 mg of protein/mL) were incubated for 60 min at 23 °C with ^{125}I-glucagon (4.7 nM) in HBSS without Ca^{2+} or Mg^{2+} and no additions (○), 0.5 mM Mg^{2+} (●), 40 mM Mg^{2+} (□), or 5 mM EDTA (■). Samples were then diluted into hormone-free medium containing the same concentration of Mg^{2+} or EDTA. Hormone binding immediately after dilution and after 60 min of dissociation in the presence of various concentrations of GTP was assayed. Data are presented as the fraction of ^{125}I-glucagon specifically bound after 60 min of dissociation relative to binding at the zero time point.

the fraction of hormone in high molecular weight fractions was significant ($p < 0.005$).

It is not valid to quantitatively compare hydrodynamic to binding experiments since the proportion of high- to low-affinity binding and specific to nonspecific binding varies with hormone concentration. The high concentration of ^{125}I-glucagon used in gel filtration experiments permitted recovery of sufficient solubilized radioactivity for hydrodynamic characterizations but emphasizes lower affinity interactions. Lower concentrations of ^{125}I-glucagon preferentially characterized higher affinity receptor interactions. However, the effects of Mg^{2+} or EDTA on the proportion of hormone in high molecular weight receptor–N_s complexes (Figure 4, panels A–C) and receptor affinity for glucagon (Figure 3, panel A) correlated qualitatively.

GTP nearly eliminated elution of ^{125}I-glucagon in higher molecular weight receptor–N_s complexes (Figure 4, panel D). Addition of 0.5 mM Mg^{2+} (panel E), 2.5 mM Mg^{2+} (not shown), or 40 mM Mg^{2+} (panel F) to membranes incubated with GTP did not significantly change the proportion of higher molecular weight complexes, suggesting that these reagents did not change binding by altering receptor–N_s interactions. Thus, hydrodynamic measurements, together with receptor binding assays (Figure 3, panel B), indicate that Mg^{2+} and EDTA can act on the glucagon receptor.

To determine if Mg^{2+} and EDTA regulate the sensitivity of hormone–receptor complexes to dissociation by GTP, membranes were incubated with these reagents and ^{125}I-glucagon before initiation of hormone dissociation in medium containing various concentrations of GTP. The fraction of hormone remaining specifically bound after 60 min of dissociation is shown as a function of nucleotide concentration in Figure 5. In the absence of GTP, about 92.5% of the bound hormone was retained by control membranes or membranes incubated with 0.5 mM Mg^{2+} after dissociation. GTP decreased the fraction of hormone remaining bound with an EC_{50} of 270 nM. EDTA (5 mM) or Mg^{2+} (40 mM) enhanced dissociation in the absence of nucleotide, and about 70% and 82%, respectively, of the hormone remained bound after 60 min. The EC_{50} for GTP-enhanced dissociation in the presence of 5 mM EDTA increased to 500 nM whereas 40 mM Mg^{2+} decreased the EC_{50} to 30 nM. Addition of 2.5 mM Mg^{2+} to control membranes did not affect ^{125}I-glucagon dissociation in the absence of GTP, and 92.5% of the hormone remained bound after 60 min. The EC_{50} for nucleotide-enhanced hormone dissociation in the presence of 2.5 mM Mg^{2+} (37 nM), however, was similar to that observed in the presence of 40 mM Mg^{2+} (not shown).

Discussion

In the absence of GTP, maximal binding of ^{125}I-glucagon to rat liver plasma membranes occurs without added Mg^{2+} (Figure 1). Titration of tightly bound Mg^{2+} with EDTA or addition of high concentrations of Mg^{2+} diminishes binding. In the presence of GTP, a greater concentration of Mg^{2+} (2.5 mM) is necessary to observe maximal glucagon binding. The EC_{50} (0.3 mM) for the effect of Mg^{2+} is similar to the estimated intracellular concentration of Mg^{2+} in several types of cells (0.2–1.5 mM; Rink et al., 1982; Terasaki & Rubin, 1985; Kushmerick et al., 1986) including rat hepatocytes (0.37 mM Mg^{2+}; Corkey et al., 1986). These observations suggest that in the physiological environment within cells (which includes GTP), Mg^{2+} contributes to the regulation of glucagon binding and hormonal stimulation of adenylyl cyclase. Moreover, these regulatory effects may occur at multiple sites.

The binding of [^{125}I-Tyr^{10}]glucagon or a mixture of iodinated glucagon isomers, consisting predominantly of [^{125}I-Tyr^{13}]glucagon, is regulated similarly by divalent cations. This is not surprising as various iodoglucagon isomers bind to receptors similarly (Hagopian & Tager, 1984). The dose response of binding to divalent cations is bell-shaped (Figure 1) which makes it possible to observe similar glucagon binding in the presence of low (added EDTA) or high concentrations of Mg^{2+}. The sensitivity of binding to exogenous Mg^{2+} or EDTA depends on the concentration of Mg^{2+} in the membranes (Figure 2), however, and consequently may vary with the procedure used to prepare plasma membranes or between laboratories.

Previous observations also suggest that divalent cations regulate glucagon binding. Rodbell et al. (1971a,b) demonstrated that EDTA promotes dissociation of ^{125}I-glucagon from rat liver plasma membranes and that GTP decreases the level of steady-state binding in the presence, but not the absence, of EDTA. Also, Mg^{2+} binds to and facilitates activation of N_s (Iyengar, 1981; Iyengar & Birnbaumer, 1981; Sternweis et al., 1981) and increases β-adrenergic agonist binding to membranes from a variety of cells (Williams et al., 1978; Bird & Maguire, 1978; Heidenreich et al., 1982).

Receptors coupled to regulatory proteins bind hormone with high affinity whereas uncoupled receptors bind glucagon with lower affinity. GTP promotes dissociation of receptor–N protein complexes by activating N_s and thereby diminishes receptor affinity for hormone (Rodbell, 1980; Gilman, 1984; Birnbaumer et al., 1985). ^{125}I-Glucagon–receptor complexes from rat liver plasma membranes fractionate on gel filtration columns as high molecular weight, GTP-sensitive complexes in which receptors are coupled to regulatory proteins and lower molecular weight, GTP-insensitive complexes in which receptors are not coupled to N_s (Welton et al., 1977; Lipson et al., 1986a,b). In this study, gel filtration and binding measurements show that as Mg^{2+} or EDTA diminishes receptor affinity in the absence of GTP (Figure 3), a smaller fraction

of bound ^{125}I-glucagon is in high molecular weight, GTP-sensitive complexes (Figure 4). Since there is no measurable decrease in the number of higher affinity receptors (Figure 3, panel A), these data suggest that divalent cations regulate the affinity of glucagon receptor–N_s complexes for hormone. Thus, while GTP affects receptor–N_s coupling, divalent cations determine how avidly such complexes bind glucagon.

In the presence of GTP, Mg^{2+} (up to 2.5 mM) increases ^{125}I-glucagon binding (Figure 1) without significantly increasing the proportion of hormone in higher molecular weight, coupled complexes (Figure 4). Although there is no direct evidence that the low molecular weight peaks in Figure 4E,F contain glucagon bound with differing affinities, the observation that Mg^{2+} alters the affinity of uncoupled receptors (Figure 3) implies the presence of a cation binding site in the glucagon receptor. This possibility is supported by the ability of EDTA to decrease ^{125}I-glucagon binding in the presence of GTP with an EC_{50} of 50 μM (Figure 1). Previous studies have shown that N_s contains a divalent cation binding site (Birnbaumer, 1985). Our observations indicate that divalent cations may also act on the glucagon receptor.

EDTA or high concentrations of Mg^{2+} decrease receptor affinity for hormone (Figure 3), decrease the proportion of hormone in receptor–N_s complexes (Figure 4), and enhance hormone–receptor dissociation in the absence of GTP (Figure 5). However, EDTA and Mg^{2+} change the sensitivity of receptor–regulatory protein complexes to GTP in opposite ways. This effect may be rationalized by considering that, in addition to acting at the level of the receptor, Mg^{2+} accelerates the activation of N_s by guanine nucleotides (Iyengar, 1981). This process may be inhibited by EDTA. While the Mg^{2+} concentrations (>20 mM) used to activate N_s in vitro are supraphysiological, the nucleotide sensitivity of hormone dissociation was also enhanced by 2.5 mM Mg^{2+} (not shown). This supports the hypothesis that regulation of glucagon receptor affinity by Mg^{2+} could be an important physiological process.

Glucagon, GTP, and Mg^{2+} play interdependent roles in regulating the hormone-sensitive adenylyl cyclase system. Glucagon reduces the K_m of N_s for Mg^{2+} and enhances N_s activation by guanine nucleotides (Iyengar & Birnbaumer, 1982), GTP alters the concentration of Mg^{2+} necessary to elicit maximal glucagon binding (Figure 1), and Mg^{2+} accelerates activation of N_s (Iyengar, 1981) and facilitates GTP-promoted hormone dissociation (Figure 5). These observations illustrate that regulation of interactions among constituents of the glucagon-sensitive adenylyl cyclase system involves a dynamic balance between hormone, GTP, and Mg^{2+} and suggests that changes in the concentrations of these species can differentially alter cellular responsiveness to glucagon.

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