THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 249, No. 1, Issue of January 10, pp. 59–65, 1974
Printed in U.S.A.

Evidence for Interdependent Action of Glucagon and Nucleotides on the Hepatic Adenylate Cyclase System

(Received for publication, July 5, 1973)

MARTIN RODBELL, MICHAEL C. LIN, AND YORAM SALOMON

From the Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland 20014

SUMMARY

The kinetic characteristics of glucagon action and binding as functions of hormone and nucleotide concentrations have been investigated in the hepatic adenylate cyclase system. Either App(NH)p (5′-adenylylimidodiphosphate), 0.1 mM, or ATP, 2 mM, was used as substrate. The formation of adenosine 3′:5′-monophosphate (cyclic AMP) in response to glucagon exhibits lag times as long as 4 min in the presence of about 0.1 nM glucagon. The lag times diminish as the hormone or substrate concentrations increase. Addition of GTP (0.1 mM) abolishes the lags at all concentrations of glucagon tested in the presence of 0.1 mM App(NH)p and causes a decrease in the concentration of glucagon required for half-maximal activation of the enzyme. In the presence of GTP, the apparent K_m for glucagon action ranges from 0.2 to 0.5 nM glucagon; in its absence, the K_m ranges from 2 to 7 nM.

Pretreatment of the enzyme system with glucagon for 15 min prior to addition of substrate also abolishes the lags seen at low concentrations of the hormone. When the pretreated enzyme system is incubated with limiting concentrations of substrate (0.1 mM App(NH)p) in the absence of free hormone, the velocity of enzyme activity is dependent upon GTP. Stimulatory effects are observed with 30 nM GTP; GTP exerts maximal effects at 1 μM.

The lag times observed in onset of glucagon activation appear to reflect a change in state of the enzyme induced by glucagon binding to its receptor. The time dependency is changed simply by increasing the concentration of glucagon; GTP acts subsequent to or attendant upon this change in state induced by glucagon. Glucagon binding exhibits Hill coefficients of about 1.5, suggesting cooperative interactions between two or more subunits of the system.

GTP, in addition to its effects on glucagon action, stimulates the rate of dissociation of glucagon from its receptor and, as a consequence, causes a decrease in the steady state levels of bound glucagon. The release of hormone seems not to be causally related to enzyme activation since the rates of dissociation are increased by addition of 0.1 mM GTP without concomitant increases in enzyme velocity.

A hyperbolic relationship is observed between the levels of receptors occupied by glucagon and adenylate cyclase activity generated in the presence of GTP. Complete activation of the enzyme requires full occupation of the specific binding sites in hepatic plasma membranes (about 3 pmoles per mg of membrane protein). This finding shows that all of the specific binding sites for glucagon in these membranes represent receptors functionally and structurally linked to the adenylate cyclase system.

The finding that glucagon and GTP activate adenylate cyclase by a concerted or interdependent mechanism is discussed in relation to the characteristics of the adenylate cyclase system as an allosteric regulatory enzyme system.

Adenylate cyclase systems in mammalian cells are activated by biogenic amines, prostaglandins, and a variety of peptide hormones. These systems, localized primarily in the plasma membrane, are considered to be the first stage in the regulation of target cell metabolism by the stimulatory agents. It was first suggested by Robinson and co-workers (1) that adenylate cyclase is a complex organized system containing regulatory and catalytic subunits in analogy to other regulatory enzyme systems. Subsequent studies (for recent review see Ref. 2) indicate that these adenylate cyclase systems consist of at least two distinct components that are responsible, respectively, for specific recognition of a hormone (receptor or regulatory unit) and for the catalytic conversion of ATP to cyclic AMP.[1] The enzyme system has not been purified in a hormonally sensitive state. Knowledge of these systems is mostly limited to information obtained from studies of the binding of hormones and the dose-response curves generated by these agents on the membrane form of the enzyme.

Several studies have shown that hormones bind to specific sites on target cell membranes containing adenylate cyclase systems responding to these hormones (3–10). Although specificity of binding is an essential feature of receptors, such findings cannot be used alone as definitive evidence for functional receptors linked to adenylate cyclase. It has been shown that adrenocorticotropic hormone (9), glucagon (10), and epinephrine (11) bind reversibly to specific binding sites in target cell plasma membranes to give macroscopic equilibrium constants that appear to bear a relationship to the dose-response curves generated

[1] The abbreviations used are: cyclic AMP, adenosine 3′:5′-monophosphate; App(NH)p, 5′-adenylylimidodiphosphate.

by the additions of these hormones on adenylate cyclase. Based on Scatchard plots of adrenocorticotropic hormone binding to adrenal membranes, Roth *et al.* (12) suggested that adenylate cyclase may be activated by adrenocorticotropic hormone through receptors having high and low affinities for the hormone and over distinct dose ranges that reflect the number and affinity of the binding sites for the hormone. This suggestion was based on the assumptions that each binding site contributes independently to stimulation and that a linear relationship exists between receptor occupancy and activity of the enzyme. However, the latter assumption seems not to be valid for the glucagon-sensitive system in hepatic membranes. For example, glucagon activates this system without lag to give constant rates of activity whereas binding of glucagon to its specific binding sites continues to increase subsequent to activation (13). Birnbaumer and Pohl (14) have analyzed the kinetic relationship between glucagon binding and action on the hepatic system and concluded that these processes cannot be fitted to either the occupation or rate theories (15) of hormone action. They concluded that only a small fraction of the specific binding sites is linked to adenylate cyclase (16). In offering this suggestion, they also assumed that occupation of these sites bears a proportional relationship to adenylate cyclase activity.

An alternate possibility to explain the divergent characteristics of hormone binding and response is that binding leads to cooperative interactions between the components of the enzyme system. Response reflects allosteric transitions between different states of the enzyme upon receptor occupancy. In accordance with the theoretical considerations of Monod *et al.* (17) and Koshland *et al.* (18), if hormone induces an allosteric process then binding of the hormone and the velocity of substrate utilization need not bear a proportional relationship. For example, conformational transitions induced by the hormone prior to the rate-determining step may not all be rapid and readily reversible. Rate-determining steps may be involved at sites other than the receptor and catalytic sites of the enzyme system. In addition, it cannot be assumed that the rate-determining steps in the decomposition of substrate enzyme complexes are the same for all molecular species of the enzyme system.

Previous studies of the glucagon-sensitive system in hepatic plasma membranes have shown that activation of adenylate cyclase by glucagon is enhanced markedly in the presence of GTP at concentrations ranging from about 10 nM to 1 μM (19); ATP at 1000-fold higher concentrations exerts similar effects on glucagon action (13). Other adenylate cyclase systems sensitive to glucagon (20), prostaglandins (21, 22), oxytocin (23), thyroid-stimulating hormone (22), epinephrine (24), and the multireceptor system in fat cells (25) are similarly affected by GTP. Since GTP is not a substrate for adenylate cyclase, its effects are likely to be at sites distinct from the catalytic site. GTP, over the same concentration range required for its effects on glucagon action, also causes increased rates of dissociation of glucagon from its specific binding sites in hepatic membranes (26). The effects of GTP on glucagon binding are not competitive with respect to hormone concentration (26) which suggests that GTP acts at distinct sites from the hormone binding sites.

Based on the above observations, it has been suggested (27) that glucagon and GTP act allosterically to produce states of the enzyme that have increased reactivity of the catalytic site for substrate. The finding that GTP (or ATP) affects both binding and action of the hormone suggests that a simple relationship between hormone binding and action may not apply, particularly if the putative states induced by glucagon and the nucleotides have differing activities depending upon the relative binding of these agents to their respective sites.

As a possible means of distinguishing between the various steps involved in hormone and nucleotide action on the hepatic adenylate cyclase system, we have investigated the time course of enzyme activation as a function of glucagon and nucleotide concentrations both before and after allowing glucagon to react with the system. These studies will show that glucagon and the nucleotides activate the enzyme by an interdependent or concerted process and that this process has characteristics similar to those of other allosteric regulatory enzyme systems.

EXPERIMENTAL PROCEDURE

Materials—[α-^{32}P]ATP, App(NH)p, and [α-^{32}P]App(NH)p were obtained from International Chemical and Nuclear Corp.; cyclic [^{3}H]AMP was purchased from New England Nuclear Corp. ATP, cyclic AMP, GTP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co. Oxoid membranes were from Amersham-Searle. Crystalline porcine glucagon was obtained from Eli Lilly and Co. Bovine serum albumin was obtained from Pentex. ^{125}I-glucagon was prepared as described previously (28).

Preparation of Hepatic Membranes—Partially purified plasma membranes from rat livers were prepared by a modification of the procedure of Neville (29) as previously described (30) and stored in liquid nitrogen.

Assay for Adenylate Cyclase Activity—Assays were performed in the following incubation medium: 2 mM [α-^{32}P]ATP (20 to 50 cpm per pmole) or 0.1 mM [α-^{32}P]App(NH)p (200 to 500 cpm per pmole), 5 mM $MgCl_2$, 1 mM cyclic AMP, 25 mM Tris-HCl, pH 7.6, and 5 mg per ml of serum albumin. In all experiments where ATP was used and in indicated experiments where App(NH)p was the substrate, an ATP regenerating system consisting of 20 mM creatine phosphate and 100 units per ml of creatine phosphokinase was included. Both the assay medium and the membrane suspension were prewarmed to 30° for 1 or 2 min prior to the assay. The reaction was initiated by addition of 150 to 300 μg of membrane protein in 100 μl of 20 mM Tris-HCl, pH 7.6, to a final volume of 0.6 ml of incubation mixture. Aliquots of 100 μl were withdrawn at various times and transferred to test tubes containing 100 μl of stopping solution (2% sodium dodecyl sulfate, 4 mM ATP, 1.4 mM cyclic AMP at pH 7.5). Cyclic AMP was determined by a modification (31) of the method of Krishna *et al.* (32). To improve the recovery of cyclic AMP, Dowex 50-X4 (200 to 400 mesh) from Bio-Rad Laboratories was used instead of the previously described use of Dowex 50-X8.[2] Assay blanks, containing no enzyme, were processed similarly. Even at the lowest activity measured, the ratio of cyclic AMP formed was 2 to 3 times that given by the blank, the count for which ranged from 15 to 25 cpm per 10^6 cpm added as labeled substrate to the incubation medium. The results obtained from duplicate determinations agreed within 5%. Protein concentration was determined according to Lowry *et al.* (33) with serum albumin as standard.

Pretreatment of Hepatic Membranes with Glucagon—Three to six milligrams of membrane protein were incubated with native or ^{125}I-glucagon in a final volume of 10 ml of 20 mM Tris-HCl, pH 7.6, containing 1% serum albumin. After 15 min incubation at 25°, the mixture was centrifuged at 4° for 10 min at 30,900 × *g*. The pellet was rinsed once with 5 ml of 1% serum albumin in 20 mM Tris-HCl, pH 7.6, and was suspended in the same medium to

[2] J. P. Harwood and G. Krishna, unpublished observations.

give a concentration of about 3 mg of protein per ml. In experiments with labeled glucagon, the amount of ^{125}I-glucagon bound was measured by counting the pellet directly or with the use of an Oxoid membrane filtration technique described previously (28).

RESULTS

Effects of GTP or ATP on Time Course of Hormonal Activation as a Function of Glucagon Concentration—Previous studies (19) have shown that the effects of GTP on the activity and response of the hepatic adenylate cyclase system to glucagon are more readily observed in the presence of low substrate concentrations (0.1 mM or less). At these concentrations, App(NH)p is a suitable substrate for investigating the time course of enzyme activity since, unlike ATP, it is not hydrolyzed by nucleotide phosphohydrolyases present in hepatic membranes (19) and is therefore maintained for longer periods of incubation. As shown in Fig. 1, cyclic AMP production in the absence of glucagon, which is referred to as basal activity, proceeded without lag and at a constant rate for at least 5 min of incubation with 0.1 mM App(NH)p as substrate. Addition of 0.2 nM glucagon at zero time or at any time subsequent to incubation of the enzyme with "cyclase" reagents (not shown) did not result in detectable increases in enzyme activity until about 3 min after glucagon addition (Fig. 1, *Panel A*). In five experiments of this type, the lag time for activation using 0.2 nM glucagon ranged from 3 to 4 min. The lag diminished progressively to about 30 s with increasing concentrations of glucagon (see *inset*, Fig. 1, *Panel B*). Once established, the increased rates observed with glucagon remained constant for 3 to 5 min. From these rates, the concentration of glucagon required for half-maximal activation was calculated. In the experiment described in Fig. 1, half-maximal activation was attained with 2 nM glucagon (see *inset*, Fig. 1, *Panel A*); the apparent K_m ranged from 2 to 5 nM glucagon in five experiments.

The relatively long lag time for onset of detectable activation with 0.2 nM glucagon was not due to diffusion-limited access of glucagon to its receptor. As shown in Fig. 1 (*Panel B*), addition of GTP (0.1 mM) reduced the lag time for activation at all concentrations of glucagon tested to about 30 s or less. In addition to diminishing the lag time for activation with glucagon, GTP decreased the concentration of glucagon required for half-maximal activation to 0.5 nM (0.2 to 0.5 nM in five experiments) as shown in the *inset* to Fig. 1 (*Panel A*). Maximal activation required concentrations of glucagon in excess of 10 nM in the absence or presence of 0.1 mM GTP.

Previous studies have shown that ATP, at concentrations of 10 μM or higher, mimics the effects of GTP on the glucagon-sensitive hepatic and islet cell adenylate cyclase systems (13, 20). It was of interest, therefore, to compare the time course of glucagon activation seen above with 0.1 mM App(NH)p in the presence or absence of GTP to that obtained in the presence of relatively high concentrations of ATP (2 mM). As shown in Fig. 2 (*Panel A*), lag times in the onset of glucagon activation were observed with 2 mM ATP; at 0.17 nM glucagon the lag time was between 1 and 2 min or approximately one-half the lag time observed with 0.1 mM App(NH)p as substrate and with this concentration of glucagon (Fig. 1, *Panel A*). This finding is consistent with ATP acting not only as substrate but also at the same site(s) as GTP but with a lower affinity than the guanyl nucleo-

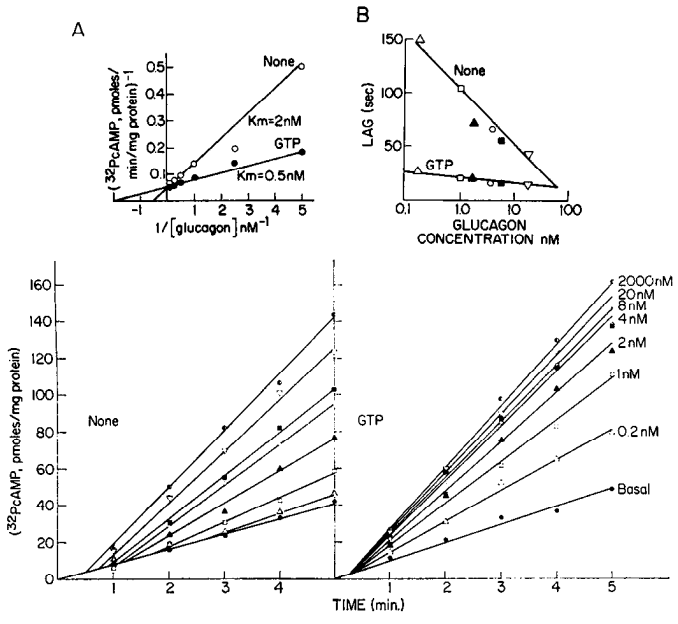

FIG. 1. Time course of glucagon action on adenylate cyclase in the absence and presence of GTP. Adenylate cyclase activity was measured with 0.1 mM [α-^{32}P]App(NH)p as substrate in the absence (*Panel A*) and in the presence (*Panel B*) of 0.1 mM GTP. The concentrations of glucagon are shown in *Panel B;* the same symbols for the respective concentrations are used in *Panel A*. Rates were obtained from the slope of each curve. The increase in rate due to addition of glucagon was used in the Lineweaver-Burk plot (*inset A*) to determine the apparent K_m for glucagon. The lag time required to reach constant rate at each glucagon concentration was obtained from the intercept of the rate curves with the curve given by basal activity. Lags were plotted as a function of glucagon concentration (*inset B*).

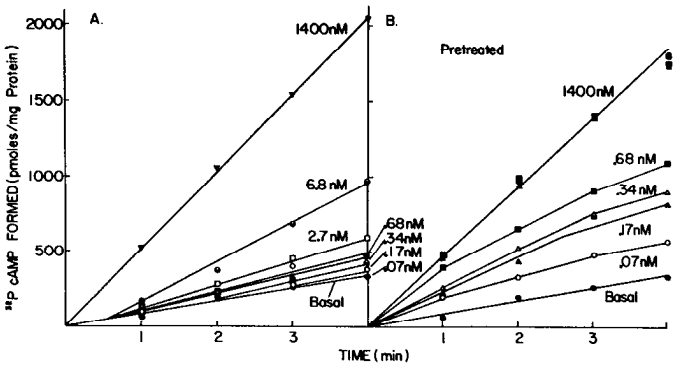

FIG. 2. Effects of pretreatment of hepatic membranes with glucagon on the time course of adenylate cyclase activity. Hepatic membranes (5 mg of protein) were incubated in a final volume of 10 ml of 20 mM Tris-HCl, pH 7.6, containing 1% serum albumin. In the experiments described in *Panel A*, no glucagon was added to the medium, whereas in *Panel B* glucagon was added to portions of the membrane suspension at concentrations ranging from 0.07 to 0.68 nM. Membranes exposed to glucagon during this phase are termed "pretreated." After 15 min incubation at 25°, the suspensions were centrifuged at 4° for 10 min at 30,900 × *g*. The pellets were rinsed once with 5 ml of 1% serum albumin in the Tris buffer and were suspended in the same medium to give a concentration of 3 mg of protein per ml. The washed membranes were assayed for adenylate cyclase activity in the presence of 2 mM [α-^{32}P]ATP and other ingredients stated under "Experimental Procedure." In *Panel A*, glucagon was added to the assay medium at the indicated concentrations to membranes not previously exposed to glucagon; activities were compared to membranes pretreated with the same concentration of glucagon (*Panel B*). As indicated by the *top time curve* in *Panel B*, the same maximal activities (with 1.4 μM glucagon) were obtained with each set of pretreated membranes and were essentially identical with the maximal rates obtained with nonpretreated membranes (*top curve* in *Panel A*).

tides. It will be noted that lag times of between 3 and 4 min were observed with 0.07 nM glucagon, the lowest hormone concentration tested with 2 mM ATP as substrate.

Effects of Pretreatment with Varying Concentrations of Glucagon—The finding that the lag time for glucagon activation is diminished as a function of glucagon concentration raised the possibility that glucagon binding to its receptor induces new states of the enzyme system by a time- and concentration-dependent process. To test this possibility, the membranes were incubated, in the absence of "cyclase" reagents, for 15 min at 25° with glucagon at concentrations ranging from 0.07 nM to 0.68 nM. Previous studies have shown that glucagon binds to its specific binding sites by a slowly reversible process under "non-cyclase" incubation conditions (9, 26). After pretreatment with glucagon, the membranes were washed to remove unbound hormone prior to assaying for adenylate cyclase activity in the presence of 2 mM ATP. Control membranes, similarly processed without glucagon, were used to determine basal activity as well as activity with added glucagon at concentrations indicated (Fig. 2, *Panel A*). As illustrated in Fig. 2, membranes pretreated with glucagon at all concentrations tested displayed immediate increases in activity over that of basal activity (*Panel B*) in contrast to the lag times noted with control membrane (*Panel A*). Moreover, the concentration of glucagon required during pretreatment for half-maximal activation of the enzyme (maximal activation was obtained by addition of 1.4 μM glucagon to samples of control and pretreated membranes) was about 0.4 nM glucagon in contrast to 5 to 7 nM glucagon when the hormone was added simultaneously with substrate (Fig. 2, *Panel A*).

The finding that pretreatment with glucagon alone is sufficient to abolish the lag times for activation suggests that the lag times reflect a change in enzyme states due to glucagon interaction with its receptor. This process is rate-limiting at low concentrations of the hormone and in the presence of substrate (App(NH)p or ATP) at concentrations ranging from 0.1 mM to 2 mM.

It will also be noted in Fig. 2 (*Panel B*) that the enzyme activity in pretreated membranes declined, but not to basal level, after 1 or 2 min of incubation.

Nucleotide-dependent Activation Subsequent to Hormone Pretreatment—The findings reported thus far indicated that either pretreatment with glucagon or addition of 0.1 mM GTP both abolished the lag time for activation of the enzyme and diminished by 5- to 10-fold the concentration of glucagon required for half-maximal activation. Two questions arose. Does pretreatment with glucagon abolish the effects of GTP and what is the relationship between the quantity of hormone bound to its specific binding sites and the degree of activation of the enzyme?

The first question was investigated as follows. Membranes were pretreated with 0.5 nM ^{125}I-glucagon and washed to remove free hormone as described previously. Labeled glucagon was used to monitor the amount of bound glucagon during pretreatment and incubation under "cyclase" conditions. Activity was assayed using 0.1 mM App(NH)p as substrate in order to test for the effects of GTP at concentrations ranging from 30 nM to 0.1 mM; preliminary experiments showed that the regenerating system was required for effects of GTP to be seen at 30 nM. In the experiment described in Fig. 3 (*Panel A*), 0.45 pmole of glucagon per mg of membrane protein remained bound to the specific binding sites following the pretreatment and washing procedures. Pretreated membranes showed an immediate increase over basal activity (obtained with control membranes incubated in absence of glucagon) as is the case when 2 mM ATP was used as substrate (Fig. 2). Although the lag was abolished

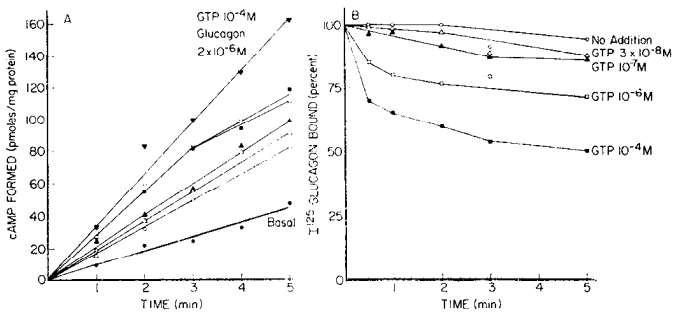

FIG. 3. Effects of GTP on glucagon-pretreated hepatic membranes. *Panel A*, effects on the adenylate cyclase activity. Hepatic membranes were incubated with 0.5 nM ^{125}I-glucagon for 15 min at 25°, sedimented, and washed as described under "Experimental Procedure." Control membranes (no glucagon added) were similarly treated. The membranes were suspended in 20 mM Tris-HCl, pH 7.6, containing 1% serum albumin to give a membrane concentration of about 3 mg of protein per ml. Aliquots of the membrane suspensions were assayed for adenylate cyclase activity as described under "Experimental Procedure" using 0.1 mM App(NH)p as substrate with the regenerating system (1 mM creatine phosphate) and the indicated concentrations of GTP. *Panel B*, effects on dissociation of bound glucagon. The same membranes pretreated with ^{125}I-glucagon (*Panel A*) were assayed for bound hormone by transferring 100 μl of washed membranes to tubes containing 1 ml of ice-cold 20 mM Tris-HCl, pH 7.6, containing 1% serum albumin. The contents were immediately filtered on Oxoid membranes. Hepatic membranes pretreated with 0.5 nM ^{125}I-glucagon in the presence of 2.0 μM unlabeled glucagon were used to correct for nonspecific binding. A level of 100% bound glucagon represents 12,000 cpm per 43 μg of membrane protein. The specific activity of ^{125}I-glucagon was 6.5×10^5 cpm per pmole. The remainder of the pretreated membrane was added to the same incubation media described in *Panel A* for assaying adenylate cyclase activity except that labeled substrate was replaced with unlabeled App(NH)p. At each incubation time, aliquots of 100 μl were assayed for bound hormone as described above. Both the measurements of enzyme activity and glucagon dissociation were carried out simultaneously. Unless otherwise indicated, identical symbols were used in both panels to indicate the same concentrations of GTP.

by pretreatment, activity of the hormone-occupied system was increased further by the addition of GTP; as little as 30 nM GTP caused a significant increase in rate. GTP at 1 μM gave 80% of maximal activation (*upper curve* in Fig. 3, *Panel A*). In agreement with previous findings (19), maximal effects were observed with 1 μM GTP; addition of 0.1 mM GTP did not cause further increases in enzyme activity. It should be emphasized that these effects of GTP were observed in the absence of free glucagon. Addition of a maximal concentration of glucagon (2 μM) caused a 20% increase in activity.

GTP, at each concentration tested, did not cause significant increases in activity of control, untreated membranes. Thus, under the conditions described in Fig. 3, the effects of GTP appear to be related to the hormone-occupied or pretreated enzyme system. Since the effects of the nucleotide occurred subsequent to binding of the hormone and the change in state of the enzyme produced by glucagon during pretreatment, the conclusion can be drawn that GTP acts interdependently with the hormone-induced forms of the enzyme system.

It will be noted in Fig. 3 (*Panel A*) that the rate of enzyme activity of the pretreated enzyme system declined after 3 min of incubation in the presence of 1 μM or 0.1 mM GTP to a new level of activity which was significantly above that of untreated membranes (basal activity). As shown in Fig. 3 (*Panel B*), increasing concentrations of GTP resulted in increased dissociation of

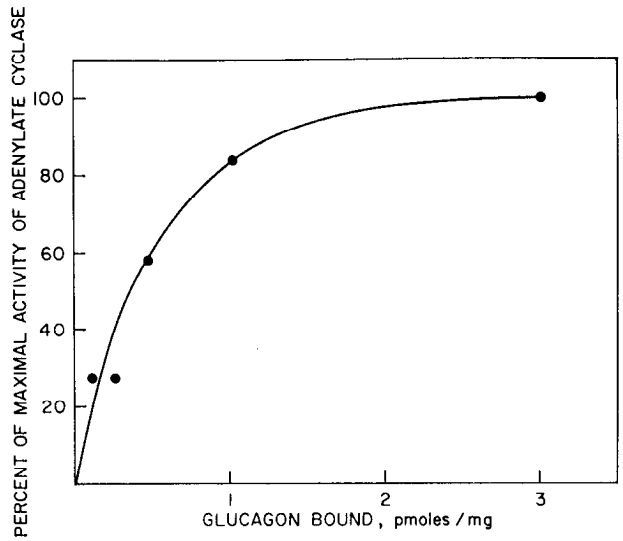

FIG. 4. Adenylate cyclase activity as a function of bound glucagon. Hepatic membranes were pretreated with various concentrations of ^{125}I-glucagon (50 pM to 0.1 μM), washed, and assayed for adenylate cyclase activity and for bound hormone by the procedures described in the legend to Fig. 3. Adenylate cyclase activity was assayed in the presence of 1 μM GTP, using 10 μM [α-^{32}P]ATP as substrate. Activity was determined at 30-s time intervals and the rates were calculated from the linear portion of the curves. In all cases, rates were constant with time for at least 60 s. Maximal activity (set at 100%) was obtained by addition of 2.0 μM glucagon to pretreated membranes or to untreated membranes processed in the same manner.

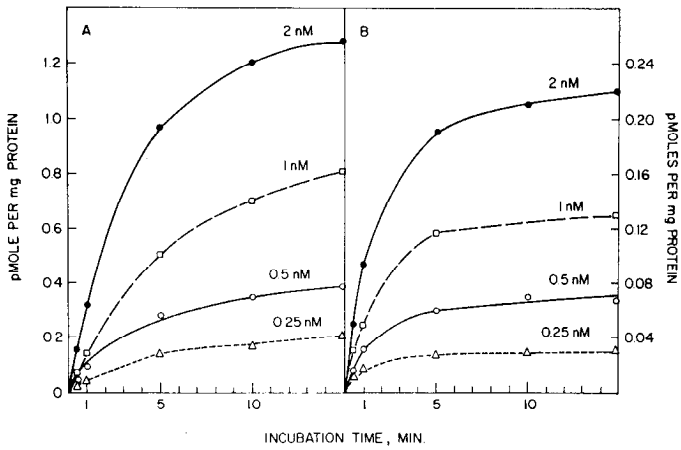

FIG. 5. Binding of ^{125}I-glucagon to hepatic membranes. Hepatic membranes (50 μg of protein) were incubated in a final volume of 100 μl of 20 mM Tris-HCl, pH 7.6, containing 1% serum albumin and the indicated concentrations of ^{125}I-glucagon (*Panel A*). In the experiments described in *Panel B*, the same membrane preparation was incubated in the Tris-albumin medium containing 2 mM ATP, 0.5 mM GTP, 5 mM $MgCl_2$, and the same concentrations of ^{125}I-glucagon used in *Panel A*. Incubations were carried out at 30° for the indicated time periods. Binding was assayed by filtration of the membranes on Oxoid membranes as previously described (32); nonspecific binding obtained by addition of 2.0 μM unlabeled glucagon with labeled hormone was subtracted from each value. The amount of glucagon bound was calculated from the specific activity of the labeled hormone (6×10^5 cpm per pmole).

^{125}I-glucagon from its specific binding sites. No relationship was observed between the degree of dissociation and the increased rates of activity due to nucleotide action; 0.1 mM GTP enhanced the rate of dissociation without increasing enzyme activity over that observed with 1 μM GTP. Furthermore, in the presence of 1 μM to 0.1 mM GTP, a decline in enzyme activity occurred 1 or 2 min after the rapid phase of hormone dissociation induced by the nucleotide.

Receptor Occupation versus Degree of Enzyme Activation—Pretreatment experiments with ^{125}I-glucagon (0.07 to 100 nM) were carried out in the manner described above. The amount of labeled glucagon remaining bound after pretreatment and washing was determined prior to enzyme assay. A separate aliquot of the washed membrane suspension was assayed for adenylate cyclase over a period of 3 min in the presence of 1 μM GTP and 10 μM ATP as substrate. Initial rates of enzyme activity were determined and expressed as the percentage of maximal activation rates given in the presence of 2.0 μM glucagon. As illustrated in Fig. 4, a hyperbolic relationship was obtained between activity and the levels of labeled hormone bound. Maximal activation occurred with 3 pmoles of glucagon bound per mg of membrane protein. This value is nearly the same reported in previous studies when hepatic membranes were incubated with saturating concentrations of labeled glucagon (9, 26). It appears, therefore, that all of the glucagon-specific binding sites in hepatic plasma membranes are functionally linked to adenylate cyclase and can be designated as receptors involved in hormonal activation of the enzyme system.

Similar results to those reported in Fig. 4 were also obtained with 2 mM ATP as substrate in the absence of GTP. However, it should be emphasized that the relationship between the fractional binding of glucagon and the fractional activity (at submaximal hormone concentrations) varies according to the concentration of nucleotide (GTP or substrate), as evidenced by the differences in apparent K_m of glucagon action in the absence and presence of GTP (*cf.* Fig. 1, *Panels A* and *B*). Thus, receptor occupancy *versus* activity or dose-response curves for glucagon action on adenylate cyclase are a complex function of the concentrations of both glucagon and the nucleotides.

Time Course of Glucagon Binding in Absence and Presence of Nucleotides—Previous studies (13) have shown that activation of the hepatic adenylate cyclase system by glucagon is essentially immediate when activity is assayed in the presence of 3.2 mM ATP whereas binding of the hormone continues to increase with time under identical incubation conditions. Fig. 5 shows the time course of ^{125}I-glucagon binding at hormone concentrations approximating those used in the experiments described in Figs. 1 and 2. In order to emphasize the effects of nucleotides on binding, these experiments were carried out under "non-cyclase" conditions (absence of substrate and GTP) and under "cyclase" conditions where both 0.5 mM GTP and 2 mM ATP were included.

In the absence of the nucleotides, the time required for binding of the hormone to attain equilibrium ranged from 5 to 10 min. Since the longest time required for onset of glucagon activation under similar conditions (0.1 mM App(NH)p does not influence the time course of binding) was 4 min, it is apparent that a constant activity was established before the equilibrium in binding was achieved.

Binding of labeled hormone in the presence of the nucleotides was reduced markedly (note that the *ordinate* in *Panel B* has a different scale from that in *Panel A*). At 2 nM glucagon, the amount of hormone bound was reduced by nearly 80%. As previously shown (26), the decrease in binding is due to increased rates of dissociation which are not compensated by increased rates of association of the hormone to its receptor; the net effect is a decrease in the steady state levels of bound hormone. Although constant levels of hormone bound are achieved faster than seen

in the absence of the nucleotides, activation of the enzyme under these conditions precedes the times required to attain steady state levels.

The binding data in Fig. 5 and from two other experiments of the same type were plotted according to Hill (34) for hormone concentrations ranging from 0.1 nM to 5 nM. Both in the absence and presence of the nucleotides, Hill coefficients ranging from 1.3 to 1.6 (average, 1.5) were obtained. These findings suggest that glucagon binding is a cooperative process and that two or more subunits are involved in this process.

DISCUSSION

The studies presented here indicate that glucagon and nucleotides (GTP or ATP) activate the hepatic adenylate cyclase system by a concerted or interdependent process. The effects of these agents on the kinetic characteristics of the activation process can be explained by ligand-induced conformational changes of the enzyme system. The lags in onset of glucagon action and the observation that the onset of activation by glucagon precedes the time required for binding to attain equilibrium provide indirect evidence that glucagon binding to its receptor brings about cooperative interactions between the components of the enzyme system. Consistent with this suggestion is the observation that glucagon binding exhibits Hill coefficients of about 1.5. The changes in state induced by glucagon occur independently of the actions of GTP (or ATP) as evidenced by the loss of lags in activation when the enzyme system is simply pretreated with the hormone. Moreover, GTP enhanced activation after pretreatment with glucagon.

As shown here and in previous studies of the hepatic adenylate cyclase system (19, 26), GTP enhances the response of the hepatic system to glucagon and increases the rate of dissociation of glucagon from its receptor. ATP mimics these effects of GTP at approximately 1000-fold higher concentrations. The possibility has not been ruled out that commercial preparations of adenine nucleotides are contaminated with GTP (less than 0.1% contamination would be sufficient to explain the effects of 2 mM ATP). In any case, GTP acts on the system at sites which have a high affinity for GTP relative to ATP. GTP affects the response of adenylate cyclase systems to a number of hormones and prostaglandins (20–25); in some systems, ITP is equally or more effective than GTP (22). Although with a lower affinity, β,γ-methylene-guanosine triphosphate also enhances the response of the hepatic system to glucagon (19), indicating that the terminal high energy phosphate of GTP is not utilized in this process.[3] This finding taken in conjunction with the low concentrations of GTP (about 10 nM to 1 μM) required for activation suggests that the nucleotide acts allosterically rather than through, for example, phosphorylation of the enzyme system.

Previous studies have shown that GTP enhances basal activity of the hepatic system in the presence of 0.1 mM App(NH)p as substrate (19). In the present study, effects of GTP on basal activity were slight or not observed under similar incubation conditions. The basis of the variable effects of GTP on basal activity is under investigation. Preliminary studies indicate that GDP inhibits the effects of GTP on activity obtained in the presence of glucagon.[3] Since most of the studies with GTP were carried out in the absence of a regenerating system, it is possible that the formation of GDP by nucleotide phosphohydrolases contributes to the variable effects of GTP on basal activity. In any case, GTP consistently causes marked increases in enzyme activity in the presence of glucagon, which suggests that glucagon either causes an increased affinity of the nucleotide binding sites for GTP or induces the formation of states that have increased reactivity with the nucleotide.

Enhancement of enzyme activity by GTP is observed after occupation of the receptor by glucagon and formation of the glucagon-induced states of the enzyme system (Fig. 3). This finding provides strong evidence that the actions of GTP occur subsequent to or attendant upon the binding and actions of glucagon on the enzyme system.

GTP increases the rate of dissociation of glucagon from its receptor by a noncompetitive process (26) which, as shown here, does not correlate with the effects of the nucleotide on enzyme activity. A time delay of 1 or 2 min was observed between the initial rapid phase of hormone dissociation induced by GTP and the decline in enzyme activity due to the reduction in steady state levels of occupied receptors (Fig. 3). These findings suggest that GTP acts allosterically with respect to glucagon binding and that the states induced by GTP and glucagon are not rapidly reversible.

In analogy to the effects of 2,3-diphosphoglycerate on hemoglobin structure and function (35, 36), it is possible that GTP and glucagon induce different conformational states of the adenylate cyclase system depending upon the relative concentrations of these ligands. If the binding of these ligands causes cooperative interactions between the receptor and catalytic components, the sensitivity and response of the enzyme to GTP and glucagon cannot be predicted simply from the amount of these ligands bound to their respective sites. This point seems to be demonstrated by the finding that the apparent K_m of glucagon action on adenylate cyclase is altered depending on the concentration of GTP (or substrate) (*cf.* Fig. 1); lower hormone concentrations are required to produce half-maximal activation as the concentration of nucleotide is increased, and *vice versa*. The slow transition times observed at low hormone and nucleotide concentrations indicate that internal equilibrium between the various states of the enzyme is not achieved during activation of the system. If, as our evidences indicate, we are dealing with an allosteric enzyme system in which at best two ligands are required for activation, one would not expect a simple correlation between equilibrium binding constants for hormone-receptor interactions and the kinetic constants obtained from hormone action.

An important outgrowth of the present study is the finding that occupation of all of the glucagon-specific binding sites in hepatic membranes is required for maximal activation of the adenylate cyclase system. Thus, the binding sites can be equated with functional receptors and are likely to be structurally linked to the enzyme system. This finding has obvious implications for future studies of the mechanism of hormone and nucleotide action on the enzyme system.

In summary, glucagon and GTP activate the hepatic adenylate cyclase system by a concerted or interdependent mechanism. The kinetic characteristics of the activation process have features in common with other allosteric regulatory enzymes displaying cooperative interactions of its components in response to ligand binding. It is suggested that glucagon and GTP induce conformational changes in the enzyme system. Of possible physiological significance is the finding that the sensitivity and response of the system to glucagon are functions of the concentration of GTP (or substrate). At a fixed and low circulating concentration of glucagon, reduction in substrate or GTP concentrations in the hepatic cell might give the appearance of a hormone-resistant cell. It should be of interest to examine this possibility.

[3] M. C. Lin and Y. Salomon, unpublished observations.

Acknowledgments—We gratefully thank our colleagues Drs. James P. Harwood, Constantine Londos, and Boanerges Rubalcava for their participation in the discussions of this study and for their many helpful suggestions. We also thank Mr. Thomas Demar for excellent technical assistance.

REFERENCES

1. Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1967) *Ann. N. Y. Acad. Sci.* **139**, 703
2. Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1970) in *Fundamental Concepts in Drug-Receptor Interaction* (Danielli, J. F., Moran, J. F., and Triggle, D. J., eds) p. 59, Academic Press, New York
3. Tomasi, V., Koretz, S., Ray, T. K., Dunnick, J. K., and Marinetti, G. V. (1970) *Biochim. Biophys. Acta* **211**, 31–42
4. Lefkowitz, R. J., and Haber, E. (1971) *Proc. Nat. Acad. Sci. U. S. A.* **68**, 1773–1777
5. Dunnick, J. K., and Marinetti, G. V. (1971) *Biochim. Biophys. Acta* **249**, 122–134
6. Lefkowitz, R. J., Haber, E., and O'Hare, D. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69**, 2828–2832
7. Lefkowitz, R. J., Sharp, G. W. G., and Haber, E. (1973) *J. Biol. Chem.* **248**, 342–349
8. Lefkowitz, R. J., Roth, J., Pricer, W., and Pastan, I. (1970) *Proc. Nat. Acad. Sci. U. S. A.* **65**, 745–752
9. Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) *J. Biol. Chem.* **246**, 1861–1871
10. Marx, S. J., Fedak, S. A., and Aurbach, G. D. (1972) *J. Biol. Chem.* **247**, 6913–6918
11. Schramm, M., Feinstein, H., Naim, E., Lang, M., and Lasser, M. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69**, 523–527
12. Roth, J., Pastan, I., Lefkowitz, R. J., Pricer, W. E., Freychet, P., and Neville, D. M., Jr. (1971) in *Proceedings of the Second International Symposium on Structure-Activity Relationships of Protein and Polypeptide Hormones* (Margoulies, M., and Greenwood, F. C., eds) pp. 228–237, Excerpta Medica, Amsterdam
13. Birnbaumer, L., Pohl, S. L., Rodbell, M., and Sundby, F. (1972) *J. Biol. Chem.* **247**, 2038–2043
14. Birnbaumer, L., and Pohl, S. L. (1973) *J. Biol. Chem.* **248**, 2056–2061
15. Paton, W. D. M. (1961) *Proc. Roy. Soc. London B Biol. Sci.* **154**, 21–69
16. Rodbell, M. (1972) in *Glucagon* (Lefebvre, P. J., and Unber, R. H., eds) p. 61, Pergamon Press, New York
17. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118
18. Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966) *Biochemistry* **5**, 365–385
19. Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krans, H. M. J. (1971) *J. Biol. Chem.* **246**, 1877–1882
20. Goldfine, I. D., Roth, J., and Birnbaumer, L. (1972) *J. Biol. Chem.* **247**, 1211–1218
21. Krishna, G., Harwood, J. P., Barber, A. J., and Jamieson, G. A. (1972) *J. Biol. Chem.* **247**, 2253–2254
22. Wolff, J., and Cook, G. H. (1973) *J. Biol. Chem.* **248**, 350–355
23. Bockaert, J., Roy, C., and Jard, S. (1972) *J. Biol. Chem.* **247**, 7073–7081
24. Leray, F., Chambaut, A. M., and Hanoune, J. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1385–1391
25. Harwood, J. P., Low, H., and Rodbell, M. (1973) *J. Biol. Chem.* **248**, 6239–6245
26. Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) *J. Biol. Chem.* **246**, 1872–1876
27. Rodbell, M. (1973) *Fed. Proc.* **32**, 1854–1858
28. Rubalcava, B., and Rodbell, M. (1973) *J. Biol. Chem.* **248**, 3831–3837
29. Neville, D. R. (1968) *Biochim. Biophys. Acta* **154**, 540–552
30. Pohl, S. L., Birnbaumer, L., and Rodbell, M. (1971) *J. Biol. Chem.* **246**, 1849–1856
31. Rodbell, M. (1967) *J. Biol. Chem.* **242**, 5744–5750
32. Krishna, G., Weiss, B., and Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther.* **163**, 379–385
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
34. Hill, A. V. (1910) *J. Physiol. (London)* **40**, IV-VIII
35. Benesch, R., and Benesch, R. E. (1969) *Nature* **221**, 618–622
36. Weber, G. (1972) *Biochemistry* **11**, 864–878