Impaired Contractile Response to Beta Adrenoceptor Stimulation in Diabetic Rat Hearts: Alterations in Beta Adrenoceptors-G Protein-Adenylyl Cyclase System and Phospholamban Phosphorylation

SATOSHI GANDO, YUICHI HATTORI, YASUHIRO AKAISHI, JUN NISHIHIRA and MORIO KANNO
Department of Pharmacology (S.G., Y.H., Y.A., M.K.) and Central Research Institute (J.N.), Hokkaido University School of Medicine, Sapporo 060, Japan
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ABSTRACT
The aim of this study was to explore the cellular mechanisms underlying the impaired contractile response to beta adrenoceptor stimulation in diabetic hearts. Chronic diabetes was induced in rats by a streptozotocin injection. Four to six weeks later, papillary muscles isolated from diabetic hearts exhibited marked reductions in the positive inotropic responses to isoproterenol, norepinephrine and epinephrine. The contractile responses to forskolin, 3-isobutyl-1-methylxanthine and dibutylic cyclic AMP were also prominently depressed. The density of beta adrenoceptors was decreased by 50%. However, competitive binding studies with isoproterenol showed no difference in the proportion of beta adrenoceptors with high-affinity binding between control and diabetic myocardial membranes. Determination of the levels of the alpha subunits of G_s and G_i by immunoblotting revealed markedly less expression of G_i in diabetic myocardium. The abilities of isoproterenol, sodium fluoride, 5’-guanylyl imidodiphosphate and forskolin to stimulate adenylyl cyclase were preserved well in membranes prepared from diabetic hearts. Nevertheless, neither stimulation of beta adrenoceptors with isoproterenol nor direct activation of adenylyl cyclase with forskolin evoked any significant increase in the degree of phosphorylation of phospholamban in diabetic hearts. These results suggest that impaired contractile response to beta adrenoceptor stimulation is not caused by an alteration in the beta adrenoceptors-G_s-adenylyl cyclase system, but is possibly caused by an alteration in cellular function beyond the step of adenylyl cyclase activation.

Endogenous catecholamines play an important role in regulating myocardial function. The receptors through which catecholamines exert their actions on myocardium are predominantly of beta adrenoceptors. There is evidence for impaired cardiac responsiveness to beta adrenoceptor stimulation in experimental animals with diabetes mellitus. This has been shown in isolated hearts (Vadlamudi and McNeill, 1984) as well as in atrial and ventricular muscles (Heyliger et al., 1982; Goyal et al., 1987; Sato et al., 1989). In the patients with diabetes mellitus, the frequent occurrence of cardiomyopathy, which is characterized as heart failure independent of atherosclerotic coronary artery disease, valvular disease or hypertension, is well established (Kannel et al., 1974). One of the important features found in the failing human heart is the decreased response to beta adrenoceptor stimulation (Bristow et al., 1982). Thus, cardiac dysfunction in diabetes documented by numerous clinical studies may involve alterations in beta adrenoceptor-mediated cardiac response.

The mechanisms contributing to diminished beta adrenoceptor stimulation have been extensively investigated, but the results of these studies are not always consistent. In diabetes, the norepinephrine concentration in myocardium has been found to increase (Paulson and Light, 1981; Fushimi et al., 1984; Ganguly et al., 1986), to decrease (Neubauer and Christensen, 1976) or to be unchanged (Kaul and Grewal, 1980; Akiyama et al., 1989). Yoshida et al. (1985, 1987) have reported that norepinephrine turnover is depressed in diabetic rat hearts, whereas Ganguly et al. (1986) have shown an increased turnover, uptake and synthesis of norepinephrine in diabetic hearts. In accordance with the diminished functional responses, reductions in the number of myocardial beta adrenoceptors have been shown in many

ABBREVIATIONS: ICYP, iodocyanopindolol; GppNHP, 5’-guanylyl imidodiphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IBMX, 3-isobutyl-1-methylxanthine; PVDF, polyvinylidene difluoride filter; DBcAMP, dibutylic cyclic AMP; SR, sarcoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid.
prior studies (Savarese and Berkowitz, 1979; Heyliger et al., 1982; Williams et al., 1983; Sundaresan et al., 1984; Atkins et al., 1985; Bitar et al., 1987; Nishio et al., 1988; Sato et al., 1989). A functional uncoupling of myocardial beta adrenoceptors from adenylyl cyclase activation or altered G protein function has also been demonstrated (Guttsche, 1983; Atkins et al., 1985; Cros et al., 1986; Wielchau et al., 1994). However, there appears to be no consistency regarding the onset time of alterations in beta adrenoceptors and their signal transducing systems. Furthermore, one report indicates that the decrease in the number of myocardial beta adrenoceptors does not necessarily result in an altered beta adrenoceptor-mediated response of diabetic myocardium (Durante et al., 1989). Thus, the exact nature of linkage between the functional depression in the cardiac responses to catecholamines and the reduced number of beta adrenoceptors or their uncoupling from the succeeding signal transducing systems in diabetes has not been clearly defined.

The purpose of the present study was to clarify the mechanisms underlying the diminished positive inotropic response to beta adrenoceptor stimulation found in experimental diabetic rat hearts. We characterized changes in the myocardial beta adrenoceptors-G protein-adenylyl cyclase system in rats with streptozotocin-induced diabetes. Our current study evaluating the agonist-independent activation of myocardial adenylyl cyclase and the amounts of cardiac G proteins will provide information about whether postreceptor elements are altered in diabetic myocardium. We also determined whether the levels of phosphate labeling of phospholamban in intact hearts during beta adrenoceptor stimulation are altered in diabetes, which would serve as a valuable step in identifying the cellular locus for reduced contractile function of diabetic myocardium in response to beta adrenoceptor stimulation.

Materials and Methods

Induction of diabetes. Male Wistar rats, 8 weeks old and 200–250 g in body weight, were randomly assigned to two groups. One group of rats (diabetic group) received a single tail-vein injection of streptozotocin (45 mg/kg) under light anesthesia with diethyl ether. Streptozotocin was dissolved in a citrate solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.5). Another group (control group), the membrane suspension (100 μg) was filtered through a single layer of cheesecloth and retained. The preparations were allowed to equilibrate for at least 60 min before the experiments were begun.

Membrane preparation. Control and diabetic rats were sacrificed as stated above. Their hearts were removed and rinsed in ice-cold Tris-HCl buffer. Ventrices were dissected free of connective tissue, fat, major vessels and atria. The tissues were minced with scissors and homogenized in 5 volumes of ice-cold Tris-HCl buffer by use of a polytron for 15 s. The buffer composition (pH 7.4, 4°C) was (mM): Tris-HCl, 75; MgCl2, 25; EDTA, 5; ethyleneglycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid, 1. The homogenates were centrifuged at 1,000 × gmax for 10 min at 4°C. The supernatant was filtered through a single layer of cheesecloth and retained. The pellet was suspended in 5 volumes of cold Tris-HCl buffer and centrifuged again. Membrane fractions in the supernatant were concentrated by centrifugation at 100,000 × gmax for 30 min at 4°C. The final pellets were resuspended in cold Tris-HCl buffer and stored at −80°C until used. Protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Radioiodin binding study. Beta adrenoceptors were identified by use of the radioligand (-)-[125I]ICYP (2147–2291 Ci/mmol, New England Nuclear, Boston, MA) in saturation isotherm experiments described previously (Hattori et al., 1987). The membrane fractions were diluted further in an incubation medium (Tris-HCl, 75 mM; MgCl2, 25 mM, pH 7.4) to give a final protein concentration of 0.5 to 1.0 mg/ml. [125I]ICYP and all drugs used in this study were prepared in the incubation medium. For saturation experiments, an aliquot of the membrane suspension (100 μl) was incubated with various concentrations of [125I]ICYP (12.5–1600 pM) in a final volume of 200 μl. Agonist competition experiments were determined by incubation of 100 pM [125I]ICYP with increasing concentrations of isoproterenol (10 pM–1 mM) in the absence or presence of 100 μM Gpp(NH)p. Incubations were carried out for 30 min at 37°C and terminated by adding 5 ml of the cold incubation medium (4°C) to the incubation mixture, followed by a rapid filtration over Whatman GF/C glass fiber filters. Each filter was washed three times with an additional 5 ml of the incubation medium (4°C). The radioactivity of the wet filters was determined in a gamma counter at an efficiency of 75%. All values in binding experiments are the average of triplicates. Nonspecific binding was defined as binding in the presence of 10 μM propranolol.

Adenylyl cyclase assay. Adenylyl cyclase activity was determined in an assay that monitors the conversion of [α-32P]ATP to [γ-32P]cyclic AMP according to the method of Salomon et al. (1974). The incubation mixture contained 40 mM Tris-HCl (pH 7.5), 0.05 mM cyclic AMP, 0.05 mM ATP, an ATP-regenerating system (5 mM creatine phosphate and 50 U/ml creatine phosphokinase), 0.25 mg/ml bovine serum albumin, 0.5 mM IBMX, 5 mM MgCl2, 1 mM dithiothreitol, 1 U/ml adenosine deaminase, [α-32P]ATP (1 μCi per assay; 30 Ci/mmol, New England Nuclear, Boston, MA) and membrane protein (50–100 μg per assay). Various agents to stimulate adenylyl cyclase activity were included in the incubation mixture. The final volume was 100 μl. Assays were performed in triplicate for frequency, 5 ms in duration and 1.5 times the threshold voltage delivered by a pair of spiral platinum electrodes connected to an electronic stimulator (Sanei-Sokki, RTB1202, Tokyo, Japan) through an isolation unit (Sanei-Sokki, 5361). Isometric tension developed in the preparation was measured with a force transducer (Sanei-Sokki, RTB1202) and recorded on a thermal array recorder (Nihon Kohden, RTA-1200, Tokyo, Japan) through a preamplifier (Nihon Kohden, RP-5). The preparations were allowed to equilibrate for at least 60 min before the experiments were begun.

The concentration-response curves for the positive inotropic effects of isoproterenol, norepinephrine and epinephrine were determined in a cumulative manner by increasing the concentrations in steps of 0.5 log units. The EC50 value, i.e., the concentration required to produce 50% of the maximal response induced by the agonist, was determined from log-probit plots of the individual response versus concentrations and was expressed as the negative logarithm (pD2 value). Only one full concentration-response curve was made per preparation.

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Organ bath experiments. Four to six weeks after treatment with streptozotocin or buffer, rats were anesthetized with diethyl ether. The hearts were rapidly excised and transferred to a dissection bath filled with oxygenated Krebs-Henseleit solution at room temperature. The composition of the solution (pH 7.4) was (mM): NaCl,119; KCl, 4.8; CaCl2, 1.3; MgSO4, 1.2; KH2PO4, 1.2; NaHCO3, 24.9; glucose, 10.0. The left ventricular papillary muscles were carefully dissected from the hearts. The muscle was mounted under 0.5 g of resting tension in a water-jacketed organ bath containing 10 ml of Krebs-Henseleit solution. We confirmed that this resting tension produced 90% maximal force development in papillary muscles from both control and diabetic animals, based on resting tension/developed tension curves. The solution in the bath was bubbled with 95% O2 and 5% CO2, and its temperature was maintained at 35 ± 1°C. The muscle was stimulated by rectangular pulses of 1 Hz in

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10 min at 37°C, and the results are expressed as picomoles of cyclic AMP per milligram of protein per 10 min. The assay was linear with regard to time and to protein concentration. Isolation of [32P]cyclic AMP was accompanied by sequential Dowex and Alumina chromatography with use of [3H]cyclic AMP (26 Ci/mmol; Amersham, London, England) as a recovery marker. The average recovery of cyclic AMP was about 60%.

Assessment of Gs and Gi. Membrane proteins were dissolved in an equal volume of sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.0025% bromphenol blue, boiled at 100°C for 2 min and subjected to SDS-PAGE on 10% polyacrylamide gel according to the procedure of Laemmli (1970).

Immunoblot analysis was performed by electrophoretic transfer of proteins onto a PVDF. The PVDF was blocked for 4 h at room temperature in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20, pH 7.5) containing 4% nonfat dry milk. Thereafter, the PVDF was incubated overnight with specific rabbit antisera (RM1, recognizing Gsa; AS7, recognizing Giα2, and Giβ) at 1:750 dilution in blocking solution. After extensive washing with blocking solution, the PVDF was incubated with goat anti-rabbit colloidal gold conjugate solution diluted at 1:25 in dilution buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% bovine serum albumin, 0.02% NaN3, 0.05% Tween 20, 0.4% gelatin, pH 9.0) at room temperature overnight. Immunolabeled G proteins and the intensity of each specific band were analyzed by free software NIH image produced by Wayne Rasband. (National Institutes of Health, Bethesda, MD).

32P labeling of phospholamban in perfused heart. The hearts were taken from control and diabetic rats and perfused with Krebs-Henseleit buffer by the Langendorf technique as described previously (Gando et al., 1995). The composition of Krebs-Henseleit buffer (pH 7.4) was (mM): NaCl, 119; CaCl2, 1.3; KCl, 4.8; MgSO4, 1.2; KH2PO4, 0.234; NaHCO3, 27.2; glucose, 10.0. The buffer was gassed with 95% O2 and 5% CO2, and the temperature of perfusate was kept constant at 37°C. After 10 min of perfusion, the circuit was switched to a recirculating system containing 40 ml of the same buffer to which 1.5 mCi of [32P]ATP (30 Ci/mmol; Amersham, London, UK) was added. After 10 min of perfusion with the radioactive buffer, the circuit was returned to the drip-through system with use of the nonradioactive buffer. The hearts were then perfused with the nonradioactive buffer for 2 min and were challenged with 100 nM isoproterenol, 10 μM forskolin or vehicle buffer for 4 min. At the end of the challenge, the atria were removed, and the hearts were immediately frozen with clamps which had been cooled with liquid nitrogen. The frozen samples were stored under liquid nitrogen until further analysis. The specific activity of [γ-32P]ATP determined for each heart and was expressed as picomoles of [32P] incorporation per milligram of protein.

Plasma and tissue catecholamine assay. Blood samples and ventricular tissues were obtained from control and diabetic rats. The tissues were immediately frozen with clamps which had been cooled with liquid nitrogen. The frozen samples were weighed and then homogenized in 2.5 ml of 0.4 N HClO4 containing 2 mM EDTA by means of a microhomogenizer (Niti-on, Tokyo, Japan) for 30 s. The homogenate was centrifuged at 15,000 × gmax for 10 min at 4°C. The pellet was again centrifuged after the addition of 2.5 ml of 0.4 N HClO4 containing 2 mM EDTA. The supernatant was collected and then assayed for the determination of norepinephrine. The norepinephrine levels in plasma and myocardium were determined by a high-pressure liquid chromatographic procedure, and were expressed as norepinephrine per ml of plasma and per gram of wet tissue.

Chemicals. Streptozotocin, l-isoproterenol hydrochloride, l-epinephrine bitartrate, dl-propranolol hydrochloride and IBBMX were purchased from Sigma Chemical Co. (St. Louis, MO). l-Norepinephrine bitartrate was purchased from Wako Pure Chemical Industries (Osaka, Japan), endothelin-1 (human) was from Peptide Institute, Inc. (Osaka, Japan), Gpp(NH)p was from Boehringer Mannheim GmbH (Mannheim, Germany) and forskolin was from Research Biochemicals (Natick, MA). DBcAMP was a gift of Duichi Pharmaceutical Co. (Tokyo, Japan), and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl)pyridine-5-carboxylate) was from Bayer AG (Leverkusen, Germany). All materials for SDS-gel electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA) or Wako. Other chemicals used in this study were of the highest purity available from Sigma, Wako or Nacalai Tesque, Inc. (Kyoto, Japan).

Data analysis and statistics. All values are presented in terms of means ± S.E. Comparisons of variables obtained by various treatments with basal values were made by a one-way analysis of variance with a repeated measures design, and if any significant difference was found the Scheffe’s multiple comparison test was applied. Student’s t test was used to make comparisons between control and diabetic groups. Nonparametric data were analyzed by the Mann-Whitney U test or Wilcoxon signed-rank test. A P value < .05 was considered statistically significant.

In the radioligand binding assay, the equilibrium dissociation constant (Kd) and the maximum binding capacity (Bmax) were determined by Scatchard analysis. Analysis of the curve for the isoproterenol-induced displacement of [3H]ICYP by a nonlinear curve-fitting method was performed by use of the LIGAND program.

Results

General features of animals. The general features of diabetic rats and age-matched control animals are summarized in table 1. All rats injected with streptozotocin developed severe diabetes as indicated by increased serum glucose levels (range, 565–615 mg/dl). Serum glucose levels in diabetic rats were elevated approximately 3.5-fold as compared with controls; the seemingly high values in controls may reflect the sympathetic response to ether anesthesia. Body

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TABLE 1

<table>
<thead>
<tr>
<th>Control (n = 52)</th>
<th>Diabetic (n = 52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>298 ± 2</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>185 ± 2</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>168 ± 2</td>
</tr>
</tbody>
</table>

a All values are expressed as means ± S.E.

* P < .001 vs. the corresponding control values.
weights and heart weights were significantly lower in diabetic rats than in age-matched control animals. However, the heart weight to body weight ratios were slightly higher in diabetic rats (0.62 vs. 0.80 mg/g).

**Positive inotropic responses to beta adrenoceptor agonists and other agents.** The basal force of contraction of left ventricular papillary muscles isolated from diabetic rats (326 ± 36 mg; n = 52) was not significantly different from that of muscles from age-matched control animals (262 ± 24 mg; n = 52). Isoproterenol, norepinephrine and epinephrine all produced a concentration-dependent increase in force of contraction in both control and diabetic papillary muscles (fig. 1). However, the positive inotropic effects of these agonists were markedly depressed in diabetic papillary muscles. The sensitivities of the muscles to these agonists were essentially the same in the two groups. Thus, the pD₂ values for isoproterenol, norepinephrine and epinephrine were 7.79 ± 0.01, 6.26 ± 0.10 and 5.98 ± 0.16 in the control group, and 7.72 ± 0.11, 6.67 ± 0.45 and 5.92 ± 0.13 in the diabetic group, respectively (n = 6–8).

As shown in figure 2, the increases in force of contraction elicited by 10 μM forskolin, 100 μM IBMX and 3 mM DBCAMP were also significantly less in diabetic papillary muscles than in control papillary muscles. On the other hand, the Ca²⁺ channel agonist Bay K 8644 (100 nM) produced a small but significant increase in force of contraction, and there was no significant difference in the maximum response between papillary muscles from control and diabetic animals (26 ± 8 vs. 34 ± 12%, n = 4 for each group). In addition, the positive inotropic effect of 100 nM endothelin-1 observed in diabetic muscles (33 ± 4%; n = 8) was not significantly different from that in controls (35 ± 9%; n = 8).

**Beta adrenoceptor binding.** The binding of [¹²⁵I]ICYP to myocardial membranes was saturable and of high affinity in both control and diabetic animals. Scatchard analyses of the data resulted in a straight line, which indicates binding to a single class of sites. The number of beta adrenoceptors was significantly lower in myocardial membranes from diabetic rats (78 ± 6 fmol/mg protein; n = 13) compared with controls (138 ± 16 fmol/mg protein; n = 11, P < .01). The pKᵦ values for [¹²⁵I]ICYP were similar in control and diabetic animals (10.05 ± 0.05 vs. 9.97 ± 0.05).

Agonist competition curves with isoproterenol were biphasic in both control and diabetic myocardium (fig. 3). Further analyses of the binding data by the nonlinear curve fitting program LIGAND clearly revealed two binding sites for isoproterenol in both myocardia. The affinities for isoproterenol at these two binding sites were similar in control and diabetic myocardium. Thus, the pKi values of the high-affinity site were 8.94 ± 0.15 and 8.85 ± 0.21, and the pKi values of the low-affinity site were 6.74 ± 0.10 and 6.65 ± 0.08 in control and diabetic myocardium, respectively (n = 6 for each group).

**Fig. 1.** Concentration-response curves for the positive inotropic effects of isoproterenol (A), norepinephrine (B) and epinephrine (C) in papillary muscles from control (●) and diabetic (○) rats. Points (means ± S.E., n = 6–8) represent the net increases in force of contraction expressed as a percentage of basal values recorded before the addition of the agonists. *P < .05, **P < .01, ***P < .001 vs. the corresponding control values.

**Fig. 2.** Effects of 10 μM forskolin, 3 mM DBCAMP and 100 μM IBMX on force of contraction in papillary muscles from control (open bars) and diabetic (hatched bars) rats. Bars (means ± S.E., n = 5–7) represent the net increases in force of contraction expressed as a percentage of basal values recorded before the addition of the agonists. **P < .01, ***P < .001 vs. the corresponding control values.
Furthermore, the proportion of high-affinity binding sites was 37 ± 5% in diabetic myocardium, a value which was not significantly different from that obtained in control myocardium (40 ± 4%). With the addition of 100 μM GppNHp, the curves for both control and diabetic myocardium moved to the right and were best fit to a single low-affinity site model with no high-affinity site (fig. 3).

Adenylate cyclase activity. Basal adenylate cyclase activity in myocardial membranes prepared from diabetic animals was significantly higher than in those from controls (199 ± 13 vs. 135 ± 14 pmol cyclic AMP/mg protein/10 min, n = 6 for each group, P < .01). In the following text, adenylate cyclase activity stimulated with various agents is therefore expressed as a net increase in stimulation (basal subtracted) to adjust for differences in basal activity. Isoproterenol-stimulated adenylate cyclase activity was similar in control and diabetic myocardium through a wide range of isoproterenol concentrations (fig. 4). Stimulation of adenylate cyclase activity with sodium fluoride or GppNHp was higher in diabetic myocardium than in control myocardium (fig. 5). Similarly, adenylate cyclase activity stimulated directly with forskolin was significantly increased in diabetic myocardium (fig. 5).

Assessment of Gs and Gi. To determine whether the changes in adenylate cyclase activity in diabetic myocardium are attributable to an altered balance between Gs and Gi, we measured the levels of these G proteins in myocardial membranes from control and diabetic rats by Western blotting with specific antibodies. Figure 6A shows a representative immunoblot in which lanes 1 and 2 each contained membrane proteins prepared from control and diabetic rat hearts. The Gs antiserum identified three particular protein bands in control myocardial membranes; the lowest band had an apparent molecular mass of 45 kdaltons, the intermediate band of 47 kdaltons and the highest band of 52 kdaltons. Diabetes affected strikingly differently the three Gs signals. The 45-kdalton band was not detectable in diabetic myocardial membranes. The 47-kdalton band for diabetic membranes was similar to control. The 52-kdalton band revealed a slight but significant decrease of 29 ± 6% (n = 4) compared with the control in diabetes (fig. 6B). However, the total amount of Gsual was the same in control and diabetic myocardial membranes (81 ± 8% of control, n = 4, P > .2). The antiserum AS/7 had approximately equal affinity for G11a, and G2a, and little cross-reactivity with G13a (Simonds et al., 1989). As presented in figure 6A, it identified one single
proteins with a molecular mass of 40/41 kdaltons, which is referred to as $G_{i2,0}$. We found a markedly lower level of the $G_{i2,0}$ protein in diabetic myocardial membranes (Fig. 6B). Quantitative analysis of immunoblots showed a decrease in $G_{i2,0}$ by 65 ± 6% ($n = 4$, $P < .001$).

**Phospholamban phosphorylation.** Figure 7A shows a representative autoradiogram of membrane vesicles prepared from control rat hearts after $^{32}$P incorporation into phospholamban in control and diabetic hearts. Points (means ± S.E., $n = 6$) represent the net increases in cyclic AMP production (basal subtracted). $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs. the corresponding control values.

In the present study, the density of myocardial beta adrenoceptors was reduced by 50% in diabetic rats compared with controls, whereas the affinity for the antagonist $[^{125}I]ICYP$ was unchanged. This finding is in good agreement with many reports of diminished myocardial beta adrenoceptor numbers, with no change in affinity, in streptozotocin-induced diabetic rats. Furthermore, we found that the positive inotropic effects elicited by forskolin, IBMX and DBcAMP were also attenuated in diabetic papillary muscles. On the other hand, diabetic muscles responded normally to Bay K 8644 and endothelin-1, both of which are known to produce a positive inotropic effect in a manner independent of intracellular cyclic AMP (Thomas et al., 1985; Hattori et al., 1993). It thus appears that the cyclic AMP-related positive inotropic effects are specifically impaired in diabetic rat myocardium.

**Discussion**

In accordance with prior studies showing a decreased contractile responsiveness to beta adrenoceptor stimulation in diabetic myocardium (Heyliger et al., 1982; Vadlamudi and McNeill, 1984; Goyal et al., 1987; Sato et al., 1989), the positive inotropic responses to isoproterenol, norepinephrine and epinephrine were markedly diminished in papillary muscles from streptozotocin-induced diabetic rats. Furthermore, we found that the positive inotropic effects elicited by forskolin, IBMX and DBcAMP were also attenuated in diabetic papillary muscles. On the other hand, diabetic muscles responded normally to Bay K 8644 and endothelin-1, both of which are known to produce a positive inotropic effect in a manner independent of intracellular cyclic AMP (Thomas et al., 1985; Hattori et al., 1993). It thus appears that the cyclic AMP-related positive inotropic effects are specifically impaired in diabetic rat myocardium.

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In our diabetic models, however, normal levels of plasma and myocardial norepinephrine were measured. Thus, it appears that chronic activation of beta adrenoceptors is not the sole expla-
nation for beta adrenoceptor down-regulation in diabetic myocardium, or at least that the mechanisms by which heart failure results in myocardial beta adrenoceptor down-regulation are not operative in diabetes.

It has been reported that in vivo insulin treatment is capable of reversing the altered inotropic response to myocardial beta adrenoceptor stimulation in streptozotocin-induced diabetic rats (Fein et al., 1981; Pfaffman, 1980; Ramandham et al., 1987). Furthermore, reversal of reduction in the number of beta adrenoceptors has been observed after insulin replacement (Ramandham et al., 1983, 1987; Williams et al., 1983). These reports suggest that streptozotocin-induced alterations in myocardial beta adrenoceptor responsiveness and number are a consequence of the resulting diabetic state and independent of direct cardiotoxic effects of streptozotocin.

The key finding in the present radioligand binding study is that the proportion of beta adrenoceptor binding agonist with high affinity, determined by competitive binding with isoproterenol, was not changed in diabetic myocardium. The high-affinity state of the receptors is believed to be the physiologically relevant form of the receptors, because they are functionally coupled to Gs (DeLean et al., 1980). The implication of this finding is that coupling of beta adrenoceptors with Gs is not impaired in diabetic myocardium. Inasmuch as the estimated numbers of high-affinity binding sites were approximately 55 and 29 fmol/mg protein in control and diabetic myocardium, respectively, one may argue that the less pronounced inotropic response to beta adrenoceptor stimulation in diabetic myocardium compared with control is, at least in part, caused by the involvement of a smaller number of high-affinity binding sites in diabetes. However, the functional experiments with forskolin, IBMX, and DBcAMP show that even when beta adrenoceptors and adenylate cyclase were bypassed, diabetic myocardium still exhibited an impairment of the inotropic response. Taken together, the results suggest that a potential defect in the inotropic responsiveness to beta adrenoceptor stimulation may reside at the level distal to beta adrenoceptors rather than the level of the receptors.

Diminishment of myocardial beta adrenoceptor responses
in diabetes may occur by altered G protein expression. Increased Gs expression may have mitigated adenylate cyclase activity, and thus contributed to the diminished inotropic response to beta adrenoceptor stimulation. However, the assessment of Gi by immunochemical quantification with antisera revealed a marked decrease of 65% in diabetic myocardium compared with control myocardium. This is consistent with the data of Wichelhaus et al. (1994), who reported a significant reduction in Gi expression in cardiomyocytes from diabetic rats as determined by immunoblot analysis. In contrast, Nishio et al. (1988) have shown that pertussis toxin-dependent ADP-ribosylation is increased in myocardial membranes from diabetic rats. Our pertussis toxin-in-mediated ADP-ribosylation labeling method also indicated an increased level of Gi in diabetic myocardium (Gando, Hattori and Kanno, unpublished data). It should be noted that pertussis toxin-mediated ADP-ribosylation depends on many cofactors and is very sensitive to changes in assay conditions (Bohm et al., 1991). This may account for conflicting data regarding amounts of myocardial Gi in diabetes. However, the pertussis toxin labeling may represent a functional property of Gi (Insel and Ransnas, 1988), because the toxin-mediated labeling of Gi provides information on the protein available for ADP-ribosylation. Although the level of the Gi12 protein was shown to decrease in diabetic myocardium in this study, if shifts in expression of Gi isoforms could occur in diabetes, it might have affected the measurement of the total ribosylatable substrate. Reduced levels of Gi may have contributed to the diminished contractile response to beta adrenoceptor agonists in diabetic myocardium. The reported alterations of myocardial Gi in diabetes are controversial depending on the assay methods (Nishio et al., 1988; Wichelhaus et al., 1994). In the present investigation, three different Gi alpha subunits with molecular masses of 45, 47 and 52 kdaltons were detected in rat ventricular myocardium. The influences of diabetes on each of these individual bands were clearly different. However, the total level of myocardial Gi was unaltered in diabetes. Therefore, it is concluded that the levels of myocardial G proteins may not be a key component of the decreased inotropic responsiveness to beta adrenoceptor stimulation in diabetes.

To further study the mechanisms underlying the decreased beta adrenoceptor-mediated functional responsiveness, the ability of isoproterenol to stimulate adenylate cyclase was examined in membranes prepared from both control and diabetic myocardium. The findings of decreased inotropic responsiveness and reduced beta adrenoceptor density led us to expect that beta adrenoceptor-mediated stimulation of adenylate cyclase would be decreased in myocardial membranes from diabetic rats. Instead, to our surprise, we found that isoproterenol produced a similar increase in adenylate cyclase activity in myocardial membranes from control and diabetic rats. This may be associated with a preserved proportion of high-affinity binding sites for a beta adrenoceptor agonist, which implies a tight coupling of beta adrenoceptors to Gi in diabetic myocardium. Some reports suggest a defect in the coupling of beta adrenoceptors to adenylate cyclase in diabetic myocardium (Gotzsche, 1983; Atkins et al., 1985; Wichelhaus et al., 1994). At the present time, we do not have a clear understanding of this discrepancy, but our results are consistent with the concept proposed by other investigators (Ingebretsen et al., 1983; Vadlamudi and McNeill, 1983; Smith et al., 1984) that coupling of myocardial beta adrenoceptors with adenylate cyclase is unaltered in diabetes. Furthermore, our results demonstrated that basal adenylate cyclase activity as well as activity in the presence of sodium fluoride, GppNHp and forskolin were significantly enhanced in diabetic myocardium. This most probably reflects a defect beyond the level of adenylate cyclase is associated with impaired inotropic responsiveness in diabetes.

In isolated perfused rat hearts, stimulation of beta adrenoceptors with isoproterenol and direct adenylate cyclase activation with forskolin significantly increased phosphorylation of phospholamban in SR. No significant increase in this phosphorylation was evident in diabetic rat hearts. The basal level of phosphorylation in diabetic hearts tended to be greater than that in controls, although the difference was not statistically significant. This may be related to the increased adenylate cyclase activity, possibly as a result of a reduced Gi level, in diabetic hearts. Phosphorylation of phospholamban is recognized to play a key role in acceleration of myocardial
relaxation upon beta adrenoceptor stimulation by increasing the velocity of Ca\(^{2+}\) sequestration from the myoplasm by the SR Ca\(^{2+}\) pump (Tada and Katz, 1982). This effect of phospholamban phosphorylation on the SR Ca\(^{2+}\) pump also leads to augmentation of the contractile Ca\(^{2+}\) reserve within the SR lumen, which, in turn, could enable greater Ca\(^{2+}\) release from SR during subsequent excitations, thus promoting the positive inotropic effect of beta adrenoceptor stimulation as well (Tada and Katz, 1982). Therefore, the lack of beta adrenoceptor-mediated phospholamban phosphorylation in diabetic hearts can account, at least in part, for the diminished inotropic response of diabetic myocardium to beta adrenoceptor stimulation. The interplay of several factors may underlie the defect in beta adrenoceptor-mediated phosphorylation of phospholamban in diabetic hearts, which may include alteration in protein kinase A activation and/or altered activity of protein phosphatase. A previous study reported that protein kinase A regulation of isolated SR is unchanged in diabetic rats (Lopaschuk et al., 1984). Thus, it seems less likely that the phosphorylation process of phospholamban by protein kinase A is altered in diabetes. On the other hand, it is possible that diabetes may accelerate dephosphorylation of phospholamban because of altered protein phosphatase activity. We observed that inhibition by okadaic acid of protein phosphatase activity markedly increased phosphorylation of phospholamban in diabetic hearts as well as control hearts (Gando, Hattori and Kanno, unpublished data). This finding together with the results with isoproterenol implies that diabetic myocardium may have much higher levels of protein phosphatase activity than the control myocardium, thus minimizing the physiological effects of cyclic AMP-increasing agents like isoproterenol. In addition to the well-established actions of protein kinase A, a Ca\(^{2+}\)-calmodulin-dependent mechanism is probably involved in phosphorylation of phospholamban in the intact heart secondary to an increase in intracellular Ca\(^{2+}\) concentration (Vittone et al., 1990). In electrically stimulated myocytes isolated from diabetic rat hearts, the increases in intracellular Ca\(^{2+}\) concentration in response to isoproterenol and 8-bromo-cyclic AMP have been depressed (Yu et al., 1994). Thus, the possibility of a defect in phosphorylation of phospholamban by a Ca\(^{2+}\)-calmodulin-dependent mechanism in diabetic hearts cannot be ruled out.

Activation of protein kinase A also phosphorylates L-type Ca\(^{2+}\) channels, which are one of the most important regulators for excitation-coupling. Radioligand binding studies have shown either no change, an increase or a decrease in the density of Ca\(^{2+}\) channels in cardiac membranes from diabetic rats (Lee et al., 1992; Nishio et al., 1990; Yu and McNeill, 1991). In a whole cell patch-clamp study, no change in the basal Ca\(^{2+}\) current has been demonstrated in ventricular myocytes isolated from streptozotocin-induced diabetic rat hearts (Jourdon and Feuvray, 1993). Similarly, Tsuchida et al. (1994) have reported that the basal Ca\(^{2+}\) current density and kinetic parameters of the current are unaltered, but found a decreased channel response to beta adrenoceptor stimulation in genetically diabetic rat hearts. The present results do not allow conclusions as to whether phosphorylation of Ca\(^{2+}\) channels in response to beta adrenoceptor stimulation is altered in diabetic hearts. Experiments now in progress are aimed at clarifying the effect of beta adrenoceptor stimulation on the Ca\(^{2+}\) current in streptozotocin-induced diabetic rat cardiomyocytes.

The present observations and their consequences for the function of the beta adrenoceptor system in diabetic hearts are summarized schematically in figure 8. In diabetic hearts, the number of beta adrenoceptors is reduced, whereas coupling of the receptors with G\(_{s}\) is not impaired. In protein levels, G\(_{i}\) is markedly decreased, whereas G\(_{s}\) is unchanged. The result is an unaltered beta adrenoceptor-mediated stimulation of adenylate cyclase. Thus, generation of cyclic AMP can proceed normally under beta adrenoceptor stimulation. Cyclic AMP activates protein kinase A that is capable of phosphorylating a series of proteins including phospholamban and Ca\(^{2+}\) channels. Specifically, cyclic AMP-dependent phosphorylation of phospholamban is blunted in diabetes, which may be associated with a decrease in the rate of Ca\(^{2+}\) release and/or uptake by SR, thereby leading to the diminished inotropic responsiveness to beta adrenoceptor stimulation.

In summary, the present study shows that diabetic myocardium exhibits a diminished positive inotropic action in response not only to beta adrenoceptor agonists but also to cyclic AMP-generating or -mimicking agents. The reduced number of myocardial beta adrenoceptors does not account for the functional depression. The data indicate that the diminished responsiveness is not caused by an alteration in adenylate cyclase or changes in the levels of G proteins. We conclude that the diminished functional responsiveness in diabetic myocardium is the result of a defect at the level beyond the steps of activation of adenylate cyclase but before the contractile machinery.

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Fig. 8. Diagram of the cardiac beta adrenoceptor system in diabetic rats (see text for details). Abbreviations: beta AR, beta adrenoceptors; AC, adenylate cyclase; cAMP, cyclic AMP; PKA, protein kinase A; PLB, phospholamban; AP, action potential.


Send reprint requests to: Yuichi Hattori, M.D., Department of Pharmacology, Hokkaido University School of Medicine, Sapporo 060, Japan.