Guanine Nucleotide Binding Regulatory Proteins and Adenylate Cyclase in Livers of Streptozotocin- and BB/Wor-Diabetic Rats

Immunodetection of G_s and G_i with Antisera Prepared against Synthetic Peptides

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Abstract

Adenylate cyclase in liver plasma membranes from streptozotocin-diabetic (STZ) or BB/Wor spontaneously diabetic rats showed increased responsiveness to GTP, glucagon, fluoroaluminate, and cholera toxin. Basal or forskolin-stimulated activity was unchanged in STZ rats, but increased in BB/Wor rats. No change in the α-subunit of G_i (α_i) was observed in STZ or BB/Wor rats using pertussis toxin-stimulated [^{32}P]ADP-ribosylation. Immunodetection using antibodies against the COOH-terminal decapeptides of α_T and α_{i-3} showed no change in α_i in STZ rats and a slight decrease in BB/Wor rats. Angiotensin II inhibition of hepatic adenylate cyclase was not altered in either diabetic rat. In both models of diabetes, G_s α-subunits were increased as measured by cholera toxin-stimulated [^{32}P]-ADP-ribosylation of 43–47.5-kD peptides, reconstitution with membranes from S49 cyc$^-$ cells or immunoreactivity using antibodies against the COOH-terminal decapeptide of α_s. These data indicate that STZ-diabetes increases hepatic G_s but does not change G_i or adenylate cyclase catalytic activity. In contrast, BB/Wor rats show increased hepatic G_s and adenylate cyclase. These changes could explain the increase in hepatic cAMP and related dysfunctions observed in diabetes.

Introduction

In experimental and naturally occurring forms of diabetes mellitus, the production of glucose, fatty acids, and ketone bodies exceeds their utilization, resulting in many of the features characteristic of the disease. There is considerable evidence that hyperglucagonemia and the resulting increases in hepatic glycogenolysis, gluconeogenesis, and ketogenesis are causal factors in some of these changes (see 1, 2 for review). In rats, these changes are associated with an increase in hepatic cAMP content (3–6) in both experimental diabetes (e.g., induced by exogenous insulin-antibodies, alloxan or streptozotocin [STZ],[1] and naturally occurring forms of acute diabetes (e.g., the spontaneously diabetic Bio-Breeding Worcester rat [BB/Wor]). Since it is commonly found that desensitization occurs when cells are exposed to elevated levels of hormones such as glucagon (e.g., 7) this increase in hepatic cAMP in diabetes is surprising.

There are many proteins involved in regulating hepatic cAMP production. These include the glucagon and β_2-adrenergic stimulatory receptors, the α_2-adrenergic and angiotensin II inhibitory receptors, the stimulatory (G_s) and inhibitory (G_i) guanine nucleotide binding proteins, the adenylate cyclase catalytic moiety, and cAMP phosphodiesterase. In theory, the increased hepatic cAMP in diabetes could be due to changes in one or more of these proteins. Alternatively, if no changes occurred in this system, the mere increase in the plasma concentration ratio of glucagon to insulin in diabetes could explain the increase in hepatic cAMP in this disease.

Several groups have explored the possibility that molecular components of the adenylate cyclase system are altered in diabetic liver by studying glucagon receptor binding and/or adenylate cyclase activity in liver cells and plasma membranes from normal and STZ-diabetic rats (5, 8–18). However, the results of these studies have been conflicting. For example, glucagon-sensitive and basal adenylate cyclase activities have been reported to be either increased, decreased, or unchanged in liver plasma membranes from STZ-diabetic rats (5, 10, 12, 14–18). In addition, several groups have reported that STZ treatment decreases plasma membrane marker enzymes in liver homogenates and/or membrane preparations (13, 14, 17, 18). While this has not been a consistent finding (5, 15), it may explain why some have observed decreases in total hepatic adenylate cyclase activity and glucagon receptor binding and G_i in chemically induced diabetes. The hepatotoxicity of some diabetogenic agents may also partly account for the differences (for review see 19). Another possible source of variability is the severity of the diabetic state, which varies with route of administration and dose of streptozotocin or alloxan. Obviously these variables are not a problem in the naturally occurring form of diabetes exhibited by BB/Wor rats (for review see 20, 21).

In the present communication, we have reexamined the roles of G_s, G_i, and adenylate cyclase in the increased hepatic cAMP in diabetes. G_s, G_i, and adenylate cyclase were measured in various ways in purified liver plasma membranes or homogenates from control and STZ-injected animals. In view of the recent study by Gawler et al. (16) reporting that G_i expression is abolished in experimental diabetes, we have

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1. *Abbreviations used in this paper:* BB/Wor, Bio-Breeding Worcester rat; DB-RES, diabetes-resistant rat; G-protein, guanine nucleotide binding regulatory protein; G_i, inhibitory guanine nucleotide binding protein of adenylate cyclase; G_o, a G protein of unknown function from bovine brain; G_s, stimulatory guanine nucleotide binding protein of adenylate cyclase; GTPγS, guanosine 5′-0-(γ-thiotri-phosphate); IAP, islet activating protein, a Bordetella pertussis toxin; STZ, streptozotocin; T_d, transducin.

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compared several of these parameters in animals whose hepatic G_i function has been inactivated by islet-activating pertussis toxin (IAP). Because of the problems inherent in the use of the STZ-model (see above) we have also examined these parameters, for the first time, in the spontaneously diabetic rat (BB/Wor) and its genetic counterpart the diabetic-resistant rat (DB-RES).

Our data indicate that increased expression of G_s may contribute to the increase in hepatic cAMP in the acute STZ-diabetic and spontaneously diabetic BB/Wor rat. Additionally, unlike STZ-diabetes, the naturally occurring form of diabetes exhibited by the BB/Wor rats is associated with an approximately twofold increase in basal and forskolin-stimulated hepatic adenylate cyclase activity. Thus insulin may regulate the expression of regulatory and catalytic components of the adenylate cyclase system.

Methods

Materials. [^{32}P]NAD (1,000 Ci/mmol) and [α-^{32}P]ATP (3,000 Ci/mmol) were from New England Nuclear (Boston, MA). S49 cyc$^-$ (94.15.1) cells were obtained from the Cell Culture Facility of the University of California, San Francisco. Acute spontaneously diabetic (BB/Wor) rats and their diabetic resistant counterparts (DB-RES) were obtained from Dr. Dennis L. Guberski (Department of Pathology, University of Massachusetts Medical School, Worcester, MA). At the University of Massachusetts, the urine of BB/Wor rats was analyzed twice a week to detect diabetes of spontaneous onset (termed conversion). After detection of conversion, the animals were monitored daily and stabilized by insulin injection to maintain a moderate diabetic state (i.e., +4 urine glucose and 0 ketones). Insulin doses were individualized and ranged from 1.4 to 2.6 U/d in rats weighing between 180 and 350 g. Animals were received 1–6 d after detection of conversion and were used immediately along with their DB-RES counterparts, which served as controls for BB/Wor in this study. Glucagon, insulin, and protamine zinc insulin suspension were from Eli Lilly (Indianapolis, IN). NaF and $AlCl_3$ were from Fisher Scientific Co. (Pittsburgh, PA). Streptozotocin was from either Calbiochem-Behring Corp. (La Jolla, CA) or Sigma Chemical Co. (St. Louis, MO). Bacterial toxins were from List Biological Laboratories (Campbell, CA) or Sigma. Dextrostix reagent strips were from Miles Laboratories (Elkhart, IN). Percoll was from Pharmacia Fine Chemicals (Piscataway, NJ). Sources of other materials have been given previously (22–24).

Experimental diabetes mellitus and in vivo treatment with IAP. Male Sprague-Dawley rats (225–275 g) were made diabetic (25) by a single intraperitoneal injection of streptozotocin (50 mg/kg) dissolved in 50 mM citrate buffer (pH 4.0). Urine or serum glucose levels were measured, respectively, with dextrostix or a glucose analyzer 2 (Beckman Instruments, Inc., Fullerton, CA). Animals with urine glucose concentrations > 200 mg/dl or serum glucose concentrations > 400 mg/dl 7 d after streptozotocin were deemed diabetic.

Abolition of G_i activity was obtained 24 h after intraperitoneal injection of IAP (25 mg/100 g body wt) as previously described (22).

Plasma membrane purification, hepatocyte isolation and cAMP measurement. Experimental treatment groups (i.e., control, diabetic, insulin-replaced) typically consisted of five animals (with the exception of IAP-treated groups which consisted of 1–2 animals per group). One or two of these animals were used for hepatocyte studies and the remainder were used for plasma membrane preparation. Liver plasma membranes prepared by Percoll density gradient centrifugation (26) were resuspended in 25 mM Tris HCl and 1 mM EGTA (pH 7.4), and then frozen in liquid N_2 for storage at −70°C. Membranes from control and experimental groups were prepared on the same day. Membrane protein was determined using BCA assay (Pierce Chemical Co., Rockford, IL).

Liver parenchymal cells from control and diabetic rats were prepared (24) simultaneously and cAMP measurements were carried out as described elsewhere (27).

Adenylate cyclase activity. The method of Salomon (28) was used to assay adenylate cyclase in liver plasma membranes. The assay was performed at 30°C in a final volume of 50 μl for 10–15 min with the following components: 0.5 mM [α-^{32}P]ATP (70–150 cpm/pmol), 20 mM creatine phosphate, 50 U/ml creatine phosphokinase, 25 mM Tris HCl (pH 7.4), 5 mM magnesium acetate, 0.05 mM cAMP, 1 mM DTT, 0.1 mg/ml BSA, 0.9–1.5 mg membrane protein/ml and other additives as indicated. Activity measured in the absence of other additives is termed basal activity. The assay was initiated by the addition of membranes and terminated by the addition of 100 ml of medium (pH 7.5) containing 2% SDS, 45 mM ATP, and 1.3 mM cAMP. Addition of [^{3}H]cAMP for the estimation of [^{32}P]cAMP recovery preceded boiling for 5 min and column purification as described by Salomon (28). Radioactive content was determined using a Beckman LS3801 scintillation counter. Results shown are representative of two to six experiments on different membrane batches.

Bacterial toxin catalyzed [^{32}P]ADP incorporation into G_s and G_i. Conditions for toxin-catalyzed [^{32}P]ADP ribosylation of membranes were optimized for each toxin as recommended by Ribeiro-Neto et al. (29). The assay mixture for cholera toxin catalyzed [^{32}P]ADP-ribosylation of G_s consisted of the following in 250 μl: 10 μM [^{32}P]-NAD (50–60 μCi/tube), 50 mM Na-Hepes (pH 7.4), 400 mM NaH_2PO_4 (pH 7.4), 10 mM $MgCl_2$, 10 mM thymidine, 1 mM EGTA, 1 mM ATP, 0.1 mM GTPγS, 80 μg/ml of cholera toxin A-subunit and 100 μg of membrane protein. The mixture was incubated for 15 min at 30°C at which time more ATP, GTPγS, and [^{32}P]NAD were added (22). After an additional 15-min incubation the assay was terminated by the addition of 750 μl of ice-cold 10% TCA and incubation on ice.

IAP-catalyzed [^{32}P]ADP incorporation into plasma membrane G_i has been described (22). In some experiments cholate-solubilized hepatocyte proteins were used as substrate in place of plasma membranes. For this purpose hepatocytes from control or diabetic animals were adjusted to equal wet weights (~ 30–40 mg/ml) and 1 ml of suspended cells was centrifuged at 11,000 *g* for 1 min. The cell pellet was solubilized in 0.5 ml of 1% sodium cholate (pH 8.0), 1 mM EDTA, and 1 mM DTT. Unsolubilized material was removed by centrifugation at 100,000 *g* for 1 h at 4°C. Assayed proteins were precipitated with TCA as described above. Precipitated proteins from the cholera toxin and IAP studies were solubilized for SDS-PAGE, and autoradiography of the dried gels was performed as described previously (22, 30). Quantitation of radioactivity incorporated into peptides was determined by scintillation spectrometry or by laser densitometry of autoradiographs and computer integration of the peaks.

S49 cyc$^-$ assay for G_s activity. G_s activity was determined by measuring the ability of 1% cholate solubilized liver membranes (1.5 mg/ml) to stimulate MgATP-dependent adenylate cyclase activity in plasma membranes from S49 cyc$^-$ lymphoma cells, which lack endogenous G_s (31–33). Briefly, S49 cyc$^-$ lymphoma cells were harvested and plasma membranes prepared by the method of Ross et al. (34). The adenylate cyclase reconstitution assay for G_s activity used was an adaptation of the method of Sternweis et al. (35). Cholate solubilized liver plasma membranes (60 μl) were incubated with 25 μl of AMF for 10 min at 0°C. AMF consists of ATP (1 mM), $MgCl_2$ (10 mM), NaF (10 mM), and $AlCl_3$ (0.25 mM). Subsequently, 15 μl of this mixture was mixed with 25 μl of cyc$^-$ plasma membranes on ice and allowed to incubate for 30 min on ice. During this period G_s reconstitutes with the adenylate cyclase of the cyc$^-$ plasma membranes. Next, 20 μl of Tris (150 mM, pH 8.0), ATP (2 mM), $MgCl_2$ (15 mM), NaF (10 mM), $AlCl_3$ (0.25 mM), potassium phosphoenolpyruvate (9 mM), pyruvate kinase (30 μg/ml), and BSA (0.3 mg/ml) were added, and the sample was incubated for 10 min at 30°C. At this point, 40 μl of assay cocktail was added to the sample and the mixture was incubated for 30 min at 30°C. Assay cocktail consisted of Tris (125 mM, pH 8.0), ATP (0.25 mM), [α-^{32}P]ATP (~ 10^{-6} cpm), $MgCl_2$ (20 mM), EDTA (2.5 mM),

isobutylmethylxanthine (0.25 mM), NaF (10 mM), $AlCl_3$ (0.25 mM), potassium phosphoenolpyruvate (7.5 mM), and pyruvate kinase (25 μg/ml). The reaction was terminated and cyclic AMP quantitated as described above for adenylate cyclase (28).

Antiserum production. In initial studies antisera were raised to synthetic peptides corresponding to internal sequences of several G protein α-subunits including the peptide termed "α-common" (36). However, in agreement with the findings of Mumby et al. (37), these antisera reacted poorly or not at all with rat liver membrane G-proteins. Synthetic peptides corresponding to the COOH-terminus of T_d have been used to raise antibodies to T_d and various G_is including the 41-kD pertussis toxin substrate in rat liver (16). Therefore we synthesized peptides corresponding to the COOH-terminal decapeptide of several G-protein α-subunits including: α_{i-3} (38, 39), α_s (38, 40), α_o (38, 40), α_T (41), α_{i-1}, and α_{i-2} (38, 39). Since the latter two have identical COOH-terminal decapeptide sequences (38, 39) the peptide corresponding to this sequence is termed $\alpha_{i-1,2}$.

The peptides were synthesized as undecapeptides with an NH_2-terminal *cys* (in order to increase cross-linking efficiency to keyhole limpet hemocyanin) using the solid-phase method of Merrifield (42) on a Beckman 990 peptide synthesizer. HF cleavage of the peptide from the carrier resin was performed on ice (1 h) in the presence of 10% anisole and 1% ethanedithiol. The resulting mixture was washed three times with ether and the peptide was extracted with 10% acetic acid. After lyophilization to dryness, peptides were purified on a 200-ml G-10 column equilibrated with 10% acetic acid and lyophilized. The composition of each peptide was verified by HPLC amino acid analysis as described by Bidlingmeyer et al. (43) after precolumn derivatization of the acid hydrolyzed peptide with phenylisothiocyanate.

The synthetic peptides were cross-linked to the carrier protein keyhole limpet hemocyanin as described by Green et al. (44) except that as coupling-reagent we employed a soluble analogue of m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (sulfo-MBS; Pierce Chemical Co.).

Three rabbits were immunized per peptide using the protocol of Green et al. (44) and animals were bled 4 wk after the initial injection and 7–10 d after booster injections every 5 wk.

ELISA and immunoblots. Production of antibodies and cross-reactivity with other peptides were measured using an ELISA and the Vectastain ABC kit for detection of rabbit IgG (Vector Laboratories, Burlingame, CA) as previously described by Van Eldik et al. (45). The assay was conducted in 96-well microtitration plates at an antigen concentration of 200 ng peptide/well. Binding of antibodies to endogenous liver plasma membrane proteins was detected following PAGE of SDS solubilized liver plasma membranes. Transfer of the protein to nitrocellulose and immunodetection were performed as described by Mumby et al. (36, 37).

Results

Effect of streptozotocin, IAP-injection, and naturally occurring diabetes on liver plasma membrane markers. Liver plasma membranes were isolated from Sprague-Dawley control, STZ-diabetic (7 d following a single injection of streptozotocin to Sprague-Dawley rats), IAP-treated (24 h after a single injection of IAP into Sprague-Dawley rats), BB/Wor-diabetic (1–6 d following detection of diabetes) and DB-RES rats (which served as controls for the BB/Wor). IAP-treated animals were studied in view of the paper by Gawler et al. (16) in which G_i activity was reported to be abolished by diabetes. We have previously shown that the IAP-treatment used in these studies results in ADP-ribosylation of over 97% of liver plasma membrane G and prevents the ability of angiotensin II to decrease glucagon-elevated cAMP level in liver cells (22), a measure of G_i activity (46).

The IAP-treatment protocol had no significant effect on plasma membrane maximal binding capacity (B_{max}) of α_1-adrenergic, V_1-vasopressin, or angiotensin II receptors, which may serve as plasma membrane markers (22). When plasma membranes from STZ-diabetic rats were compared to control membranes a 20–30% decrease was observed in α_1-adrenergic receptor maximal binding capacity with no change in affinity for [^{3}H]prazosin. However, no significant changes were observed in the binding parameters of angiotensin II or vasopressin receptors. Furthermore, no alterations were observed in the mass measurements of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate in the STZ-diabetic membranes.[2] Table I shows that neither STZ-diabetes (5, 15 cf. 13, 14, 17, 18) nor spontaneous diabetes significantly altered 5′-nucleotidase activities of liver homogenates or plasma membranes when compared with their respective controls.

Basal and forskolin-sensitive adenylate cyclase activity. Table II shows that adenylate cyclase activity in response to 100 μM forskolin was not significantly different between control and STZ-diabetic animals (cf. 14, 16). In agreement with this finding, basal activity was also not altered by STZ-diabetes (Table II). Further, no difference was observed when adenylate cyclase was assayed in the presence of ATP and Mn^{2+}, which may be a more accurate reflection of the catalytic component per se (Table III). In contrast both basal and forskolin-sensitive adenylate cyclase were elevated in acute diabetic BB/Wor-diabetic animals as compared to DB-RES (Tables II and III).

GTP and glucagon stimulation of adenylate cyclase. Neither streptozotocin- or IAP-treatment caused any significant effect on the EC_{50} for GTP (Fig. 1) and GTPγS (data not shown) stimulation of adenylate cyclase activity. However, maximal and submaximal concentrations of these guanine nucleotides elicited greater adenylate cyclase activity in membranes from animals receiving either treatment. The increases in a series of experiments (50–100 μM nucleotide) were smaller in membranes from STZ-treated rats (133±7% of control, $n = 15$ animals, $P < 0.05$, t test) than in membranes from IAP-treated rats (196±20% of control, $n = 3$ animals, $P < 0.05$, t test).

GTP-sensitive adenylate cyclase activity was also increased in BB/Wor rats (Fig. 1) relative to DB-RES, although no effect was observed on the EC_{50} for this compound. The magnitude of the increase in GTP-sensitive adenylate cyclase (50–100 μM GTP) activity observed in the BB/Wor-diabetics was larger than the increase in basal activity in a series of experiments (i.e., 210±12% for GTP vs. 166±17% for basal, $P < 0.05$, t test) and therefore may not be due just to a change in adenylate cyclase per se.

Fig. 2 shows that neither STZ- nor IAP-treatment affected the EC_{50} for glucagon activation of adenylate cyclase, which ranged from 3 to 10 nM in four different experiments. However, as with GTP, glucagon responses (measured in the presence of 50 μM GTP) were larger in membranes from IAP-treated and STZ-diabetic animals. In the absence of GTP, the glucagon response was greatly reduced (15). The increment in cAMP production in IAP-treated membranes in response to

2. Blackmore, P. F., C. J. Lynch, S. B. Bocckino, B. Bouscarel, S. J. Taylor, G. Augert, and J. H. Exton, unpublished observations.

Table I. 5'Nucleotidase Activity in Rat Liver Homogenates and Purified Liver Plasma Membranes

Experimental group (strain)	Homogenate activity (n)*	Plasma membrane activity (n)‡
	mg P_i/mg protein/h	*mg P_i/mg protein/h*
Control (Sprague-Dawley)	0.037±0.005 (8)	1.81±0.19 (9)
STZ-diabetic (Sprague-Dawley)	0.030±0.006§ (8)	1.40±0.21§ (9)
DB-RES Control (Wistar-derived)	0.023±0.001 (5)	1.36±0.11 (5)
BB/Wor-diabetic (Wistar-derived)	0.021±0.002§ (5)	1.24±0.10§ (5)

* *n* represents the number of animals tested.
‡ *n* represents the number of different membrane batches tested containing two to four animals per batch.
§ Not significantly different from genetic control as determined by Student's *t* test ($P > 0.05$).

glucagon plus GTP (234±12%, $n = 3$ animals) was comparable to the increase in response to GTP alone. In STZ-diabetic membranes the glucagon (plus GTP) responses were significantly higher than the responses to GTP alone [(160±15% for 1 μM glucagon plus 50 μM GTP in four studies vs. 133±7% for 50–100 μM GTP alone in 11 studies) ($P < 0.05$, *t* test)].

Fig. 2 also shows that hepatic adenylate cyclase of BB/Wor-diabetic rats showed increased responsiveness to glucagon relative to DB-RES rats (238±12% for 100 nM glucagon from five determinations, $P < 0.05$, *t* test). In agreement with the findings with the STZ model, in BB/Wor-diabetic membranes, the glucagon (plus GTP) responses were also significantly higher than the responses to GTP alone (238±12% vs. 210±12%, respectively) and no effect was observed on the EC_{50} for glucagon which ranged from 2 to 5 nM in these animals.

Fluoroaluminate and cholera toxin A-subunit stimulation of adenylate cyclase activity. In Fig. 3, the effects of NaF on adenylate cyclase were measured in the presence of 250 μM

*Table II. Basal and Forskolin-stimulatable Adenylate Cyclase Activity in Liver Plasma Membranes from Control and Diabetic Animals**

Experimental group (strain)	Adenylate cyclase activity: Basal	Adenylate cyclase activity: Forskolin-stimulated (100 μM)
	pmol cAMP/mg protein/15 min	
Control (Sprague-Dawley)	150±11	10,900±520
STZ-diabetic (Sprague-Dawley)	140±4‡	11,000±580‡
DB-RES Control (Wistar-derived)	122±9	6,460±400
BB/Wor-Diabetic (Wistar-derived)	203±32§	12,900±1043§

* Basal and forskolin-stimulated activity was determined in the absence of GTP and in the presence of 5 mM magnesium acetate.
‡ Not significantly different from genetic control as determined by Student's *t* test ($P > 0.05$).
§ Significantly different from genetic control as determined by Student's *t* test ($P < 0.05$).

*Table III. Magnesium- and Manganese-stimulatable Adenylate Cyclase Activity in Liver Plasma Membranes from Control and Diabetic Animals**

Experimental group (strain)	Adenylate cyclase activity: $MgSO_4$ 10 mM	$MgSO_4$ 100 mM	$MnSO_4$ 10 mM	$MnSO_4$ 100 mM
	pmol cAMP/mg protein/15 min			
Control (Sprague-Dawley)	257±6	656±17	237±7	652±5
STZ-diabetic (Sprague-Dawley)	246±7‡	648±15‡	244±2‡	655±15‡
DB-RES control (Wistar-derived)	196±4	440±10	165±2	460±30
BB/Wor-Diabetic (Wistar-derived)	370±4§	780±12§	371±10§	938±11§

* Activities were measured in the absence of GTP. Data are representative of two studies on separate membrane batches.
‡ Not significantly different from genetic control as determined by Student's *t* test ($P > 0.05$).
§ Significantly different from genetic control as determined by Student's *t* test ($P < 0.05$).

$AlCl_3$. These experiments were conducted in the absence of GTP. The response to fluoroaluminate was biphasic in all of the experimental groups. The responses were maximal at 5–7.5 mM NaF and decreased at higher concentrations. The inhibition at higher NaF may be attributable to an effect on G_i or a salt effect. As expected, IAP-treatment had no significant effect on adenylate cyclase activity stimulated by maximal concentrations of fluoroaluminate, since this ion is capable of overcoming the inhibitory effects of IAP.

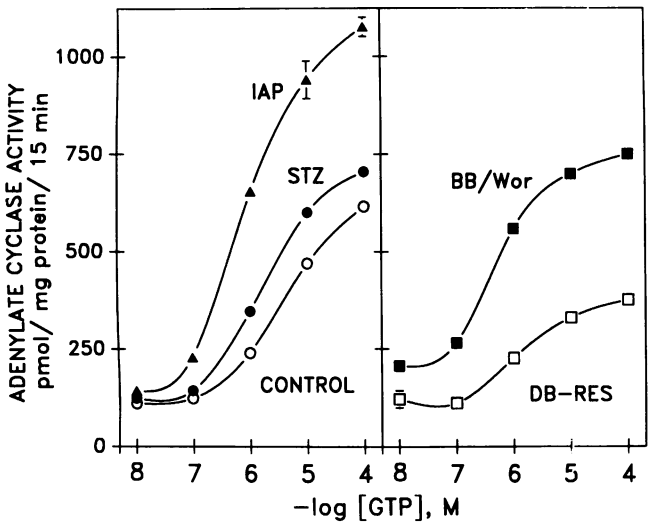

Figure 1. GTP stimulation of adenylate cyclase activity. Adenylate cyclase activity was measured in liver plasma membranes prepared from left: Sprague-Dawley control (○), 7-d STZ-diabetic (●), and 24 h IAP-treated (▲) rats or right: DB-RES control (□) and BB/Wor-diabetic (■). The assay was conducted in the absence or presence of the indicated concentrations of GTP. Each point is the mean of triplicate determinations and is representative of three or more studies on separate membrane batches. Bars represent the standard error which exceeds the size of the symbol.

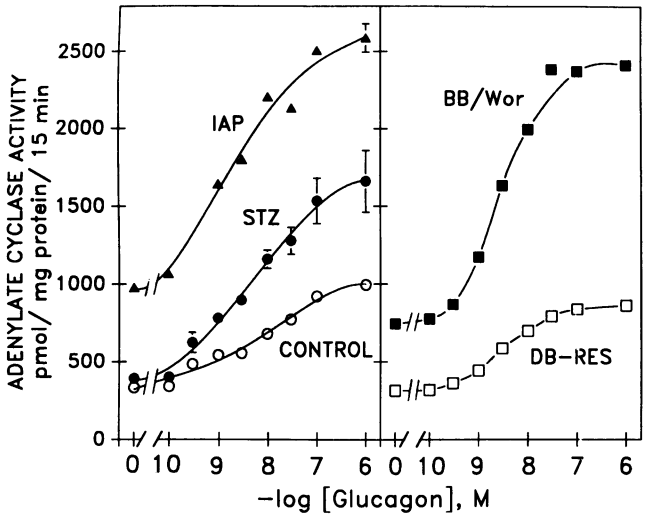

Figure 2. Glucagon stimulation of adenylate cyclase activity. Adenylate cyclase activity was measured in response to 50 μM GTP and the concentrations of glucagon shown (diluted in 10 mM Tris HCl and 1 mg/ml BSA. (*Left*) Liver plasma membranes were from Sprague-Dawley control (○), 7-d STZ-diabetic (●) and 24 h IAP-treated (▲) rats. (*Right*) Liver plasma membranes were from DB-RES control (□) and BB/Wor-diabetic (■) rats. Each point is the mean from triplicate determinations and a representative of three or more studies on separate membrane batches. Bars represent the standard error which exceeds the size of the symbol.

In contrast, membranes from STZ-diabetic animals exhibited elevated adenylate cyclase activity in response to all concentrations of NaF tested (Fig. 3). BB/Wor-diabetic rats also showed increased responsiveness to fluoroaluminate compared with DB-RES. The extent of this increase was larger at submaximal (e.g., from 299 to 361%) than at maximal concentrations of NaF (e.g., 211–244%).

Fig. 4 shows the concentration-dependent stimulation of adenylate cyclase by the A-subunit of cholera toxin. These experiments were performed in the presence of sodium phosphate, GTP, and NAD. Sodium phosphate is necessary for optimal modification of G_s by cholera toxin (29), but has inhibitory effects on adenylate cyclase. For this reason a lower, less inhibitory, concentration of sodium phosphate (100 mM) was employed in these studies, as compared to 400 mM used in [^{32}P]ADP ribosylation experiments. As compared to control, membranes from STZ-diabetic animals displayed an increased response to every concentration of cholera toxin A-subunit tested (Fig. 4, Table IV). In agreement with the findings with GTP and glucagon, cholera toxin produced even greater increases in membranes from IAP-treated rats (Fig. 4). The effect of STZ-diabetes on cholera toxin-stimulated adenylate cyclase activity could be partly reversed by insulin replacement (Table IV) supporting the idea that this effect is due to loss of insulin. Sodium phosphate and NAD reduced the elevation in BB/Wor hepatic adenylate cyclase typically observed in the presence of GTP alone (from 166±16% to 128±4% above the activity observed in DB-RES). This was increased to 170–180% above the DB-RES controls in the presence of all concentrations of cholera toxin A-subunit tested (Fig. 4). Thus the changes in fluoroaluminate- and cholera toxin- as well as GTP- and glucagon-stimulated activities were in excess of the increases in basal cyclase activity of the BB/Wor rat.

Angiotensin II inhibition of adenylate cyclase activity and of cellular cAMP content. Angiotensin II inhibition of adenylate cyclase activity has been used as a measure of G_i function in liver membranes (46). Angiotensin II inhibition of hepatic

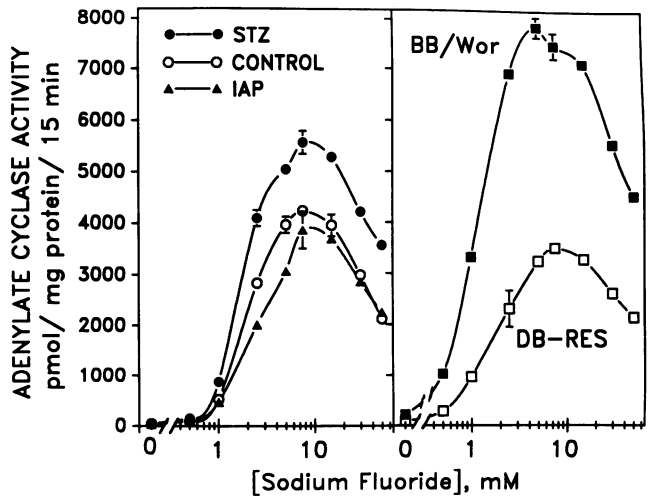

Figure 3. Fluoroaluminate stimulation of adenylate cyclase. (*Left*) Adenylate cyclase activity was measured in the presence of 250 μM $AlCl_3$ and the concentrations of NaF shown in membranes from Sprague-Dawley control (○), 7-d STZ-diabetic (●) and 24-h IAP-treated (▲) rats. (*Right*) DB-RES control (□) and BB/Wor-diabetic rats (■). GTP was not present. Each point is the mean from triplicate determinations with bars representing the standard error where it exceeds the size of the symbol. This experiment is representative of three studies on each model using different membrane batches.

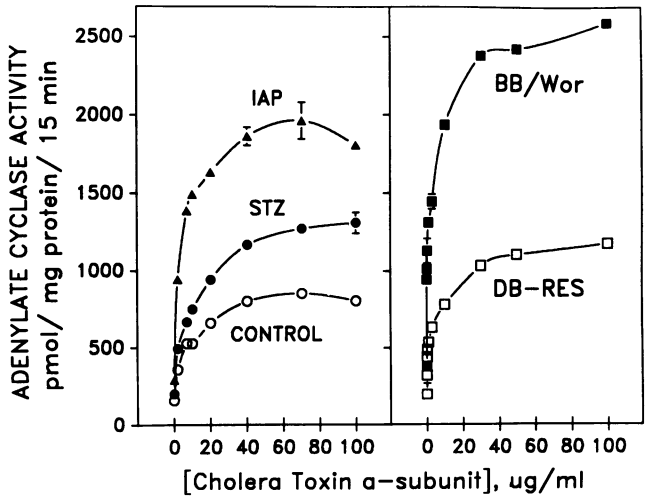

Figure 4. Stimulation of adenylate cyclase by the A-subunit of cholera toxin. Adenylate cyclase was measured in the presence of 100 mM sodium phosphate (pH 7.4), 5 mM NAD, 1 mM GTP and various concentrations of cholera toxin, A-subunit. Membranes were from left panel: control (○), 7 d STZ-diabetic (●) and 24-h IAP-treated (▲) rats or right panel: DB-RES (□) and BB/Wor (■). Each point is the mean from triplicate determinations with bars representing the standard error where it exceeds the size of the symbol. This experiment is representative of three studies on each model using different membrane batches.

*Table IV. Insulin Replacement Reverses the Effect of STZ-Diabetes on Cholera Toxin-Stimulated Adenylate Cyclase**

Experimental group	Animals per group	cAMP formed	% Control
		pmol/mg protein/15 min	
Control	3	1,540±64	100±4
STZ-diabetic	3	2,141±75	139±4
Insulin-replaced‡	4	1,734±85	113±5

* Adenylate cyclase activity was measured in the presence of 70 mg/ml of cholera toxin A subunit and other additives as described in Fig. 2 *B* legend. Data are means±standard errors from three experiments on separate membrane batches.

‡ Insulin replacement was begun 3 d after streptozotocin injection in Sprague-Dawley rats showing signs of diabetes (see Methods). These animals (200–270 g) were injected on four subsequent days with 10 U of protamine zinc insulin per day (13) and sacrificed for membrane preparation on the eighth day.

adenylate cyclase is optimally measured under conditions of high salt and only in membranes previously washed in hypotonic, EDTA containing buffer (46–48). While the exact reasons for this have not yet been elucidated, salt concentrations that decrease α_1-adrenergic and V_1-vasopressin binding affinity, increase the affinity of angiotensin II receptors for agonist and increase the effect of GTP analogues on agonist binding (22). Another index of G_i activity is angiotensin II inhibition of cAMP accumulation in hepatocytes, which is attenuated in cells prepared from animals treated with IAP (22).

Since the effects of diabetes on adenylate cyclase stimulated by guanine nucleotide, glucagon, fluoroaluminate, and cholera toxin were consistent with partial loss but not abolition (16) of G_i in diabetes, we studied the ability of angiotensin II to inhibit adenylate cyclase in membranes and cells prepared from diabetic animals. In contrast to what was expected from the findings of Gawler et al. (16), no effect of either STZ or BB/Wor-diabetes on the ability of angiotensin II to inhibit GTP-stimulated adenylate cyclase activity (Fig. 5) was observed, although as expected, this effect was attenuated in membranes from IAP-treated nondiabetic rats. In agreement with the membrane studies (Fig. 5), neither form of diabetes had any significant effect on the ability of angiotensin II to lower glucagon-elevated cAMP levels in hepatocytes (Fig. 6). In summary, the results of the adenylate cyclase experiments (Figs. 1–6, Tables I–III) were more consistent with an increase in G_s activity in STZ-diabetes or an increase in the catalytic activity of adenylate cyclase and in G_s activity in BB/Wor-diabetes rather than a physiologically important loss of G_i.

IAP- and cholera toxin-catalyzed [^{32}P]ADP ribosylation of G_s and G_i. The incorporation of ^{32}P into G_i or G_s stimulated by repetitive addition of [^{32}P]NAD in the presence of IAP or cholera toxin A subunit under conditions optimized for either toxin was used as an index of the levels of G_i and G_s in liver membranes. Fig. 7 (*A*) shows that, as previously reported (22), IAP-treatment resulted in a > 97% reduction of labeling of a 41-kD peptide in Sprague-Dawley rat liver plasma membranes, (presumably one or more species of α_i). Fig. 7 (*A*) also shows that radiolabeling of G_i was not significantly different in membranes from STZ-diabetic animals (i.e., 104±8% when compared to control, n = 5 separate batches of membranes

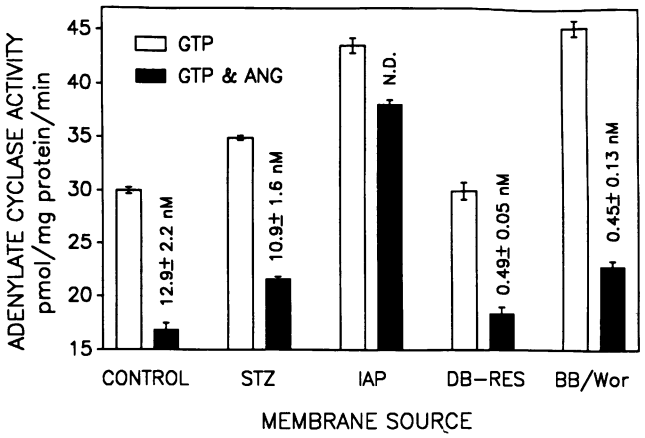

Figure 5. Angiotensin II inhibition of adenylate cyclase activity in liver plasma membranes. To enhance the effect of angiotensin II on GTP-stimulated adenylate cyclase activity, 1 ml of frozen liver plasma membranes (3–4 mg/ml) were thawed and resuspended in 14 ml of medium containing 10 mM Tris HCl, 5 mM EDTA and 0.05% β-mercaptoethanol (46, 48, 49). Membranes were adjusted to 1 mg protein/ml in buffer containing 200 mM LiCl, 10 mM Tris HCl and 1 mM EDTA (49). Adenylate cyclase activity was measured for 10 min in the presence (*filled bars*) or absence (*open bars*) of angiotensin II (100 nM) and in the presence of 0.1 mM GTP, 200 mM LiCl (28), 1 mM EDTA and other additions described in Methods. The data are means and standard errors from triplicate determinations and are representative of two such studies on separate membrane batches. The numbers above the bars represent the IC_{50}±SE for angiotensin II inhibition of adenylate cyclase activity determined from separate concentration-response studies (not shown) employing nine concentrations of angiotensin II between 0.1 nM and 10 μM. IC_{50} values were not determined (ND) in membranes from IAP-treated animals.

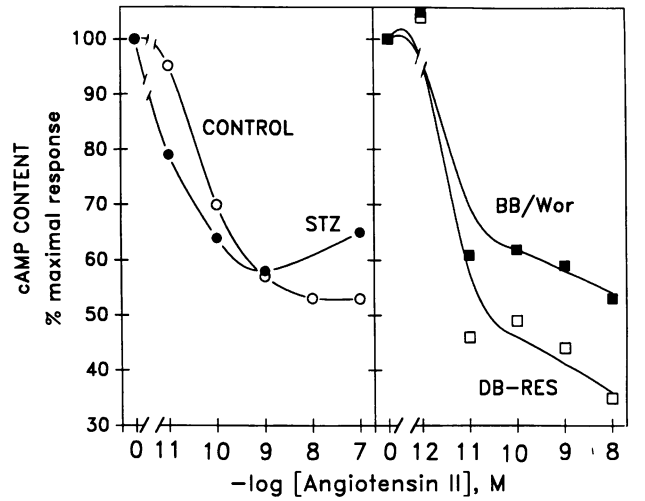

Figure 6. Angiotensin II inhibition of cAMP formation in hepatocytes. Angiotensin II inhibition of glucagon-stimulated cAMP formation. cAMP was measured in hepatocyte suspensions incubated for 5 min with various concentrations of angiotensin II in the presence of 10 nM glucagon. Mean glucagon-stimulated values were: (*left*) 4,045 and 5,570 fmol/mg wet wt in Sprague-Dawley control (○) and STZ-diabetic (●) cells, respectively, and (*right*) 9,847 and 15,723 fmol/mg wet wt, respectively, in DB-RES controls (□) and BB/Wor-diabetic (■) cells. Results are from a single experiment representative of two studies on each model.

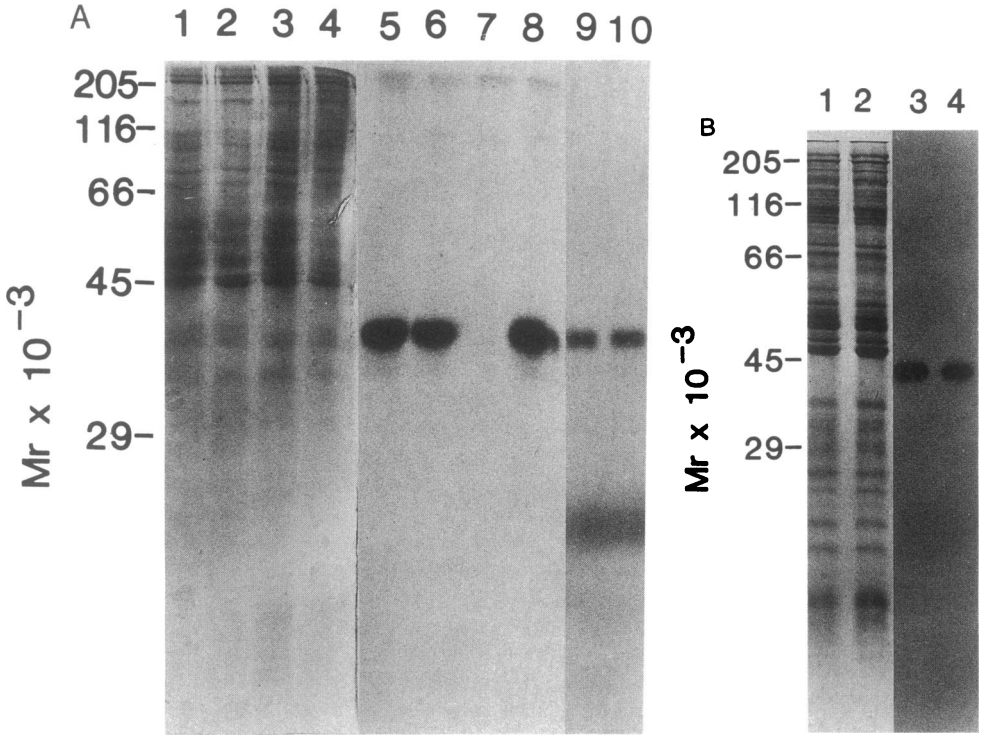

Figure 7. [^{32}P]ADP ribosylation of liver plasma membrane and homogenate proteins by IAP. IAP-catalyzed [^{32}P]ADP ribosylation of isolated liver plasma membranes and 100,000 *g* supernatants from cholate-solubilized hepatocytes was performed with three repetitive additions of [^{32}P]NAD as described previously (16) prior to polyacrylamide gel electrophoresis of SDS-solubilized proteins and autoradiography. (*A*) Lanes *1–4* show a Coomassie blue-stained gel and lanes *5–8* are the corresponding autoradiograph. These are representative of five studies on separate membrane batches. Lanes *1* and *5* represent Sprague-Dawley control membranes, lanes *2* and *6* are membranes from 7-d STZ-diabetic animals, lanes *3* and *7* are membranes from 24 h IAP-treated animals and lanes *4* and *8* are membranes from another group of diabetic animals. Lanes *9* and *10* are autoradiographs of 100,000 *g* supernatants from cholate-solubilized hepatocytes, [^{32}P]ADP-ribosylated in the presence of IAP. Hepatocytes were prepared simultaneously from control (lane *9*) and diabetic (lane *10*) rats and adjusted to equivalent wet weight before solubilization and radiolabeling. (*B*) Lanes *1–2* show a Coomassie blue-stained gel and lanes *3–4* are the corresponding autoradiographs from an experiment representative of four studies on separate membrane batches. Lanes *1* and *3* represent DB-RES control membranes and lanes *2* and *4* represent BB/Wor-diabetic membranes.

from diabetic animals). Similarly, no change was observed in G_i radiolabeling in cholate-solubilized hepatocytes prepared from diabetic rats (Fig. 7 *A*).

In contrast, a 40±8% average decrease was observed in G_i radiolabeling in nine separate batches of liver membranes from BB/Wor-diabetic rats relative to DB-RES rats (Fig. 7 *B*). According to the findings of Pobiner et al. (46) a 40% loss of G_i would not be sufficient to influence angiotensin II inhibition of adenylate cyclase activity due to spare G_i in this tissue. Our data from Figs. 5 and 6 support this notion, since we did not observe any change in angiotensin II inhibition. Interestingly, despite the decrease in G_i radiolabeling in the BB/Wor-diabetic, we could detect no change in α_{i-3} immunoreactivity (see below).

We consistently observed a significant increase in ^{32}P incorporation from [^{32}P]NAD into two (43 and 47.5 kD) peptides (presumably α-subunits of G_s) stimulated by the A-subunit of cholera toxin (Fig. 8, top). The increase in the radiolabeling was the same for both peptides and ranged from 125 to 200% in a series of experiments (n = 7 separate membrane batches). This effect could be partly reversed by insulin replacement (Table V). A significant increase was also observed in the radiolabeling of these peptides in membranes from BB/Wor rats (Fig. 8, *bottom*), however the extent of the increase was not the same for both peptides (i.e., 153±6% vs. 120±5% increase in the 43- and 47.5-kD peptides respectively, n = 4 separate membrane batches).

Heyworth and co-workers (49) have reported that a 25-kD liver plasma membrane peptide (called G_{ins}) is radiolabeled in the presence of cholera toxin and [^{32}P]NAD. In agreement with this finding we also observed the radiolabeling of a peptide in this molecular weight range in the presence of [^{32}P]-NAD and cholera toxin A subunit. The labeling of this peptide was not altered by STZ or BB/Wor-diabetes. This lower M_r peptide comigrated with cholera toxin A subunit (Fig. 8) and was radiolabeled in the absence of plasma membranes (data not shown).

S49 cyc⁻ reconstitution assay for G_s activity. Table VI shows that the ability of cholate-solubilized liver membranes to reconstitute with and stimulate S49 cyc⁻ membrane adenylate cyclase in the presence of fluoroaluminate was significantly greater in both diabetic models compared to their respective controls.

Immunological studies

We synthesized peptides containing sequences corresponding to the C-terminal decapeptides of several G-protein α-subunits including α_{i-1}, α_{i-2}, and α_{i-3} (38, 39), α_T (41), α_o (38, 40), and α_s (38, 40). Another decapeptide, designated $\alpha_{1-3(ser)}$, was synthesized in which serine replaced the cysteine located four residues from the carboxy-terminus of α_{i-3} which is the site of IAP-catalyzed ADP-ribosylation. A synthetic decapeptide variant of α_{i-2}, designated $\alpha_{i-1,2(arg,asn)}$, was also made in which arginine and asparagine replaced the lysine and aspartate located six and five residues from the carboxy terminus, respectively.[3] Antisera to the peptides linked to keyhole limpet he-

3. This corresponds to the carboxyterminus of a protein initially termed α_H (50), but which should be classified as α_{i-2} (51).

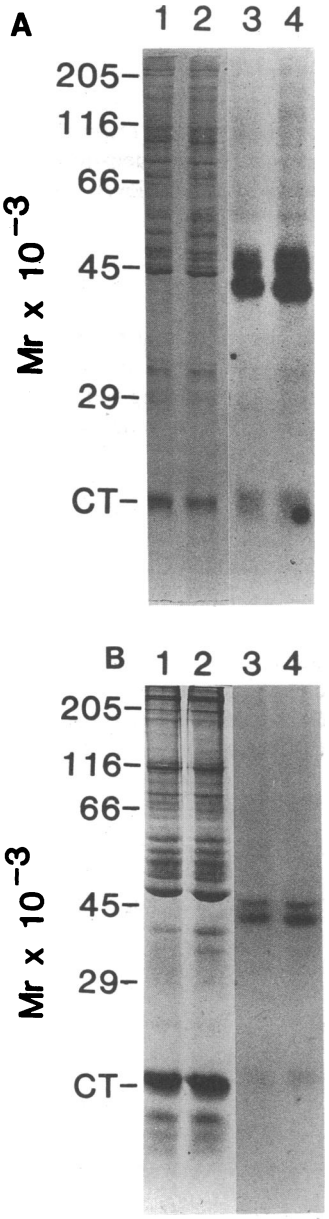

Figure 8. [^{32}P]ADP-ribosylation of liver plasma membrane proteins by cholera toxin A-subunit. Cholera toxin A subunit-catalyzed [^{32}P]ADP-ribosylation of liver plasma membranes was performed as described in Methods before polyacrylamide gel electrophoresis of SDS-solubilized proteins and autoradiography. (*A*) Lanes *1, 2* are Coomassie blue stained gels from an experiment which is representative of seven studies on separate membrane batches. Lanes *3, 4* are the corresponding autoradiograph. Lanes *1, 3* are Sprague-Dawley control membranes and lanes *2, 4* are membranes from STZ-diabetic animals. (*B*) Lanes *1, 2* are Coomassie blue-stained gels from an experiment representative of 4 on separate membrane batches and lanes *3, 4* are the corresponding autoradiograph. Lanes *1, 3* are DB-RES control membranes and lanes *2, 4* are membranes from BB/Wor-diabetic rats.

mocyanin (44) were raised in rabbits as described in Methods. This approach has been used for a number of G-proteins (16, 36, 37, 52).

Fig. 9 shows the peptide specificity and immunoreactivity of antisera prepared against four of these peptides α_{i-3}, α_o, α_s, and α_T. Antiserum 940, prepared against the α_{i-3} peptide showed similar reactivity, at dilutions of 1/100 or less, toward all of the peptides except α_s. At greater dilutions, antiserum 940 was less effective at recognizing α_T, α_o, and $\alpha_{i-1,2(arg,asn)}$ peptides compared to α_{i-3}, $\alpha_{i-3(ser)}$ and $\alpha_{i-1,2}$ (Fig. 9 *A*). Antiserum 939, prepared against the α_T peptide [identical to the peptide employed by Gawler et al. (16)] displayed a broad range of reactivities. The order of immunoreactivity for this antiserum, at dilutions of 1/100 to 1/1,000, against the various peptides tested was $\alpha_{i-1,2(arg,asn)} > \alpha_T \geq \alpha_{i-1,2} > \alpha_{i-3} > \alpha_{i-3(ser)} > \alpha_o > \alpha_s$ (Fig. 9 *B*). The poor reactivity of this antiserum to G_o is in agreement with the findings of Gawler et al. (16) using antiserum AS/7. However as exemplified by antiserum 918 (Fig. 9 *C*) antiserum raised against the α_s peptide was highly specific for this peptide relative to the others. Antiserum 929 raised against peptide α_o produced a weak reaction that was relatively specific for the α_o, α_{i-3}, and $\alpha_{i-3(ser)}$, but not the other peptides (Fig. 9 *D*).[4]

Fig. 10 shows the reaction of these antisera and preimmune serum with liver plasma membrane proteins separated by SDS-PAGE. Anti-α_o serum and preimmune serum did not react significantly in immunoblots with any proteins in the 38–49-kD range. The negative finding with anti-α_o serum is in agreement with studies indicating that G_o is poorly expressed in liver (37, 53, 54). In contrast, anti-α_{i-3} and anti-α_T sera showed strong reactivity towards a 41-kD protein. This was also true for anti-$\alpha_{i-1,2}$ and anti-$\alpha_{I-3(ser)}$ sera, whereas anti-$\alpha_{i-1,2(arg,asn)}$ serum recognized many proteins (all data not shown). There was also some reaction of the anti-α_T serum with proteins in the 45–48-kD range (Fig. 10), however this was inconsistently observed (e.g., Fig. 11). The anti-α_s serum reacted with two proteins of 43- and 47.5-kD weight (Fig. 10). These correspond to the two cholera toxin substrates found in rat liver plasma membranes and are presumed to be two species of α_s.

We observed no significant change in the level of the 41-kD protein detected by anti-α_T and anti-α_{i-3} sera in membranes from either STZ-diabetic rats (three different membrane batch preparations) or BB/Wor diabetic rats (five different mem-

*Table V. Insulin Replacement Reverses the Effect of STZ-Diabetes on Cholera Toxin-stimulated [^{32}P]ADP Ribosylation of G_s**

Experiment No.	Experimental group	^{32}P incorporated into G_s α-subunits	% Control
		cpm	
1	Control	1,242	100
	STZ-diabetic	1,602	129
	Insulin-replaced‡	1,360	110
2	Control	1,392	100
	STZ-diabetic	2,352	169
	Insulin-replaced	1,238	89
3	Control	969	100
	STZ-diabetic	1,508	156
	Insulin-replaced	1,350	139

* [^{32}P]ADP ribosylation in the presence of cholera toxin A subunit was performed as described in Methods. The 43–47.5 kD regions of the polyacrylamide gels were excised and the radioactivity measured by scintillation spectrometry. The mean of the distribution underlying the STZ-diabetic values from the three experiments shown is greater than that of control as determined by Student's *t* test (two samples with paired observations, $P < 0.05$). In contrast the insulin-replaced values were not found to be significantly different from control, $P > 0.05$).

‡ Insulin replacement was performed as described in Table IV legend.

4. We also raised peptide antisera to other regions of $G_{s\alpha}$, $G_{o\alpha}$, and $G_{i\alpha-2}$, and to a sequence common to many G-protein α-subunits (36, 37). However, in agreement with Mumby et al. (37), these cross-reacted too weakly with the rat liver plasma membrane proteins to be useful in quantitating the G-proteins.

Table VI. Fluoroaluminate-stimulable Cyc⁻-Reconstituting Activity of Cholate Solubilized Liver Plasma Membranes

Experiment No.	Experimental group (strain)	Cyc⁻ adenylate cyclase activity	% Control
		pmol cAMP/mg solubilized liver membrane protein/ 15 min	
1	Control (Sprague-Dawley)	371±19	100±5
	STZ-diabetic (Sprague-Dawley)	463±12‡	125±3‡
2	DB-RES Control (Wistar-derived)	1,575±94	100±6
	BB/Wor-diabetic (Wistar-derived)	2,038±58‡	129±4‡

* Cyc⁻ protein concentration was 0.38 and 0.55 mg/ml in experiments 1 and 2, respectively.
‡ Significantly higher than genetic control as determined by Student's *t* test ($P < 0.05$).

brane batch preparations) compared to the appropriate controls (Fig. 11). This was not due to limitations in quantitation since the amount of 41 kD protein detected was shown to be proportional to the amount of membrane protein loaded on to the gels (data not shown). These findings differ from those of Gawler et al. (16), who observed nearly complete loss of immunoreactivity to anti-α_T sera in STZ-diabetic rats (16). In contrast, immunoreactivity of the anti-α_s sera with the 43- and 47.5-kD regions of immunoblots was increased 145±12% and 162±20% in STZ- and BB/Wor-diabetic rats, respectively, compared to controls as determined by laser densitometry (Fig. 11). This supports the conclusion that G_s levels are increased in diabetic rat liver.

Discussion

Several groups have explored the possibility that the hepatic adenylate cyclase system is altered in diabetes (5, 8–18). Such an alteration seems likely since naturally occurring and experimental diabetes in rats have consistently been associated with an increase in hepatic cAMP content (3–6).

In order to investigate this problem, most groups have used the streptozotocin model of diabetes. Unfortunately, the results from these studies have been inconsistent. For example, it has been reported that glucagon receptor binding capacity in liver membranes or cells is either unchanged (9–11) or decreased (12–14) by experimental diabetes. Basal and glucagon-stimulated hepatic adenylate cyclase activity has been found to be either increased, decreased, or unchanged in STZ-diabetes (5, 10, 12, 14–18). Furthermore, GTP-sensitive adenylate cyclase or G_s-activity has been reported to be either

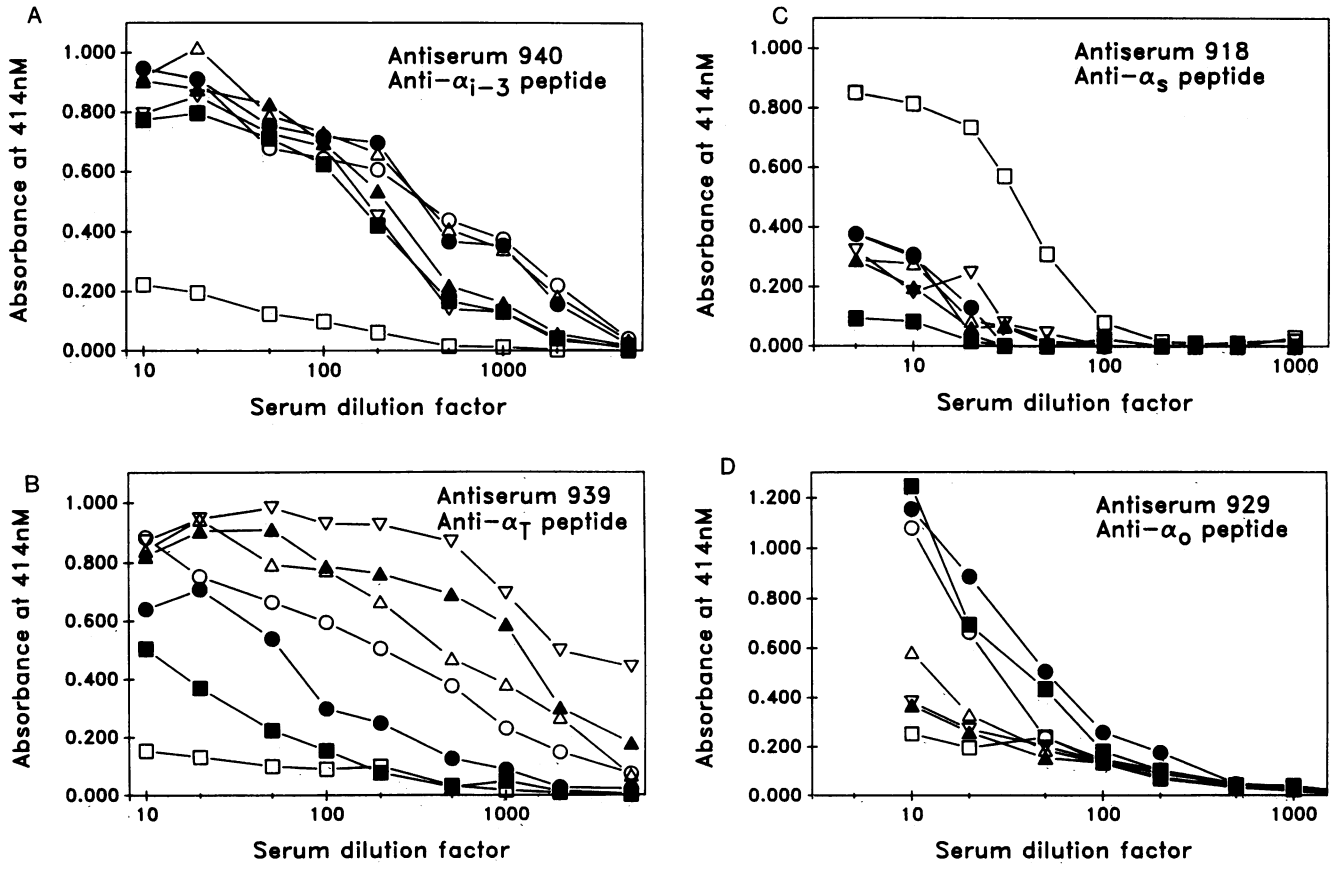

Figure 9. Specificity of antisera for various peptides. Each panel represents an ELISA experiment performed with a single antiserum from a rabbit injected with a decapeptide corresponding to the COOH-terminus of a specific G protein α-subunit (crosslinked to keyhole limpet hemocyanin). Each symbol represents the COOH-terminal decapeptide of the following G-protein α-subunits: ○, α_{i-3}; ●, $\alpha_{i-3(ser)}$; △, $\alpha_{i-1,2}$; ▲, α_T; □, α_s; ■, α_o; ▽, $\alpha_{i-1,2(arg,asn)}$. Each point is the average absorbance from duplicate determinations.

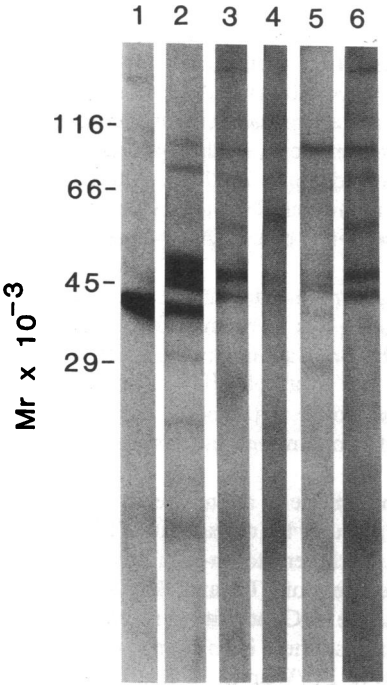

Figure 10. Immunoreactivity of antisera with liver plasma membrane proteins. 5 mg of SDS-solubilized liver plasma membrane protein were loaded on a 10% polyacrylamide gel fitted with a two-dimensional gel comb consisting of one 22 cm lane (sample) and a single 0.6 cm lane (^{14}C-protein molecular weight standards). Following separation by SDS-PAGE and transfer to nitrocellulose, the portion of the blot containing membrane protein was cut into 0.6–0.8 cm wide vertical strips and immunoblotted with antisera as follows: lane *1*, antiserum 940 (anti-α_{i-3} peptide, 1/100 dilution); lane *2*, antiserum 939 (anti-α_T peptide, 1/50 dilution); lane *3*, antiserum 927 (anti-α_s peptide, 1/50 dilution); lane *4*, antiserum 929 (anti-α_o peptide, 1/25 dilution); lane *5*, pooled pre-immune rabbit serum (1/50 dilution); lane *6*, antiserum 927 (anti-α_s peptide, 1/25 dilution). The labeled molecular weight markers are shown on the left. Autoradiographs were developed following overnight exposure at −70°C.

increased (15) or decreased (14). Alternatively, Gawler et al. (16) have provided evidence that G_i is abolished in both alloxan- and STZ-injected diabetic animals. One explanation for some of these discrepancies comes from the observation that STZ-treatment can decrease plasma membrane markers in liver homogenates and/or plasma membrane preparations (13, 14, 17, 18), although this too has been an inconsistent finding (Table I and references 5, 15). Another possible explanation for some of these inconsistencies is that the severity of the diabetic state varies with the dose and route of administration of diabetogenic agents. The hepatoxicity of this class of compounds may also account for some these differences (see 19 for review).

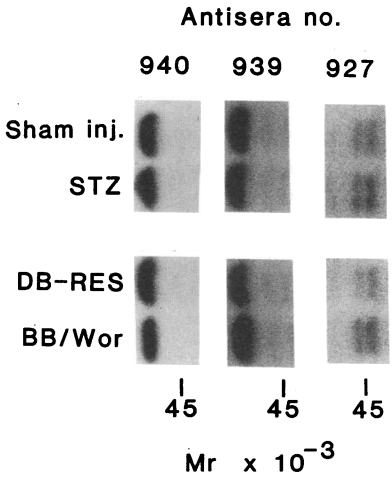

Figure 11. Immunoblots of liver plasma membranes from control and diabetic rats. Liver plasma membranes (150 μg) of from Sprague-Dawley control, STZ-diabetic or Wistar-derived DB-Res and BB/Wor diabetic rats were resolved by SDS-PAGE and transfered to nitrocellulose. The blots were treated with ^{125}I-labeled goat anti-rabbit IgG (2×10^5 cpm/ml) as described by Mumby et al. (36, 37) following incubation with peptide antisera at the following dilutions: antiserum 940 (anti-α_{i-3} peptide), 1/200; antiserum 939 (anti-α_T peptide), 1/200; antiserum 927 (anti-α_s peptide), 1/50 dilution. In this figure, only the 38–48-kD regions of the blots are shown. The position of [^{14}C]-ovalbumin is indicated. Autoradiographs were exposed overnight at −70°C.

In this study we sought to overcome these variables by examining the roles of G_s, G_i, and hepatic adenylate cyclase in both the STZ and BB/Wor-diabetes models. The BB/Wor model is a naturally occurring form of diabetes which is not subject to the problems inherent to the STZ model and may be more relevant to insulin-dependent diabetes mellitus in humans (for review see 20, 21).

STZ-diabetes. Since previous studies have not revealed increases in glucagon receptor number in liver cells or in liver membranes from diabetic animals (9–14), we hypothesized that the changes in hepatic cAMP content in STZ-diabetes (3–5) might be due to changes in the expression or activity of adenylate cyclase or of the G-proteins G_s and G_i, which regulate the activity of this enzyme. To differentiate between these possibilities we studied the effects of GTP, glucagon, forskolin, fluoroaluminate, cholera toxin, and IAP on adenylate cyclase activity.

A possible explanation of the increased adenylate cyclase responsiveness in the diabetic state is an increase in the catalytic activity of the enzyme. This appears unlikely for the STZ-diabetic rats, since there were no changes in basal (Figs. 1, 3, and Table II) or forskolin-stimulated (Table II) adenylate cyclase activity in these animals.

Another explanation is that the plasma membrane purification procedure leads to a greater enrichment of these membranes in the preparations from diabetic animals. However, we observed no significant change in the levels of plasma membrane markers, e.g., 5′nucleotidase activity, in the preparations from STZ-diabetic rats (Table I, references 5, 15; cf. 13, 14, 17, 18). Dighe et al. (14) reported that the loss of plasma membrane markers varied with the dose of STZ and presumably the severity of the diabetic state. While our animals were clearly diabetic (i.e., urine glucoses > 200 mg/dl and serum glucoses > 400 mg/dl), the diabetes may not have been severe enough to cause changes in 5′-nucleotidase and other membrane markers.[2]

Several differences were noted between the effects of IAP-treatment and STZ-diabetes on adenylate cyclase. Plasma membranes from both groups displayed an increased responsiveness to GTP, glucagon, and cholera toxin A subunit (Figs. 1, 2, 4). However, the increase was much larger in IAP-treated membranes. These results per se are consistent with the view that G_i activity is decreased though not abolished in diabetes (cf. 16). However, a number of findings do not support this conclusion as follows: (*a*) Angiotensin II inhibition of cAMP formation in membranes and of glucagon-elevated cAMP content in cells was not impaired by diabetes (Figs. 5–6), although previous studies have shown that these are G_i-mediated events (22, 46). (*b*) We observed no significant change in the amount of the α-subunit of G_i following streptozotocin treatment, as measured by [^{32}P]ADP ribosylation of the 41 kD α-subunit of G_i performed in the presence of IAP and repetitive addition of [^{32}P]NAD (Fig. 7). (*c*) We also detected no change in G_i when this was measured immunologically (Fig. 11).

This latter result is discrepant with that of Gawler et al.

(16), even though identical treatment protocols were employed, including dose of streptozotocin, treatment time, as well as species, strain, sex, and weight of the rats. We also consistently observed that diabetes did not alter basal adenylate cyclase activity, whereas they observed a 45–50% decrease which was not reversed by insulin treatment (cf. 14). We also found a greater maximal cyclase activity with glucagon in the diabetic membranes compared with the control membranes, whereas they observed the same maximal activity.

In the study of Gawler et al. (16) immunoblotting of crude rat liver plasma membranes with antiserum to the COOH-terminal decapeptide of α_T was performed to measure the α-subunit of G_i. In our study, we utilized antisera raised against this peptide and also the COOH-terminal decapeptide of $G_{i\alpha-3}$. Neither antiserum detected a difference in the $G_{i\alpha}$ content of purified liver plasma membranes from STZ rats (Fig. 11). We also employed IAP-stimulated ADP-ribosylation of purified liver membranes to measure G_i. Gawler et al. (16) and others (54) have argued that pertussis toxin should not be used for this purpose since it also recognizes T_d and G_o. However, these peptides can be differentiated by mass from G_i in SDS-polyacrylamide gels (39/40 vs. 41 kD for G_i). Furthermore, T_d is primarily expressed in retinal tissue, and G_o does not appear to be significantly expressed in liver (Fig. 10 and references 37, 53, 54). Pertussis toxin is also capable of identifying some forms of G_p (the G-protein linked to phosphatidylinositol 4,5-bisphosphate phosphodiesterase) in several tissues. However, in rat liver, G_p is not functionally modified by IAP (22, 23) suggesting that it is not a substrate.

Our data provide another explanation for the increased responsiveness of adenylate cyclase and hepatic cAMP content in STZ-diabetes, namely that there is an increase in G_s. This conclusion comes from the increase in cholera toxin stimulated [^{32}P]ADP ribosylation of the 43 and 47.5 kD α-subunits of G_s from membranes of diabetic rats (Fig. 8) the increase in cyc$^-$ reconstitutable G_s-activity (Table VI) and the increase in immunoreactivity of 43- and 47.5-kD proteins (presumed to be G_s α subunits) against antiserum raised to the COOH-terminal peptide of rat brain $G_{s\alpha}$ (Fig. 11). The ADP-ribosylation and the increased responsiveness of adenylate cyclase to cholera toxin A-subunit, were reversed by insulin therapy (Tables III and IV). An elevation of G_s is also consistent with all the hepatic adenylate cyclase data (Figs. 1–5).

BB/Wor-diabetes. Spontaneously diabetic animals also displayed increased adenylate cyclase activity relative to their Wistar-derived nondiabetic controls (DB-RES). In contrast to the STZ-diabetics, however, BB/Wor rats displayed approximately twofold increases in basal and forskolin-stimulatable adenylate cyclase. This increase accounted for the majority but not all of the increases in GTP, glucagon, fluoroaluminate, and cholera toxin-stimulated adenylate cyclase in these rats.

In agreement with the data from the STZ-model, BB/Wor-diabetics also displayed increased levels of G_s activity (Table VI) and cholera toxin stimulated [^{32}P]ADP ribosylation of the 43 and 47.5 kD liver membrane peptides, which are thought to represent the α-subunits of G_s (Fig. 8). There was also an increase in these subunits when measured immunologically using antiserum against the COOH-terminal decapeptide of $G_{s\alpha}$ (Fig. 11).

We also observed a 40% decrease in the IAP-stimulated [^{32}P]ADP-ribosylation of G_i in BB/Wor-diabetics. In contrast, no decrease in $G_{i\alpha}$ could be detected by immunological means using antisera to the COOH-terminal decapeptides of α_{i-3} and α_T (Fig. 11). This suggests that there is no change in the amount of $G_{i\alpha}$ but that its susceptibility to ADP-ribosylation is changed. This may indicate altered association between the various subunits of heterotrimeric G_i in this naturally occurring model of acute type I diabetes mellitus. Alternatively, there may be a decrease in another G protein which does not cross-react with the antisera, but can be ADP-ribosylated by the toxin.

Pobiner et al. (46) have reported that rat liver contains "spare" G_i, i.e., > 80% of G_i has to be modified before IAP affects angiotensin II inhibition of adenylate cyclase (46). Thus a 40% decrease in G_i in the livers of BB/Wor spontaneous diabetic rats, if it occurs, would not modify angiotensin II inhibition of hepatic cAMP content and membrane adenylate cyclase activity.

In summary, while an increase in adenylate cyclase per se seems to be the major contributor to increases in GTP-, glucagon-, fluoroaluminate-, and cholera toxin-stimulated adenylate cyclase activity in the livers of STZ and BB/Wor rats, it appears likely that the change in G_s-activity is responsible for part of the increase. In contrast, there is no change in G_i in the STZ rat and the decrease in BB/Wor G_i, if it occurs, is not sufficient to be physiologically relevant. With respect to the increase in hepatic cAMP observed in experimental diabetes (3–5), it should be recognized that decreased cAMP phosphodiesterase may also contribute (5). The livers of BB/Wor rats also have elevated cAMP levels (6), but it is not known whether or not the phosphodiesterase is changed.

The alterations in molecular components of the adenylate cyclase system in the livers of diabetic rats raise a number of interesting issues. The first is the mechanism(s) by which the changes occur, i.e., the site at which insulin controls the expression of these proteins. The second is whether or not these components are altered in other tissues in diabetes, e.g., adipose tissue. The third is the possible relationship between the increased responsiveness of adenylate cyclase to glucagon (and other agonists) and the metabolic dysfunctions of diabetes, e.g., enhanced lipolysis and gluconeogenesis. All of these issues represent interesting areas for future research.

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