Glucagon-stimulable Adenylyl Cyclase in Rat Liver

The Impact of Streptozotocin-induced Diabetes Mellitus

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Abstract. Glucagon receptor levels, glucagon-stimulated and other forms of adenylyl cyclase activity, and regulatory component activity of adenylyl cyclase were determined in hepatic plasma membranes of rats administered streptozotocin without and with insulin to produce varying degrees of hyperglycemia. Receptor levels were assayed by direct binding of the specific probe [^{125}I-Tyr^{10}]-iodoglucagon; regulatory component activity was assayed by the capacity to reconstitute stimulatory regulation in deficient membranes from *cyc*$^-$ S49 murine lymphoma cells. In rats given 150 mg streptozotocin, glucagon stimulation of adenylyl cyclase as well as basal, sodium fluoride, 5′ guanylylinidodiphosphate [GMP-P(NH)P] and Mn-dependent activities were reduced 50%, glucagon receptor levels but not affinity were reduced 67%, and regulatory component activity was decreased 50%. In addition, α_1-adrenergic receptors and 5′-nucleotidase were similarly reduced in diabetes. However, specifc ouabain-inhibitable Na^+,K^+-ATPase activity was not altered by streptozotocin treatment. The streptozotocin-induced changes were noted within 24 h and became maximal by 120 h after its administration. All of these decreases were partially reversed by in vivo insulin treatment. DNA, cytochrome *c* oxidase, glucose-6-phosphatase, and *N*-acetyl-β-glucosaminidase content in hepatic plasma membrane preparations were not substantially different in diabetic as compared with control animals. The data demonstrate that glucagon-mediated regulation of cyclic AMP formation is deranged in insulin deficiency owing to a combined decrease in receptors, derangement of the coupling mechanism intervening between receptor and adenylyl cyclase, and possibly, an altered basal effector system. Some of these changes appear to reflect a "desensitization-like" phenomenon which may or may not be attributable to the hyperglucagonemia of diabetes mellitus. There also appears to be a concurrent generalized decrease in several but not all plasma membrane receptor and enzymatic proteins. This may be the result of a number of processes among which is the accelerated proteolysis of uncontrolled diabetes.

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Introduction

Hyperglucagonemia is well-described in patients and in animal models with diabetes mellitus (1–4). Glucagon-stimulable adenylyl cyclase activity has been assessed in liver of animals having a variety of models of diabetes mellitus and has been conflictingly reported to be increased (5–7), decreased (1, 8, 9), or unchanged compared with nondiabetic control animals (2). Disparate changes have also been reported for hormone-stimulable adenylyl cyclase activities in other tissues (10–14). Hormone-stimulable adenylyl cyclase is formed of three major components which include: a specific hormone receptor, a catalytic component which catalyzes the reaction forming cAMP from ATP, and a regulatory or coupling system which is comprised of at least two proteins linking the hormone-receptor complexes to the catalytic component (15–20). To assess the potential effects, if any, of diabetes mellitus on glucagon-stimulable adenylyl cyclase activity in rat liver, we have used standard as well as newly developed and validated assays to measure glucagon receptors (21) and the activity of the stimulatory coupling component (22) in hepatic membranes of rats made diabetic by streptozotocin.

Methods

[α-^{32}P]ATP (~25 Ci/mmol sp act) was purchased from International Chemical and Nuclear Corporation (Irvine, CA). [Furoyl-5-^{3}H]prazosin (~17 Ci/mmol, sp act) was purchased from New England Nuclear

(Boston, MA). Phentolamine was purchased from Ciba-Geigy Corp. (Summit, NJ). Propranolol was purchased from Ayerst Laboratories (New York, NY). Streptozotocin was a gift from Upjohn Co. (Kalamazoo, MI). All other materials used were described in the preceding report (22).

Female Sprague-Dawley rats (150–200 g) from TIMCO (Houston, TX) were used in this study. The animals were made diabetic by a single intravenous injection of streptozotocin in varying dosages. Some animals were also administered insulin (neutral protein Hagedorn [NPH][1] pork) from Eli Lilly & Co. (Indianapolis, IN) according to one of four treatment schedules outlined below.

Animals and animal treatments. Group I animals received intravenous injections containing 65, 100, or 150 mg streptozotocin/kg or buffer alone, and were killed by decapitation 120 h later. Group II animals received 150 mg streptozotocin/kg at time 0 and then seven daily subcutaneous injections of 5 U of NPH insulin each, starting at 72 h. These animals were killed 240 h after the initial streptozotocin injection. Group III animals were either not treated or received 150 mg streptozotocin/kg intravenously, and were killed 24, 48, 72, or 120 h afterwards. Group IV animals received first 150 mg streptozotocin/kg intravenously at time 0, and then, seven daily subcutaneous injections of 5 or 10 U of NPH insulin each, starting at 72 h. These were killed 240 h after the initial streptozotocin injection. Induction of experimental diabetes was confirmed qualitatively by the presence of 2% glucosuria (Diastix, Ames Co., Elkhart, IN). Glucose concentrations were also determined in fasting blood taken at the time of the sacrifice by the glucose oxidase method as described previously (23).

Preparation of liver membranes. After decapitation, the livers were removed quickly and the membranes prepared according to the procedure of Neville (24) as modified by Pohl et al. (25). The membranes were stored at −80°C. S49 *cyc*$^-$ cells were grown and the membranes (*cyc*$^-$ membranes) were prepared by Dr. Ravi Iyengar (Department of Cell Biology, Baylor College of Medicine), according to described procedures (26).

Assays. Adenylyl cyclase assays, binding assays for determination of glucagon receptors using [^{125}I-Tyr^{10}]monoiodoglucagon as a specific receptor probe, and cholate extraction of and assay procedures for regulatory component of liver membrane adenylyl cyclase using reconstitution of 5′ guanylylimidodiphosphate[GMP-P(NH)P]-stimulated or isoproterenol plus guanosine triphosphate (GTP)-stimulated activities in *cyc*$^-$ membranes were all described previously (22). 5′-Nucleotidase and specific [^{3}H]prazosin binding to assess α_1-adrenergic receptors (27, 28) are described in the appropriate legends to tables and figures. DNA levels were measured fluorometrically in the whole homogenate and plasma membrane preparation by using bisbenzimide, a DNA-binding dye (29). Cytochrome *c* oxidase activity was determined by the rate of oxidation of ferrocytochrome *c* according to the method of Wharton and Tzagoloff (30). Glucose-6-phosphatase activity was assessed by the release of inorganic phosphate from glucose-6-phosphate (31). *N*-Acetyl-β-glucosaminidase (EC 3.2.1.30) activity was assessed by modification of the method of Seymour and Peters (32). Diluted samples (50 μl) were added to 450 μl of 50 mM sodium acetate buffer, pH 5.0, 0.25 M sucrose, and 0.16% Triton X-100. The final concentration of substrate, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-*D*-glucopyranoside, was 1 mM. After incubation at 37°C for up to 3 h, the reaction was quenched with 1 ml of 0.5 M sodium glycinate buffer, pH 10.5. The amount of released methylumbelliferone was measured fluorometrically. All determinations were made within the linear range for time and amount of sample assessed. Na^+,K^+-ATPase activities were determined by the linked enzyme (pyruvate kinase/lactate dehydrogenase) methodology of Pitts et al. (33). Protein was determined by the method of Lowry et al. (34) or of Bradford (35). Data from binding assays were analyzed by the method of Scatchard (36).

Results

The impact of streptozotocin-induced diabetes mellitus on glucagon-stimulable adenylyl cyclase activity in rat liver was assessed initially in rats administered three different doses of streptozotocin to produce varying degrees of hyperglycemia. 5 d after streptozotocin, control rats had fasting blood sugars of 96±5 mg/dl, and rats administered 65, 100, and 150 mg/kg of streptozotocin by intravenous injection had fasting blood sugars of 235±89, 377±21, and 423±25 mg/dl, respectively (mean±SD; n = 12 each). Another group of rats, given 150 mg/kg of streptozotocin, was started 3 d later on 5 U/d of insulin and was killed on day 10 as described for group II in Methods. Fasting blood sugars in this treatment group averaged 269±47 mg/dl. The results provided in Fig. 1 are from pooled liver membrane preparations from at least six animals in each treatment group, and these data are representative of four separate experiments. Increasing severity of diabetes mellitus produced by increasing

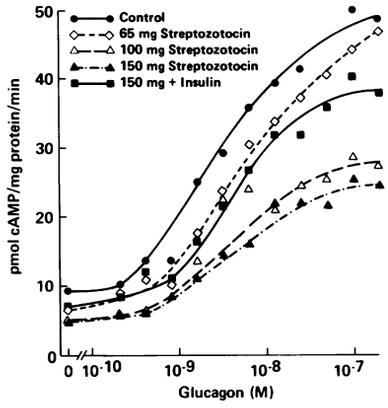

Figure 1. Effect of streptozotocin-induced diabetes mellitus, and insulin treatment on glucagon-stimulated rat liver adenylyl cyclase. Liver membranes were prepared from control, from streptozotocin-treated (65, 100, and 150 mg/kg intravenous), and from streptozotocin plus insulin-treated animals as described for groups I and II under Methods. Aliquots of these membranes (5–10 μg) were then incubated for adenylyl cyclase activity in the absence and presence of the indicated concentrations of glucagon yielding the concentration-effect curve depicted. Concentrations at which half-maximal glucagon stimulation in membranes from various treatment groups occurred were: control, 2.6 nM; 65 mg streptozotocin, 4.6 nM; 100 mg streptozotocin, 4.0 nM; 150 mg streptozotocin, 3.8 nM; and 150 mg streptozotocin plus 7 daily injections of insulin (5 U each), 4.0 nM. None of the values differ significantly from any of the others.

1. *Abbreviations used in this paper:* GMP-P(NH)P, 5′ guanylylimidodiphosphate; GTP, guanosine triphosphate; NPH, neutral protein Hagedorn.

dosages of streptozotocin decreased the maximal activity of the glucagon-responsive adenylyl cyclase. In the liver of rats given 150 mg/kg of streptozotocin, the stimulation by saturating levels of glucagon (2×10^{-7} M) was reduced by more than 50%. Insulin treatment of diabetic animals diminished but did not restore the loss of glucagon-stimulable adenylyl cyclase activity. Although in the experiment shown in Fig. 1, there was a 1.7-fold shift in the dose-response curve for glucagon stimulation of adenylyl cyclase in membranes from treated as compared with control rats; this finding was not consistent.

Using monoiodinated [^{125}I]iodoglucagon purified by high pressure liquid chromatography (21), the number and affinity of glucagon receptors were also assessed. Scatchard analysis (36) of the binding data and the resulting receptor densities and affinities in the various membrane pools are presented in Fig. 2. The data show that increasing concentrations of streptozotocin produced a progressive decrease in the number of [^{125}I]iodoglucagon-binding sites (B_{max}) without alteration in the affinity of these binding sites. Insulin treatment, although inadequate to normalize blood sugars in this treatment protocol, did reverse to a large degree the decrease in glucagon receptors noted in the diabetic group. The effect of diabetes mellitus on other adenylyl cyclase activities was also investigated (Fig. 3). Streptozotocin treatment reduced the basal and NaF-stimulated activities as well as the activities stimulated by GTP and GMP-P(NH)P in the absence and presence of glucagon. At 150 mg/kg of streptozotocin, basal adenylyl cyclase activity was diminished 47% ($P < 0.05$), fluoride-stimulable activity was diminished 37% ($P < 0.01$), and manganese-dependent activity was decreased 40% ($P < 0.05$).

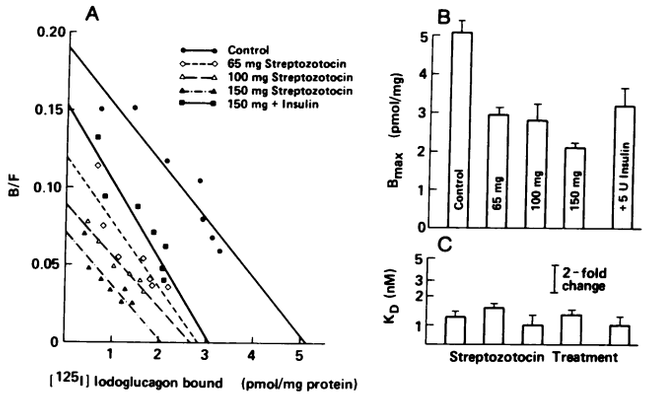

Figure 2. Effect of streptozotocin-induced diabetes mellitus and insulin treatment on the number and affinity of glucagon receptors in rat liver membranes. Aliquots of liver membranes (2–3 μg) prepared from rats treated as described for groups I and II under Methods were incubated with varying concentrations of [^{125}I]iodoglucagon in the absence (duplicates) and presence (duplicates) of 3 μM unlabeled glucagon as described under Methods. Specific binding of [^{125}I]iodoglucagon was calculated and plotted according to (*A*) Scatchard to evaluate (*B*) glucagon receptor density and (*C*) K_D values.

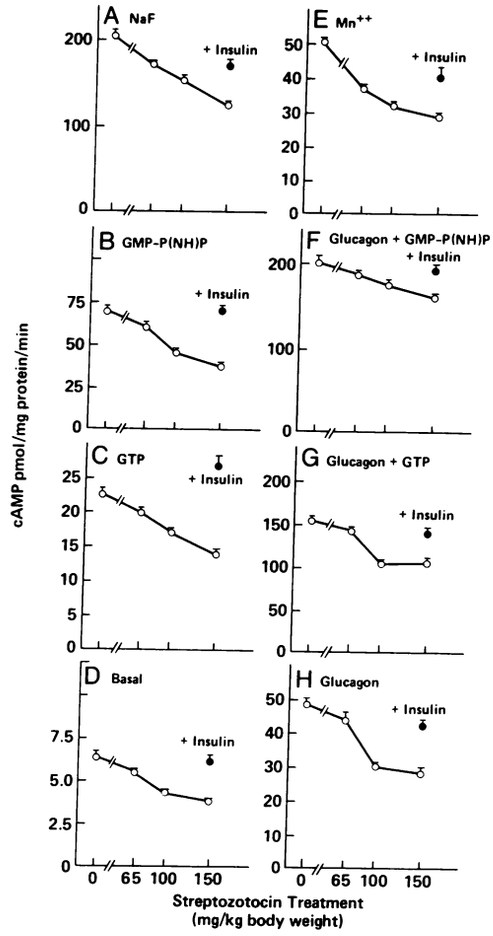

Figure 3. Effect of streptozotocin-induced diabetes mellitus and insulin treatment on liver membrane adenyl cyclase activities. Liver membranes from rats treated as described for groups I and II under Methods were assayed for adenylyl cyclase activity under standard conditions described under Methods without further additions (basal), in the presence of 10 μM GTP and 10 μM GMP-P(NH)P, 1 μM glucagon, 10 mM NaF, 1 μM glucagon plus 10 μM GTP, 1 μM glucagon plus 10 μM GMP-P(NH)P, and upon replacing $MgCl_2$ with $MnCl_2$ (Mn^{++}). Incubations were for 10 min at 32.5°C and [^{32}P]cAMP formed was quantitated.

Potential changes in regulatory component activity in liver membranes were assessed from control, diabetic, and insulin-treated diabetic animals by using reconstitution of GMP-P(NH)P-stimulated activity in membranes from S49 *cyc*$^-$ cells (Fig. 4). As shown in Fig. 4 *A*, cholate extract proteins containing regulatory component activity from liver membranes of the various treatment groups elicited concentration-dependent reconstitutions of GMP-P(NH)P-stimulable adenylyl cyclase activity in *cyc*$^-$ membranes. Fig. 4 *B* illustrates data (X±SE) from the slopes of the concentration effect lines for cholate extract-mediated reconstitutions of *cyc*$^-$ and GMP-P(NH)P-stimulable

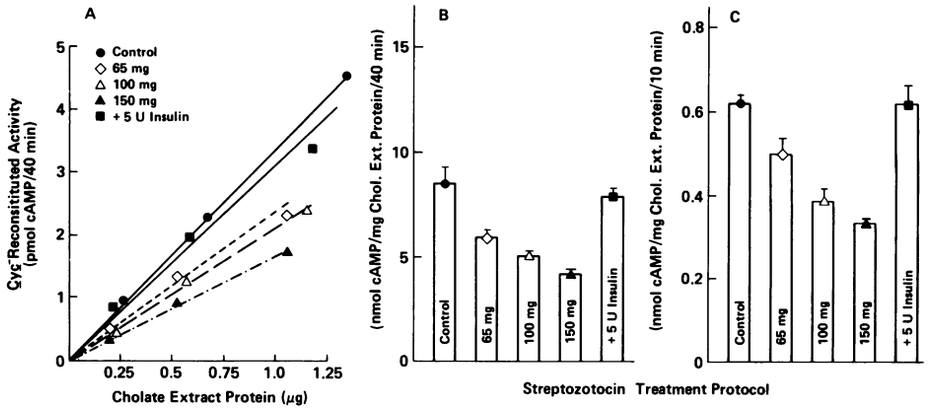

Figure 4. Effect of streptozotocin-induced diabetes mellitus, and insulin treatment on the content of regulatory component activities in rat liver membranes. Liver membranes (5–6 mg) from rats treated as described for groups I and II under Methods were extracted in a final volume of 1 ml with 1% cholate and additives described under Methods, warmed to inactivate endogenous catalytic adenylyl cyclase activity, and assayed for their capacity to reconstitute *cyc*⁻ membrane GMP-P(NH)P-stimulated and isoproterenol plus GTP-stimulated adenylyl cyclase activities. Each extract was assayed at three different concentrations after suitable dilution in KCl and β-mercaptoethanol-containing dilution medium to ensure proportionality between reconstitution of activity and cholate extract protein added. Specific activities for reconstitution of *cyc*⁻ activities were calculated, confirmed to be independent of sample dilution, and averaged to give the values reported on the figure. (*A*) Reconstitution of GMP-P(NH)P-stimulated *cyc*⁻ adenylyl cyclase activity as a function of cholate extract protein added to the reconstitution assays. (*B*) *Cyc*⁻ (GMP-P(NH)P-stimulated) reconstituting activity (x±SE) in cholate extracts assayed as shown in (*A*). (*C*) Reconstitution of isoproterenol plus GTP-stimulated *cyc*⁻ adenylyl cyclase activity by the same cholate extracts assayed in the experiment shown in (*B*). Chol. Ext., cholate extract.

activity. Both panels show that increasing doses of streptozotocin produced a dose-dependent decrease in regulatory component activity in liver membranes from diabetic animals. In the animals given 150 mg/kg of streptozotocin, this activity was reduced 50% ($P < 0.001$). Regulatory component activity was not significantly different in animals given 150 mg/kg of streptozotocin and treated with 5 U of insulin per day as compared with control animals. Streptozotocin-induced diabetes mellitus also decreased equally regulatory component activity as assessed by reconstitution of isoproterenol-responsive adenylyl cyclase (Fig. 4 *C*). This decrease was reversed by insulin. The reduced *cyc*⁻-reconstituting activity recovered from liver membranes of streptozotocin-treated rats and the increase in activity upon insulin treatment were not due to an altered heat lability of extracted reconstituting activity (data not shown). Furthermore, neither streptozotocin nor streptozotocin plus insulin treatment altered the susceptibility of regulatory component to extraction by cholate. Of the total *cyc*⁻-reconstituting activity measurable in cholate-membrane mixtures, 67%, 68%, and 65% were recovered in the cholate extracts of membranes from control, streptozotocin, and streptozotocin plus insulin-treated rats, respectively. In fact, the same decreases in regulatory component activity owing to streptozotocin-induced diabetes and its reversibility by insulin were noted also in reconstitution assays performed with the cholate-membrane mixture as a source of regulatory component (Table I), as opposed to the cholate extract alone (data not shown).

The time course of the effects of streptozotocin-induced diabetes mellitus on adenylyl cyclase activities in liver was next assessed. Rats were administered streptozotocin (150 mg/kg), and groups (n = 6) were sacrificed 24, 48, 72, and 120 h later. After 72 h, two subgroups were begun on insulin at daily doses of either 5 or 10 U for an additional 7 d (n = 6 each). Basal, GMP-P(NH)P and glucagon (alone and in combination), sodium fluoride (10 mM), and $MnCl_2$-dependent adenylyl cyclase activities were assayed (Fig. 5). Within 24 h after streptozotocin,

Table I. Time Course of Streptozotocin-induced Changes in the Regulatory Component Activities of Rat Liver Membranes

Time after streptozotocin treatment	*Cyc*⁻-reconstituted adenylyl cyclase activity
h	*nmol cAMP formed/mg liver membrane protein per 40 min*
0 (control)	7.96±0.43
24*	5.34±0.31
48*	5.05±0.24
72*	3.93±0.21
120*	2.75±0.49
Plus 5 U insulin‡	7.91±0.49
Plus 10 U insulin‡	6.94±0.13

Rats were treated with 150 mg streptozotocin/kg body weight and killed at the indicated times after streptozotocin. 5 or 10 U of insulin/d were administered from days 3 through 9 where appropriate. The liver membranes obtained from these rats were extracted with 1% cholate as described in Methods. Aliquots of the membrane-cholate extract mixtures were diluted with KCl and β-mercaptoethanol, and then, assayed at three different dilutions for total GMP-P(NH)P-stimulable *cyc*⁻-reconstituting activity as described under Methods. Specific activities for reconstitution *cyc*⁻ activities were calculated, confirmed to be independent of sample dilution under the assay conditions used, and averaged to give the values reported in the table. Values are means±SD.
* Treatment group III.
‡ Treatment group IV.

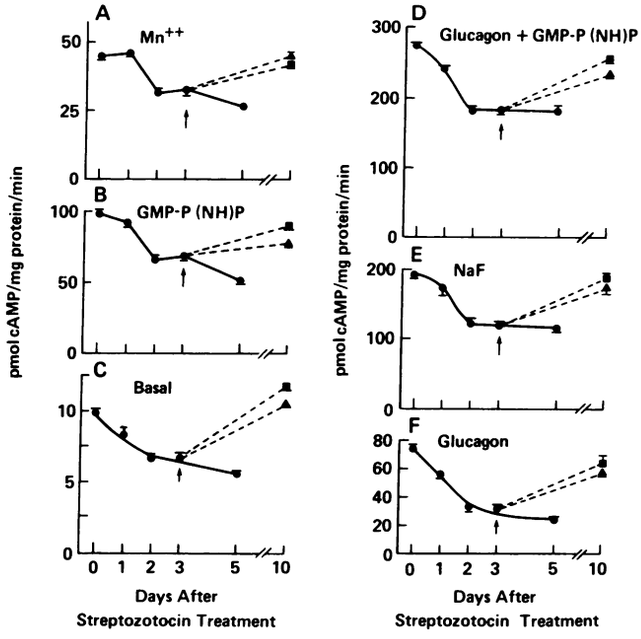

Figure 5. Time course of appearance of streptozotocin- and insulin-induced changes in liver membrane adenylyl cyclase activities. Rats were given 150 mg streptozotocin/kg body weight and killed at the indicated times without (●) or after (■, ▲) having been treated with 5 (▲) or 10 (■) U of insulin from days 3–9. Liver membranes from these rats (5–10 mg) were assayed for adenylyl cyclase activity under standard conditions described under Methods without further additives (*Basal*), in the presence of 10 μM GMP-P(NH)P, 1 μM glucagon, 10 mM NaF, 1 μM glucagon plus 10 μM of GMP-P(NH)P, and upon replacing $MgCl_2$ with 10 mM $MnCl_2$ (Mn^{++}). Incubations were for 10 min at 32.5°C and [^{32}P]cAMP formed was quantitated. Arrows indicate time of initiation of seven daily injections of insulin.

only glucagon-stimulable adenylyl cyclase was decreased significantly (from 73.4±1.8 to 55.7±2.9; $P < 0.001$). At 120 h, glucagon-sensitive adenylyl cyclase was diminished 67%, basal cyclase was decreased 43%, and fluoride- and $MnCl_2$-dependent cyclase activities were decreased 40%. Fasting blood sugars increased with the duration of streptozotocin-induced diabetes mellitus in these animals. Control rats had fasting blood sugars of 105±11 mg/dl. 24 h after streptozotocin, fasting blood sugars were 426±103 mg/dl; at 48 and 120 h, they were 436±92 and 536±91 mg/dl, respectively. Insulin treatment was begun 72 h after streptozotocin administration. This reduced the fasting blood sugars at the time of sacrifice to 236±49 and 71±10 mg/dl in the groups given 5 or 10 units of NPH insulin daily, respectively. Either insulin dosage restored basal, sodium fluoride-, manganous ion-, or GMP-P(NH)P-stimulable adenylyl cyclase activities. Glucagon- and glucagon plus GMP-P(NH)P-stimulable adenylyl cyclase activities were reduced 16 and 12%, respectively ($P < 0.05$), despite insulin. Glucagon receptor density and affinity were assessed in these same membrane preparations and analyzed by the method of Scatchard (Fig. 6 *A*). Glucagon receptor affinity was not altered at any time period following streptozotocin, regardless of insulin therapy (Fig. 6 *D*). However, glucagon receptor number was decreased 38% ($P < 0.001$) 24 h following streptozotocin (Fig. 6 *C*). A maximum decrease of 63% in [^{125}I]iodoglucagon-binding sites was observed at 72 and 120 h following the induction of diabetes mellitus. Iodoglucagon-binding sites after insulin administration were ~80% of nondiabetic levels ($P < 0.001$). Changes in regulatory component activity were also observed within 24 h after streptozotocin. This activity (Fig. 7 *A*) was decreased by 17%, and by 120 h, the activity was only about 48% of that measured in control rats ($P < 0.01$). Either 5 or 10 U/d of insulin restored completely regulatory component activity. These changes are not the result of the cholate extraction procedure since similar decreases in regulatory component activity were noted in the cholate-membrane mixtures (Table I). The loss of regulatory component activity was also assessed by hormonal reconstitution assays (Fig. 7 *B*). 120 h after streptozotocin, regulatory component activity was reduced by 54% ($P < 0.001$). Insulin administration completely reversed this decrease.

The effects of streptozotocin-induced diabetes mellitus on other plasma membrane receptors and enzymatic functions were next examined in livers of diabetic rats. α_1-Adrenergic receptor function was assessed by [^{3}H]prazosin binding studies (Fig. 8) by using standard techniques (27, 28). In addition, 5′-nucleotidase and ouabain-inhibitable Na^+,K^+-ATPase activities were also assayed. With increasing dosages of streptozotocin, and hence increasing severity of diabetes mellitus, specific binding sites for [^{3}H]prazosin decreased. At 150 mg/kg of streptozotocin, [^{3}H]prazosin binding decreased 54% ($P < 0.001$). Insulin treatment reduced this decrease by one-half. The activity of 5′-nucleotidase also decreased with increasing severity of diabetes mellitus. At 150 mg/kg of streptozotocin, 5′-nucleotidase activity was decreased by 59%. This decrease in 5′-nucleotidase activity was noted within 24 h following streptozotocin administration (Table II); insulin administration partially restored 5′-nucleotidase activity to normal. In contrast, streptozotocin treatment did not reduce ouabain-inhibitable Na^+,K^+-ATPase activity in liver plasma membrane preparations (Table III). To assess further the potential contribution of differential purification of liver plasma membranes to the decreased hormone-stimulable adenylyl cyclase in diabetic liver, homogenates and the plasma membranes were assayed for DNA, cytochrome *c* oxidase, glucose-6-phosphatase and *N*-acetyl β-glucosaminidase content (Table IV). In all instances, the contamination of the membrane preparations was 10% or less of the activity found in the homogenate. No substantial differences in diabetic as compared with the control preparations could be noted.

Discussion

Glucagon-sensitive adenylyl cyclase has been found to be increased, decreased, or unchanged in liver and in other tissues of rats made diabetic by the intravenous administration of strep-

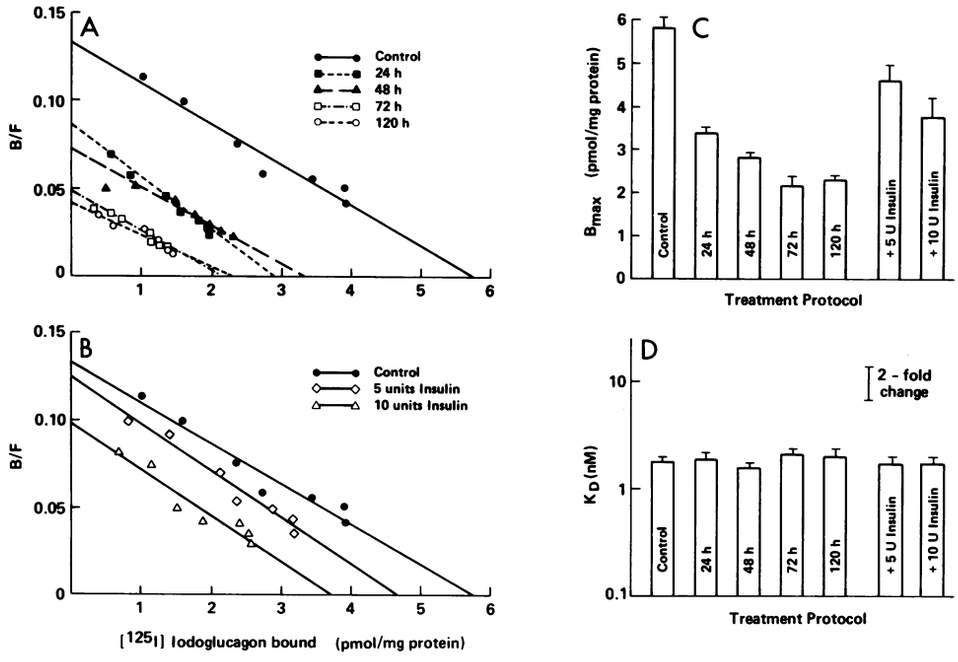

Figure 6. Time course of changes in glucagon receptor levels in rat liver membranes. Liver membranes from rats treated with streptozotocin for the indicated times (group III) and from rats that had received additional insulin treatments from days 3 through 9 and were killed on day 10 (group IV) were incubated with varying concentrations of [^{125}I]iodoglucagon in the absence (duplicates) and presence (duplicates) of 3 μM unlabeled glucagon. After 20 min at 32.5°C, free labeled hormone was separated from bound labeled hormone by filtration as described under Methods. Specifically bound glucagon was calculated and the data plotted (*A* and *B*) according to Scatchard to evaluate (*C*) receptor density and (*D*) K_D values. For the rest of the conditions, see Methods. B/F, bound/free.

tozotocin (2, 5, 6, 8, 13, 14). In a previous study, we found a decreased responsiveness of the catecholamine-sensitive adenylyl cyclase in skeletal muscle obtained from rats with streptozotocin-induced diabetes mellitus (10). The results of this study show clearly that streptozotocin-induced diabetes mellitus decreases the responsiveness of glucagon-sensitive adenylyl cyclase in rat liver. The magnitude of this defect is related to the severity and duration of the diabetic state and does not appear to derive from a nonspecific or toxic effect of streptozotocin, since partial to complete reversal of this decreased responsiveness was produced by insulin administration.

Hormone-stimulated adenylyl cyclase is comprised of three classes of functional proteins, which include hormone receptors, a catalytic subunit which catalyzes the reaction forming cAMP from ATP, and regulatory or coupling components which bear binding sites for guanine nucleotides and $MgCl_2$. Glucagon receptors are linked to the catalytic component of the system by one of these coupling proteins, the stimulatory regulatory protein (15–20). The results of the present study show clearly that at least two and possibly all three functional units of the glucagon-sensitive adenylyl cyclase are decreased in streptozotocin-induced diabetes mellitus. A decrease in the number of plasma membrane receptor binding sites for [^{125}I]monoiodoglucagon is the earliest and most substantial abnormality observed. Although this may result from a number of potential mechanisms, in view of the hyperglucagonemia which is a concomitant of insulinopenic diabetes mellitus (37, 38) such as that produced by streptozotocin (2, 8, 39–42), it is possible that this decrease may reflect, at least in part, the phenomenon of homologous desensitization (43–46). Support for such a concept is provided by the demonstration that intermittent injections of glucagon produced a diminished number of glucagon-specific binding sites in liver membranes of normal rats (22).

Changes in glucagon receptor number alone do not explain the decreases in other adenylyl cyclase activities in membranes of diabetic animals. The stimulation of adenylyl cyclase activity produced by regulators such as GMP-P(NH)P and sodium fluoride was also decreased with diabetes mellitus. Since this finding suggested an abnormality of regulatory component, its activity was therefore assessed by two different techniques. First, because regulatory component activity is required for activation of adenylyl cyclase by GMP-P(NH)P, the increase in this activity in membranes from S49 *cyc*$^-$ cells assesses regulatory component function in terms of regulation by guanine nucleotides and its interaction with the catalytic component. Second, the capability of added regulatory component to confer isoproterenol-stimulable adenylyl cyclase activity to *cyc*$^-$ membrane preparations was determined. This assesses three aspects of regulatory component function: its regulation by guanine nucleotides, its interaction with catalytic component, and its interaction with the hormone receptor. Both techniques showed equally decreased regulatory component function in membranes from diabetic animals (Figs. 4 and 7).

This finding is not consistent with mechanisms of homologous desensitization as seen in other hormone-stimulable adenylyl cyclases (43–46). In studies using intermittent glucagon injections, we found a desensitization of glucagon-stimulable adenylyl cyclase activity. This resulted primarily from a decreased glucagon receptor density; no change in regulatory component activity was noted (22). Furthermore, models of homologous

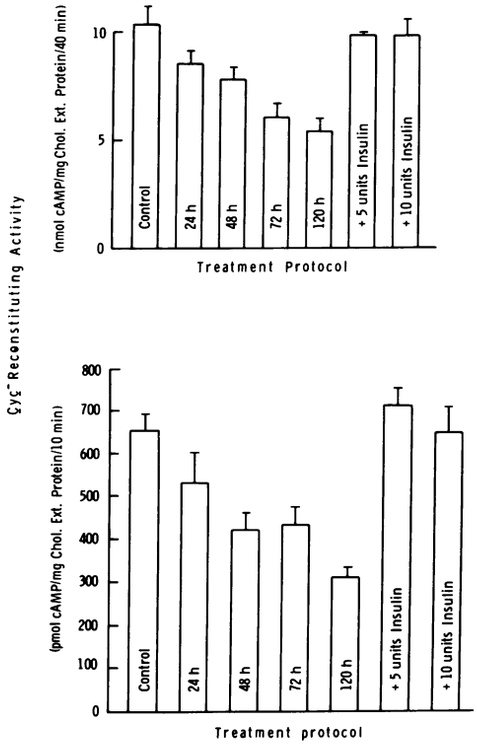

Figure 7. Time course of changes in regulatory component activities of liver membranes. Liver membranes from rats treated as described for groups III and IV under Methods were extracted with cholate and additives, warmed to inactivate endogenous coextracted adenylyl cyclase activity, and assayed at three different dilutions for capacity to reconstitute (*A*) GMP-P(NH)P-stimulated and (*B*) isoproterenol plus GTP-stimulated *cyc*⁻ adenylyl cyclase activities. Specific *cyc*⁻-reconstituting activities were calculated for each dilution of cholate extract protein added, confirmed to be independent of input protein used (0.6–0.8 μg cholate extract protein/assay at the highest concentration tested), and averaged. The resulting values, expressed as *cyc*⁻-reconstituting activity per milligram of cholate extract protein per assay time (40 min for *A* and 10 min for *B*) are represented. Chol. Ext., cholate extract.

desensitization do not explain the substantial reductions in basal, guanine nucleotide, or fluoride-stimulated adenylyl cyclase activities noted in liver of diabetic animals. It appears therefore that the adenylyl cyclase alterations in diabetes mellitus reflect two derangements: one that resembles homologous desensitization associated with decreased glucagon receptors and which possibly may be the result of the hyperglucagonemia accompanying this type of diabetes (2, 8, 39–42), and a second that leads to a reduction of all activities measured, including basal, magnesium-dependent, and manganese-dependent activities. Whether or not the catalytic component of adenylyl cyclase is decreased as well cannot be inferred since direct assessments of the quantity and functional capacity of the catalytic component of adenylyl cyclase are presently unavailable.

Other receptor and enzymatic functions in the hepatic plasma membrane were also altered by uncontrolled diabetes mellitus. Diminished [^{3}H]prazosin binding to its specific receptor sites (Fig. 8) was found indicating a loss of α_1-adrenergic receptor sites in liver of diabetic rats. Similarly, the activity of 5′-nucleotidase was also markedly decreased. The loss in both α_1-adrenergic receptor sites and 5′-nucleotidase activity parallels the severity of diabetes mellitus (Fig. 8, Table II), the time course of development of diabetes mellitus (Table II), and the decrease in basal, $MnCl_2$-dependent and fluoride-stimulated adenylyl cyclase activities (Fig. 5). Since ouabain-sensitive Na^+,K^+-ATPase activity was not reduced in these same membrane preparations (Table III), the data suggest a nonuniform loss of enzymatic and receptor functions in the plasma membrane of diabetic livers. This loss is disproportionately greater than the total protein loss in the diabetic membranes, as indicated by the decreased specific activity of these functions per milligram of membrane protein. Although it is possible that differential purification or posthomogenization proteolysis of plasma membranes from diabetic as compared with control liver might account for the observed differences, these would appear to be rather unlikely possibilities for three reasons. First, all of these seemingly unrelated receptor and enzymatic functions did not

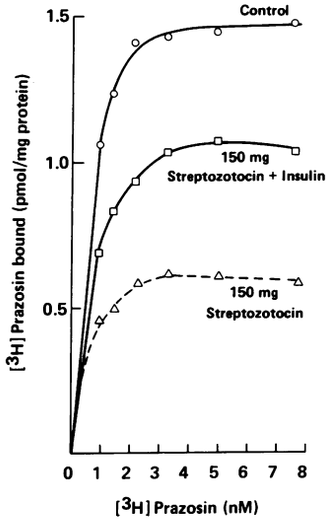

Figure 8. Effect of streptozotocin-induced diabetes and subsequent insulin treatment on α_1-adrenergic receptor levels in rat liver plasma membranes assessed by specific [^{3}H]prazosin binding. Liver membranes were prepared from control animals, from animals treated with 150 mg of streptozotocin/kg for 5 d, and from animals treated with 150 mg streptozotocin/kg and given 5 U insulin/d from days 3 through 9 as described for groups I and II under Methods. Aliquots (100–150 μg) of liver membranes were incubated in a final volume of 0.5 ml with the indicated concentrations of [^{3}H]prazosin in medium containing 5 mM $MgCl_2$, 1 mM EDTA, 100 μM propranolol, 0.8 mM ascorbic acid, 0.1% bovine serum albumin, and 25 mM Tris–HCl, pH 7.4, in the absence (duplicates) and presence (triplicates) of 10 μM phentolamine. After 15 min at 25°C, the reactions were terminated by addition of 4 ml of ice-cold 25 mM Tris-HCl, pH 7.5, followed by immediate filtration through Whatman GF/C glass fiber filters. The filters were rapidly washed twice with 4 ml of 25 mM Tris-HCl, pH 7.4, dried, and the [^{3}H]prazosin retained on them was determined by [^{3}H]-counting in a scintillation counter. Values depicted on the figure are the differences between [^{3}H]prazosin bound in the absence of phentolamine (total) and [^{3}H]prazosin bound in the presence of 10 μM phentolamine (nonspecific).

Table II. Effect of Streptozotocin Treatment and Subsequent Insulin Administration on Rat Liver Membrane 5'-Nucleotidase Activity

Streptozotocin treatment		Insulin treatment (days 3–9)	5'-Nucleotidase activity
Dose	Time		
mg	*h*		*nmol P_i release/min per mg protein*
Experiment no. 1			
Control	—	—	461±9
65	120	—	306±7
100	120	—	214±7
150	120	—	191±6
150	240	5 U/d	344±12
Experiment no. 2			
Control	—	—	640±5
150	24	—	495±3
150	48	—	390±4
150	72	—	301±10
150	120	—	188±4
150	240	5 U/d	696±4
150	240	10 U/d	592±14

5'-Nucleotidase activity was determined in liver membranes prepared from rats subjected to the different treatment schedules described under Methods. Incubations contained in a final volume of 1.0 ml: 50–100 μg liver membrane protein, 33.75 mM 5'-cAMP, 10 mM $MgCl_2$, and 0.1 M Na-glycine, pH 8.5. After 15 min at 37°C, the reactions were stopped by the addition of an equal volume of 20% TCA, precipitated protein was removed by centrifugation, and the inorganic phosphate released from 5'-cAMP was determined in aliquots of the supernatant by the method of Fiske-Subbarow (56). Values are means±SEM of four determinations.

decrease uniformly with the duration and severity of diabetes, but all were returned to control levels after insulin treatment. Second, the method of liver membrane preparation used in this study has been shown by other investigators to produce uniform and equal purification of enzymatic functions in diabetic and control livers (5, 9). Third, the data of Table IV show equal purification of diabetic and control membranes. Based on those enzymatic activities in whole homogenates and in the corresponding plasma membrane preparations, the degree of contamination amounted to 10% or less of the protein content of the control plasma membranes and this was not different in diabetic preparations.

Increased hepatic autophagy and accelerated protein degradation have been reported in diabetes mellitus (39, 47–49). Enzymatic proteins as a group tend to have much higher turnover rates than structural proteins (50). Since protein degradation appears to follow first order kinetics (51), a generalized increase in proteolysis will produce the lowest levels and the greatest decreases in those proteins that have initially the highest turnover rates. As a consequence, the decline in enzymatically active proteins will be disproportionate to the total protein loss as demonstrated by us previously (52, 53). Such a generalized increase in proteolysis might be expected as a concomitant of the insulin deficiency of diabetes mellitus (54, 55), and might possibly account for the decreased adenylyl cyclase regulatory and catalytic components produced by diabetes. Such a mechanism has been proposed for the decreased glucokinase activity in liver of diabetic rats (49). In view of the broad range of receptor and enzyme proteins decreased in diabetic plasma membranes, it seems most reasonable to hypothesize that a relatively nonselective process, such as accelerated proteolysis, may account in part for these changes.

We may, therefore, conclude that there appear to be at least two major defects producing the loss of glucagon-sensitive adenylyl cyclase activity in liver of diabetic rats. First, there is evidence for an immediate and rapid decrease in glucagon receptors which might reflect homologous desensitization. Second, there is also evidence suggesting a generalized decrease in many but not all enzymatically active proteins in the liver plasma membrane. Support for this concept of two mechanisms is provided by studies on insulin administration to rats given 150 mg/kg streptozotocin. $MnCl_2$-, fluoride-, and GMP-P(NH)P-stimulable adenylyl cyclase activities were restored to control levels (Fig. 5). Similarly, activities of 5'-nucleotidase were also nearly normal (Table II). Thus, insulin administration, although not sufficient to normalize blood sugar, was at least adequate to restore the activities of these enzyme and receptor proteins. However, glucagon receptor number was not normalized by insulin. Since relative hyperinsulinism for a prolonged period of time is generally required for adequate suppression of the

Table III. Effect of Streptozotocin Treatment With and Without Insulin Treatment on Na^+,K^+-ATPase Activity in Rat Liver Membranes

Streptozotocin treatment		Insulin treatment	Na^+,K^+-ATPase activity
Dose	Time		
mg/kg wt	*h*		*μmol P_i released/mg per h*
Control	—	—	10.0±1.0
150	120	—	13.2±1.2
150	240	5 U/d	10.5±4.5

Na^+,K^+-ATPase activity was determined in liver membranes of normal rats, rats treated with 150 mg/kg body weight streptozotocin, and rats which received 5 U insulin/d for 7 d, beginning 72 h after streptozotocin treatment. Values given are the mean±SE of at least three determinations.

Table IV. Distribution of Membrane-associated Markers in Whole Homogenates and the Hepatic Plasma Membrane Preparations of Livers from Control, Diabetic, and Insulin-treated Diabetic Rats

Treatment	DNA		Cytochrome *c* oxidase		Glucose-6-phosphatase		*N*-Acetyl-β-glucosaminidase	
	Whole homogenate	Plasma membrane preparation	Whole homogenate	Plasma membrane preparation	Whole homogenate	Plasma membrane preparation	Whole homogenate	Plasma membrane preparation
	μg/g liver	*μg/g liver*	*μmol/min per g liver*	*μmol/min per g liver*	*μmol/min per g liver*	*μmol/min per g liver*	*mmol/min per g liver*	*mmol/min per g liver*
Control	858	17.8±0.1	59.4±2.7	0.32±0.09	10.5±0.6	0.09±0.02	2.04±0.05	0.04±0.002
Streptozotocin-treated, 150 mg/kg	834±67	13.1±0.4	87.5±6.3	0.50±0.04	22.2±1.4	0.18±0.02	1.89±0.16	0.03±0.004
Insulin-treated, 5 U/d	678±20	12.8±1.1	64.2±4.8	0.20±0.01	17.2±1.9	0.11±0.01	1.23±0.07	0.01±0.001

The levels of membrane-associated markers were determined in whole homogenates and plasma membrane preparations of livers of control rats, rats treated with a single dose of 150 mg/kg streptozotocin, and rats treated with 5 U insulin/d initiated 72 h after streptozotocin treatment. Levels and activities were assessed as described more fully in Methods by using the method of Brune et al. (29) for DNA levels, a modification of the method of Seymour and Peters (32) for *N*-acetyl-β-glucosaminidase activity, the method of Aronson and Touster (31) for glucose-6-phosphatase levels, and the method of Wharton and Tzagoloff (30) for cytochrome *c* oxidase activities. Each marker was measured in at least three preparations.

hyperglucagonemia of diabetes mellitus (37, 38), it is possible that the decreased glucagon receptor number, despite insulin administration, might reflect continuing desensitization owing to sustained hyperglucagonemia, as has been found previously in insulin-treated streptozotocin-diabetic rats (2).

In summary, the data of the present study show a loss of responsiveness of the glucagon-sensitive adenylyl cyclase in liver of diabetic rats. This loss may derive from two different mechanisms including in part a homologous desensitization perhaps owing to the hyperglucagonemia which accompanies diabetes mellitus, and in part a generalized acceleration of proteolysis of a variety of enzymatic and receptor proteins in the hepatic plasma membrane.

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