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Glucagon induces disaggregation of polymer-like structures of the α subunit of the stimulatory G protein in liver membranes

(glucagon receptor/signal transduction)

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ABSTRACT **The hydrodynamic behavior of $G\alpha_s$, the α subunit of the stimulatory guanine nucleotide-binding regulatory protein (G protein), in octyl glucoside extracts of rat liver membranes was investigated. As was previously shown for G proteins similarly extracted from brain synaptoneurosomes, $G\alpha_s$ behaved as polydisperse structures with S values higher than that of heterotrimeric G proteins. At concentrations of guanosine 5′-[γ-thio]triphosphate (GTP[γS]) >100 μM, incubation with membranes led to smaller structures having S values in the range of 4–5 S. Incubation of liver membranes with glucagon also caused a marked increase in structures having these S values; glucagon action required the presence of low concentrations of GTP[γS] (maximal, 10 μM), was rapid (within 10 sec), and was not observed with vasopressin, angiotensin II, or glucagon-(19–29). When $G\alpha_s$ in its membrane-bound form was [^{32}P]ADP-ribosylated by cholera toxin and the treated membranes were extracted with octyl glucoside, >35% of the labeled $G\alpha_s$ was found in material that sedimented through sucrose gradients and contained relatively low levels of immunoreactive $G\alpha_s$. Glucagon selectively converted the apparently large molecular weight structures to the 4–5 S structures in the presence of GTP[γS], even at 1 mM (the maximal effect of the nucleotide alone), when incubated with the toxin-treated membranes. These findings suggest that the glucagon receptor selectively interacts with polymer-like structures of $G\alpha_s$ and that activation by GTP[γS] results in disaggregation. The role of the β and γ subunits of G proteins in the hormone-induced process is not clear since the polymer-like structures extracted with octyl glucoside are devoid of β and γ subunits.**

Guanine nucleotide-binding regulatory proteins, commonly termed G proteins, are a family of proteins some of which are coupled to membrane-associated receptors. These proteins directly or indirectly regulate various generators of second messengers (such as adenylyl cyclase, cGMP phosphodiesterase, and phospholipases) and regulate activities of various ion channels (1, 2). A widely held theory of how these G proteins function as transduction elements is based on the properties of extracted and purified forms containing three distinct subunits (α, β, and γ) of which the α subunit uniquely binds and degrades GTP to GDP. Release of the GTP-occupied α subunit from the heterotrimer is thought to reflect activation of G proteins by hormone-occupied receptors. Another theory of the structure and function of G proteins is not based on the properties of purified proteins but on the functional target sizes determined by irradiation of hormone-sensitive adenylyl cyclase systems in intact membranes (3). Termed "disaggregation coupling," this theory suggests that receptors coupled to G proteins are linked together as multimeric or oligomeric structures. Concerted activation of the receptor by hormone and GTP results in disaggregation of the multimers to form a "monomeric" complex of receptor and G protein. This complex was suggested to serve as the messenger or coupling agent with adenylyl cyclase and possibly other effector systems in the cell membrane.

We have reported (4) that various α subunits of G proteins extracted with octyl glucoside (OG) from rat brain synaptoneurosomes behave hydrodynamically on sucrose gradients as large polydisperse structures. These structures are devoid of β and γ subunits but, like their heterotrimeric counterparts, are sensitive to disaggregation by guanosine 5′-[γ-thio]triphosphate (GTP[γS]) or by aluminum fluoride. In the present study we report that similar large structures of $G\alpha_s$, the α subunit of the stimulatory G protein, are extracted with OG from rat liver membranes containing glucagon receptors. Glucagon, in the presence of GTP[γS], rapidly and selectively induces conversion of the large structures of $G\alpha_s$ into smaller structures that resemble those produced by high concentrations of GTP[γS] alone. These findings are consistent with the findings obtained with target analysis and lend further credence to the disaggregation–coupling theory of signal transduction.

MATERIALS AND METHODS

Reagents. All reagents, including specific antisera to $G\alpha_s$ and the β subunit of G proteins, were the same as in ref. 4. Glucagon was obtained from Peninsula Laboratories; angiotensin II and [Arg^5]vasopressin were from Sigma. Glucagon-(19–29) was kindly supplied by D. Bataille (Centre National de la Recherche Scientifique–Institut National de la Santé et de la Recherche Médical Centre de Pharmacologie, Endocrinologie, Montpellier, France).

Incubation Procedures. Plasma membrane fractions were prepared from rat liver by the Percoll fractionation procedure (5) and samples of the plasma membrane fraction were stored under liquid nitrogen. The membranes (5 mg/ml) were incubated at 30°C for 5 min, unless indicated, with or without 1 μM glucagon in incubation medium (20 mM Hepes·NaOH, pH 7.4/1 mM EDTA/0.15 M NaCl/2 mM $MgSO_4$/10 μM GTP[γS]/0.25 mM phenylmethylsulfonyl fluoride).

Gradient Fractionation. After incubation, OG was added to the membrane suspension to 1.5% (vol/vol) and the samples were immediately chilled on ice. This treatment immediately stopped hormone action. After stirring for 15 min, the samples were loaded on sucrose gradients [5–20% (wt/vol) containing 0.825% OG]. After centrifugation for 15 hr at 4°C in a Beckman SW 60 rotor, ≈20 fractions were collected and analyzed by SDS/PAGE and on immunoblots (4). Quantification of immunoblots by secondary labeling with ^{125}I-labeled donkey anti-rabbit IgG (Amersham) was performed

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Abbreviations: G protein, guanine nucleotide-binding regulatory protein; $G\alpha_s$, α subunit of the stimulatory G protein; OG, octyl glucoside; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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as described (6). As markers for relative S values ($s^0_{20,w}$), bovine serum albumin (4.4 S), bovine γ-globulin (7.7 S), and catalase (11.2 S) were added to the same gradients used with the OG extracts of membranes.

ADP-Ribosylation by Cholera Toxin. Liver membranes (2.8 mg) were incubated for 5 min at 30°C in 1 ml of medium containing 300 mM sodium phosphate (pH 7.4), 1 mM GTP, 1 mM ATP, 10 mM $MgSO_4$, leupeptin (3.75 μg/ml), aprotinin (1.5 μg/ml), 1 μM NAD^+, and cholera toxin (100 μg/ml; preactivated with 25 mM dithiothreitol for 20 min at 30°C). Membranes were subsequently washed three times by repeated centrifugation and resuspension in 50 mM Hepes, pH 7.4/0.25 mM phenylmethylsulfonyl fluoride. The treated membranes were incubated for 5 min at 30°C with or without 1 μM glucagon in the presence of 10 μM GTP[γS] and were processed as described above. In the experiments described in Fig. 5, the same batch of membranes was divided into two equal parts: one part was incubated with unlabeled NAD^+, and the other part was incubated with the same concentration of [^{32}P]NAD^+ (500 mCi; 1 Ci = 37 GBq) from New England Nuclear. Gradient fractionation, SDS/PAGE, and immunoblot analysis of both extracts were carried out as above. After immunoblot analysis, the radioactive bands were detected by autoradiography (2 days using Kodak XAR-5 plates). Bands corresponding to the positions of the immunoreactive $G\alpha_s$ subunits were scanned at 600 nm with an LKB densitometer; the readings are expressed as percent distribution of the fractions collected from the gradient (fractions 1–20) and the sedimented material (fraction 22).

RESULTS

Effects of Glucagon. Immunodetected $G\alpha_s$ extracted from rat liver plasma membranes with OG exhibited a wide range of S values on sucrose gradients including structures with hydrodynamic values >11.2 S (catalase marker) that tended to sediment (fractions 20 and above) (Fig. 1). The latter fractions, particularly the immunoreactive material that sedimented to the bottom of the centrifuge tube, proved difficult to recover quantitatively (see below). For this reason, most of the studies involving hormone and guanine nucleotide action emphasized changes in the content of $G\alpha_s$ in fractions exhibiting lower S values (fractions 1–20).

Glucagon action required incubation with intact membranes and GTP[γS] (Fig. 1*C*). Incubation of the membranes with 1 μM glucagon and 10 μM GTP[γS] for 5 min followed by extraction with OG (which effectively stops hormone action) resulted in an increase in the quantity of material in the 4.0–7.0 S range, the peak being in the region of 6 S (Fig. 1*A*). The hormone-induced changes occurred rapidly (within 10 sec) and were maximal in 1–2 min (data not shown). In contrast to $G\alpha_s$, the distribution of G β subunits was confined to a much smaller range of S values, with a peak at about 5 S and none in the sedimented fraction (data not shown); no shift in S values occurred when membranes were incubated with hormone and GTP[γS] (Fig. 1*B*).

No effect was observed with glucagon-(19–29) (Fig. 2), a proteolytically derived fragment of the hormone that inhibits a Ca^{2+} pump in rat liver by a GTP-dependent process independent of adenylyl cyclase activation (7). This finding rules out the possibility that the observed effects of glucagon are due to a separate receptor coupled to a G protein involved in regulation of a calcium pump. Vasopressin and angiotensin II, which act on receptors in rat liver that are not linked to $G\alpha_s$ and activation of adenylyl cyclase (8, 9), also failed to induce the shifts in S values observed with native glucagon (Fig. 2). This further indicates that the observed effects of glucagon are linked specifically to $G\alpha_s$ and are likely associated with its stimulatory effect on adenylyl cyclase activity.

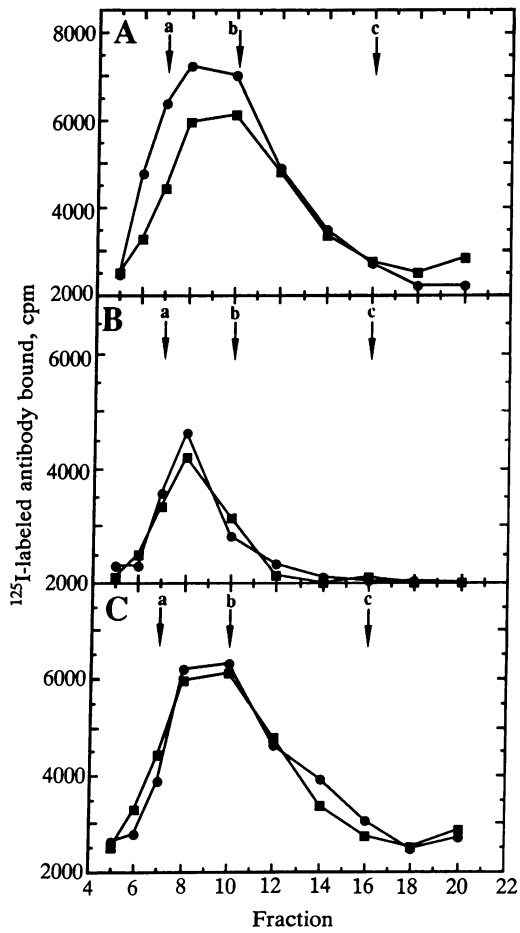

FIG. 1. Effects of glucagon on the hydrodynamic behavior of OG-extracted G proteins on sucrose density gradients. (*A*) Determined with antiserum specific for $G\alpha_s$. (*B*) Determined with antiserum against Gβ subunit. (*C*) Glucagon in absence of GTP[γS]. $s^0_{20,w}$ markers are indicated by arrows. Arrows: a, bovine serum albumin (4.4 S); b, bovine γ-globulin (7.7 S); c, catalase (11.2 S). ■, Control; ●, glucagon.

Effects of GTP[γS]. The actions of glucagon on $G\alpha_s$ are dependent on the presence of GTP[γS]. The minimal concentration of GTP[γS] required was 0.1 μM, but consistent and near-maximal effects of the nucleotide were observed in the range of 0.2–10 μM (Fig. 3). GTP, at any concentration

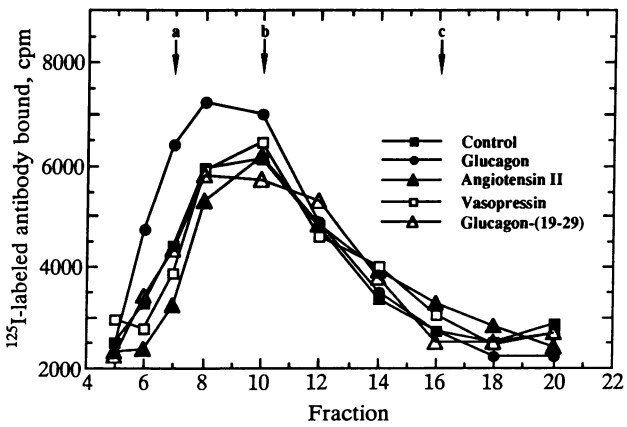

FIG. 2. Comparative effects of glucagon, glucagon-(19–29), vasopressin, and angiotensin II on the hydrodynamic behavior of $G\alpha_s$ in the presence of 10 μM GTP[γS]. Liver membranes were treated with 1 μM glucagon, 1 μM glucagon-(19–29), 1 μM angiotensin II, or 1 μM [Arg^5]vasopressin.

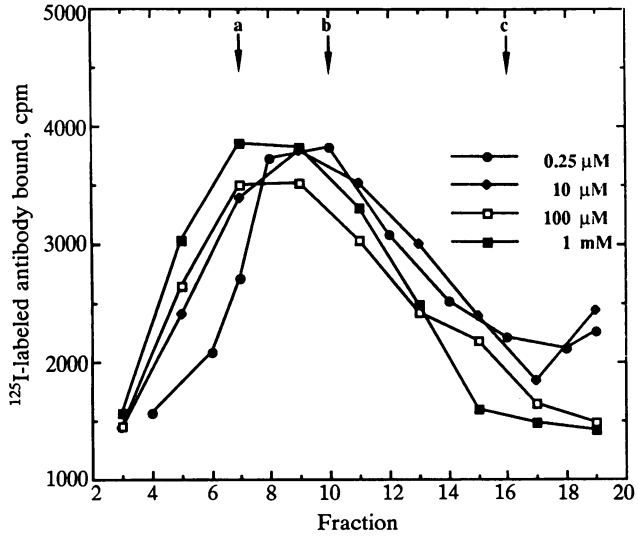

FIG. 3. Effects of GTP[γS] alone on the hydrodynamic behavior of $G\alpha_s$. Liver membranes were incubated with the indicated concentrations of GTP[γS].

tested, failed to support glucagon action. This we attribute to the high concentration of liver membranes (5 mg/ml) necessary to detect $G\alpha_s$ by the immunodetection method used in the gradient analysis and the rapid essentially complete hydrolysis of the nucleotide by the nucleotidases present in the membranes under the incubation conditions. In the absence of glucagon, conversion of $G\alpha_s$ from the high to low S values required much higher concentrations of GTP[γS]; maximal effects were observed with 1 mM GTP[γS] (Fig. 4) or at least two orders of magnitude higher than that required to observe maximal effects of glucagon in the presence of the nucleotide. Importantly, glucagon exerted its effect of $G\alpha_s$ even in the presence of 1 mM GTP[γS] (Fig. 4*D*), suggesting that the hormone acts in the presence of low concentrations of the nucleotide through a different pathway than that exerted by the nucleotide alone (see *Discussion*).

ADP-Ribosylation by Cholera Toxin. $G\alpha_s$ is a major substrate of the ADP-ribosylating unit of cholera toxin and is thought to be the dominant mediator of the physiological actions of the toxin (10). This action of the toxin enabled us to label $G\alpha_s$ and to follow the distribution of [^{32}P]ADP-ribosylated $G\alpha_s$ on sucrose gradients and the effects of glucagon thereon. Liver membranes were exposed to activated cholera toxin and [^{32}P]NAD for 5 min and then the reaction was stopped with high concentrations of unlabeled NAD. The thoroughly washed membranes were incubated with 10 μM GTP[γS] in the absence or presence of 1 μM glucagon, followed by OG extraction and gradient analysis. For comparison with results obtained by immunodetection of $G\alpha_s$, the same batch of membranes was treated with unlabeled rather than labeled NAD but otherwise in an identical fashion. As shown in Fig. 5*A*, two prominent immunoreactive bands of $G\alpha_s$ were found in essentially equal proportion in the gradient fractions from ≈4 S to 10 S. Glucagon and GTP[γS] increased the amount of $G\alpha_s$ in this range of hydrodynamic values. As stated above, only a small fraction of the total immunoreactive material was recovered in the very large structures that sedimented (fraction 21 and above), making it difficult to determine whether the increases in the soluble fraction could be accounted for by changes in the sedimented material. In striking contrast, a major portion (at least 35%)† of the total [^{32}P]ADP-ribosylated $G\alpha_s$ placed on the gradient was found in the OG-insoluble pellet (Fig. 5*B*). When the toxin-treated membranes were incubated with glucagon and GTP[γS], the total amount of labeled $G\alpha_s$ in the soluble fractions (3–6 S) increased by 50% over control whereas that in the heavily labeled sedimented fraction decreased concomitantly. In the absence of glucagon, 1 mM GTP[γS] caused nearly complete conversion of the sedimented form of $G\alpha_s$ to the lower molecular mass forms (data not shown).

Also interesting and unexpected was the finding that cholera toxin preferentially ADP-ribosylated the lower (42 kDa) band of $G\alpha_s$ (Fig. 5*B*). The upper (54 kDa) labeled band appeared as a doublet; the glucagon-induced shift on this

†The percent of total material in the sedimented fraction is likely an underestimate; the densitometric readings were not proportional to the levels of radioactivity in this fraction, which was necessarily overexposed to detect the labeled material in gradient fractions 1–20.

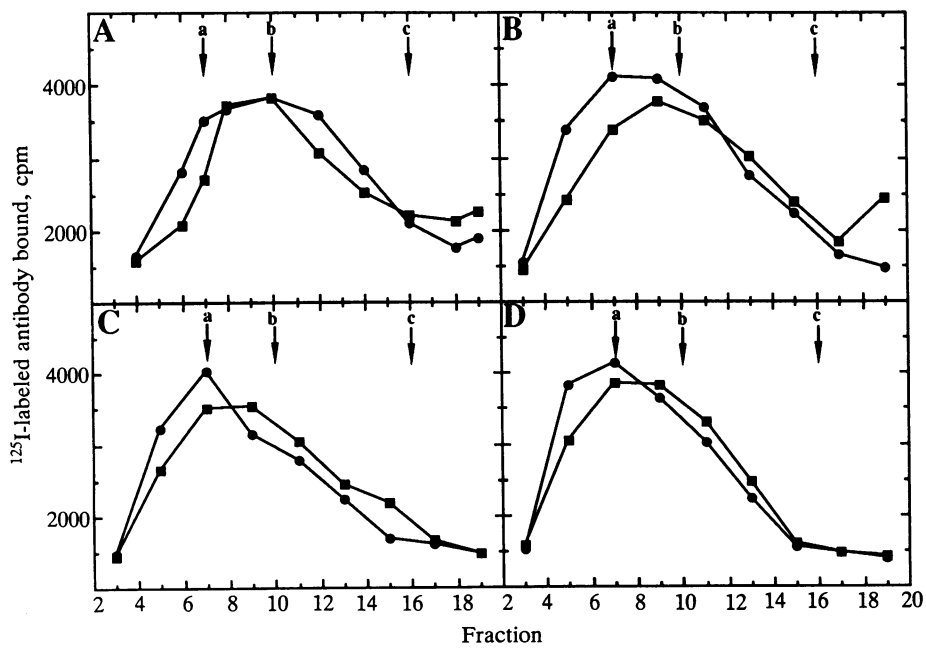

FIG. 4. Effects of glucagon on the hydrodynamic behavior of $G\alpha_s$ as a function of GTP[γS] concentration. Liver membranes were incubated with or without 1 μM glucagon in the presence of GTP[γS] at 0.25 μM (*A*), 10 μM (*B*), 100 μM (*C*), or 1 mM (*D*).

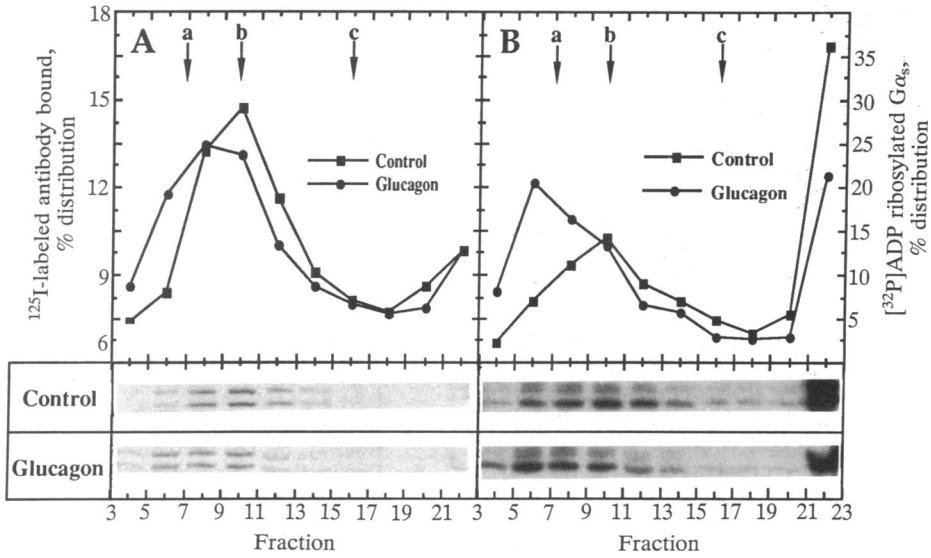

FIG. 5. Effects of glucagon on the distribution of sedimentable and soluble forms of $G\alpha_s$ extracted with OG from cholera toxin-treated liver membranes. (*A*) Distribution of immunoreactive $G\alpha_s$ and the result of immunoblots. (*B*) Distribution of [^{32}P]ADP-ribosylated $G\alpha_s$[†] with autoradiographic bands corresponding to the immunodetected bands of $G\alpha_s$ shown in *A*.

material was not as dramatic as the shift seen with the labeled 42 kDa band. An additional band of ^{32}P-labeled material was detected on the gels with an apparent molecular mass of 70 kDa; this material migrated on the gradients as a relatively sharp peak in fractions 10–12 (≈8.5 S) (data not shown). Its hydrodynamic behavior was not changed by glucagon and guanine nucleotides suggesting that it is not involved in the glucagon/G protein pathway of signal transduction.

DISCUSSION

During the past decade most of the emphasis in the field of G proteins has been placed on the heterotrimeric structures that are extracted from membranes by detergents such as Lubrol and cholate or, as for the rhodopsin-linked G proteins, by exposure of membranes to light, GTP, and hypotonic extraction (1, 2). A large body of evidence (for reviews, see refs. 1 and 2) has implicated heterotrimers as being the structures responsible for receptor-mediated actions of external signals. Although there is a persuasive concurrence of opinion, still lacking is knowledge of the structures and organization of receptors and G proteins in their native membrane environment. Nor is there a conclusive explanation of how ligand-activated receptors catalytically activate G proteins in a very rapid manner both at the onset and the offset (in cells, ranging from msec to sec), and with great amplification of the signals (cAMP, calcium ions, etc.) generated in response to the activation process.

The finding (4) that OG extracts stimulatory Gs, inhibitory Gi, and Go proteins from rat brain synaptoneurosomes in the form of large polydisperse structures containing the α subunits but not the $\beta\gamma$ complexes raised the question of whether heterotrimeric G proteins are unique transduction structures. GTP[γS], AlF_4, and other nonphysiological activators of G proteins caused these large structures to break down into smaller structures even though the $\beta\gamma$ complexes are not associated with these structures in OG extracts. Moreover, these large structures are disrupted by Lubrol or cholate, whereas they are stable in OG as well as in digitonin, saponin, and Tween 20 (unpublished observations). Interestingly, OG also stabilizes polymeric forms of actin (11). In this regard, when synaptoneurosomes are treated with the cross-linking reagent *p*-phenylenedimaleimide, all of the $G\alpha$ proteins exhibited the same range of sizes as actin and tubulin polymers on size-exclusion columns (S. Coulter and M.R., unpublished data). These findings argue strongly for the existence of polymeric forms of the G proteins in association with cell membranes.

Since OG treatment abolished hormone action, not answered directly in the present study is whether the polymer-like structures of $G\alpha_s$ interact directly with receptors. By pretreating the membranes, glucagon specifically and rapidly converted, in concert with low concentrations of GTP[γS], the sedimentable forms of $G\alpha_s$ into much smaller forms. This interconversion was most convincingly observed when $G\alpha_s$ was labeled with [^{32}P]ADP-ribosyl groups catalyzed by cholera toxin. The toxin preferentially caused the 45-kDa species of the polymer-like structures of $G\alpha_s$ to be ADP-ribosylated. Most importantly, the preferentially ADP-ribosylated structures represent only a fraction of the total $G\alpha_s$ in the liver membranes. Yet it is this fraction that selectively changes in response to glucagon action. This extraordinary selectivity suggests that only a small change in the total $G\alpha_s$ can be expected to occur in response to glucagon under physiological conditions. Although the basis of the selectivity of the toxin remains unknown, the relative abundance of labeled ADP-ribosylated 42-kDa $G\alpha_s$ provides a sensitive semiquantitative assay of the interconversion process that could prove useful in whole-cell studies.

The dependency of glucagon action on low concentrations of GTP[γS] is consistent with the guanine nucleotide dependency of glucagon action on adenylyl cyclase activity (12) and the well-known ability of many hormones to increase the binding of the nucleotide to $G\alpha_s$ and other G proteins. Relatively high concentrations of GTP[γS] alone mimicked the actions of glucagon. However, the finding that the hormone stimulated the conversion even in the presence of 1 mM GTP[γS] infers that glucagon acts through an independent process that requires only low concentrations of the nucleotide. An important component that could be responsible for this difference is the $\beta\gamma$ complex.

The $\beta\gamma$ complexes are not necessary for the GTP[γS]-induced conversion of large to small structures of $G\alpha$ proteins and exhibit hydrodynamic properties in OG that differ from the polydisperse and polymer-like structures of the $G\alpha$ proteins in OG (4). However, given their crucial role in the association of $G\alpha$ proteins with membranes and receptors, perhaps $\beta\gamma$ complexes are associated with the multimeric $G\alpha$

proteins in membranes and are involved directly in the coupling process between receptors and multimers of Gα proteins. OG extraction may disrupt this relationship; this may be one of the factors responsible for the OG-induced loss of hormone action. Suggestive evidence for an association with Gα proteins in membranes is the finding that *p*-phenylenedimaleimide, in addition to cross-linking the multimeric structures of Gα proteins, causes the appearance on sizing gels of large structures containing the β subunits (S. Coulter and M.R., unpublished data).

In conclusion, the multimeric structures of $G\alpha_s$ are sensitive to disaggregation by the combined actions of glucagon and a guanine nucleotide triphosphate. The specificity of glucagon action on disaggregation argues for a receptor-mediated process; the fact that vasopressin and angiotensin II failed to promote disaggregation of $G\alpha_s$ structures further indicates that the glucagon receptor is linked selectively to this species of G proteins in rat liver membranes. This study also has revealed that only a small subset of $G\alpha_s$ is coupled to the glucagon receptor, a specificity of coupling hitherto unrecognized. The behavior of the polymer-like structures of $G\alpha_s$ in response to glucagon and GTP[γS] is reminiscent of the changes observed by target-size analysis of the glucagon-sensitive adenylyl cyclase system in response to these agents (13). Moreover, the large functional size of the ground state of this system (1500 kDa) can now be partially accommodated by the biochemical evidence for large structures of $G\alpha_s$ being a part of the transduction process. Many questions remain, including the following questions: (*i*) What is the organization of receptors (monomers or oligomers) required for interacting with the G polymers? (*ii*) What is the stoichiometry of this interaction? Are the βγ complexes required and do they form copolymers with the Gα proteins? What are the structures (oligomers, dimers, or monomers) of the small forms of $G\alpha_s$ produced in response to glucagon action?

Finally, as suggested from the disaggregation-coupling theory of hormone action, activation of one receptor in a multimeric structure can theoretically cause many Gα proteins to be activated, which is supported with biochemical evidence (14, 15). The interactions of receptors, multimeric G proteins, and GTP in the signal transduction process have been likened (16) to the interactions of ATP, myosin, and polymeric actin that result in mechanochemical changes dynamically controlled in a "treadmill" fashion (17). Considerations of this type may lead to a better understanding of how signals can be transmitted across membranes in a dynamic reversible manner.

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