Insulin Prevention of Altered Muscarinic Receptor–G Protein Coupling in Diabetic Rat Atria

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Right atria from rats rendered diabetic by injection of streptozocin (STZ-D) for 8–10 wk are supersensitive to the negative chronotropic effects of muscarinic agonists but have decreased levels of muscarinic receptors and acetylcholinesterase activity. Insulin treatment completely prevents the development of these changes. The proportion of atrial muscarinic receptors displaying high-affinity agonist binding is lower in STZ-D rats; however, the sensitivity of high-affinity agonist binding to regulation by a guanine nucleotide (5′-guanylylimidodiphosphate) is greater in atria from diabetic rats. Again, insulin treatment eliminates these differences. These findings indicate that alterations in atrial muscarinic systems in STZ-D rats are a consequence of the elaboration of the diabetic state and suggest that an alteration of functional muscarinic receptor–G protein coupling contributes to the altered physiological responsiveness of the heart in diabetes. *Diabetes* 38:1611–16, 1989

Increased incidence of abnormal cardiac rhythm is observed in patients with diabetes mellitus (1,2). Although numerous factors contribute to normal cardiac rhythm, the maintenance of resting heart rate is predominantly regulated by the vagal-muscarinic receptor system (3,4). However, limited information is available concerning the integrity of cardiac muscarinic receptors and their contribution to cardiac disorders in diabetes.

It has been reported that carbachol sensitivity is altered in an isolated working heart preparation from streptozocin-induced diabetic (STZ-D) rats (5), and a decrease in ventricular muscarinic receptor population occurs 6 mo after the induction of the diabetic state (6). Recently, we demonstrated that isolated right atria from STZ-D rats (8–10 wk of diabetes) are supersensitive to the negative chronotropic effects of acetylcholine and related muscarinic agonists. This phenomenon is accompanied by a decrease in the density of muscarinic receptors (7,8).

The aim of this study was to further examine the effects of diabetes on the muscarinic receptor system of right atria. We characterized muscarinic receptor–binding properties and receptor coupling to guanine nucleotide–dependent transducer proteins (G proteins) and determined the influence of insulin on the development of physiological and biochemical alterations of right atrial muscarinic receptors in STZ-D.

Glucose 1 mM = 18 mg/dl

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RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were housed individually in an environmentally controlled room. Standard laboratory food and water were available ad libitum. STZ (Upjohn, Kalamazoo, MI) was dissolved in saline acidified to pH 4.5 with citrate immediately before use. Diabetes was induced in 42- to 43-day-old rats by a single injection of 65 mg/kg STZ in the lateral tail vein. Age-matched control rats received an injection of vehicle. The serum glucose level of nonfasted animals was determined at death with a Glucose Analyzer 2 (Beckman, Fullerton, CA). Seventy-two hours after injection of STZ, rats that were glycosuric were randomly divided into two groups. Untreated STZ-D rats received no further treatment; treated STZ-D rats received a daily subcutaneous injection of insulin (0.9 U/100 g body wt of protamine zinc and Iletin, Lilly, Indianapolis, IN) (9). The dose of insulin maintained blood glucose levels and body weights of treated STZ-D rats within the range of control animals.

Chronotropic responses of isolated right atria. Eight to 10 wk after STZ injection, the rats were decapitated and

their hearts rapidly removed and placed in oxygenated (95% O_2/5% CO_2) Chenoweth-Koelle solution (8). Spontaneously beating right atria were isolated, mounted on bipolar platinum surface-recording electrodes, and suspended in an organ chamber under a resting tension of 500 mg. The recording electrodes were coupled to a Beckman cardiotachometer and R711 Dynograph to record electrical firing. The organ chamber contained 30 ml of modified Chenoweth-Koelle solution at 32°C with the following composition (in mM): 120 NaCl, 5.6 KCl, 2.2 CaCl, 2.1 $MgCl_2$, 1.9 EDTA, 19 $NaHCO_3$, and 10 dextrose. Atrial preparations were allowed to equilibrate 60 min before measuring negative chronotropic responses to acetylcholine and bethanechol chloride.

Concentration-response relationships were obtained for acetylcholine and bethanechol chloride (Sigma, St. Louis, MO). After the peak chronotropic response was achieved, the preparation was washed twice and reequilibrated until the beating rate returned to the control level (~20 min) before being exposed to a higher concentration of agonist. Each tissue was exposed to only one agonist. Changes in heart rate were expressed as percent changes from control rates. The EC_{50} value was defined as the agonist concentration at which half of the maximum response was produced. The maximum response to the cholinergic agonists was a complete cessation of firing in all instances.

Muscarinic receptor–binding assays. Eight to 10 wk after injection of STZ or vehicle, the rats were killed by decapitation, and their right atria were weighed and homogenized in 10 vol of 50 mM Tris-HCl, pH 7.4, with a Brinkmann homogenizer (30 s, maximum setting, Westbury, NY). Homogenates were filtered through gauze to remove undisrupted material and centrifuged at 30,000 × *g* for 20 min. Pellets were resuspended in fresh buffer and their protein contents determined by the method of Lowry et al. (10) with bovine serum albumin as standard.

Muscarinic antagonist binding was measured with [^{3}H]-*N*-methylscopolamine ([^{3}H]MS; 60–80 Ci/mol; New England Nuclear, Boston, MA) as the probe. Tissue aliquots (20–100 μg protein) were incubated with [^{3}H]MS for 90 min at room temperature in medium containing 100 mM NaCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4. Suspensions were then filtered through glass-fiber filters (no. 32, Schleicher and Schuell, Keene, NH) with a Brandel filtration manifold (Gaithersburg, MD). Incubation tubes and filters were washed twice with 5 ml buffer, and the radioactivity content of the filters was determined by liquid-scintillation counting. Nonspecific binding was determined in the presence of 10 μM atropine.

The number and affinity of [^{3}H]MS binding sites was determined by measuring the specific binding of [^{3}H]MS at six concentrations (0.01–3.2 nM). Binding parameters were calculated by nonlinear regression analysis with a single-receptor population model

$$b = B_{max}\, C/(C + K_d)$$

where b is binding, B_{max} is receptor density, C is the concentration of free [^{3}H]MS, and K_d is the dissociation constant for [^{3}H]MS binding.

Muscarinic agonist binding was measured in carbachol-[^{3}H]MS competition studies. Carbachol (0.01–100 μM) binding was inferred from its inhibition of specific [^{3}H]MS (0.1 nM) binding (11). Carbachol was used in the equilibrium-binding studies as a representative cholinoreceptor agonist, because the rapid hydrolysis of acetylcholine would have necessitated the use of cholinesterase inhibitors. Carbachol is closely related to acetylcholine and appears to differentiate between the same populations of agonist-binding sites insofar as the proportions of high- and low-affinity agonist sites are similar when measured with either ligand (12). Binding isotherms were resolved into their constituent components by nonlinear regression analysis with a one- or two-receptor population model. The single-population model is as described above; the two-population model was

$$b = [B_h\, C/(C + K_h)] + [(1 - B_h)\, C/(C + K_l)$$

where b is fractional receptor occupancy by carbachol, C is the carbachol concentration, and B_h and $1 - B_h$ are the fractions of receptor displaying high- and low-affinity agonist binding with dissociation constants K_h and K_l, respectively. The appropriateness of the one- or two-site model was determined by an F test comparing residuals between predicted and actual values.

Acetylcholinesterase activity was determined spectrophotometrically by the method of Ellman et al. (13) with homogenates of atrial tissue. The extent of hydrolysis catalyzed by enzymes other than acetylcholinesterase was determined with specific inhibitors.

Binding parameters were determined by nonlinear regression analysis with custom software based on the Marquardt-Levenberg algorithm. Approximations of the standard deviation of binding-parameter estimations ranged from 3 to 11% in all cases. Differences in chronotropic responses to muscarinic agonists were determined by comparing EC_{50} values for the three groups. Statistical differences between parameter values for the three experimental groups were determined by analysis of variance followed by a post hoc Tukey's test. $P \leq .05$ indicated a significant difference.

RESULTS

Eight to 10 wk after the onset of diabetes, STZ-D rats weighed <50% of age-matched control rats (188 ± 18 vs. 424 ± 8 g, n = 21). The serum glucose level of the STZ-D rats was fourfold that of age-matched control rats (545 ± 14 vs. 139 ± 8 mg/dl). Daily insulin administration to STZ-D rats completely prevented both the loss in weight and the elevation of serum glucose level. Insulin-treated STZ-D rats weighed 419 ± 12 g and had a serum glucose level of 141 ± 11 mg/dl.

CHRONOTROPIC RESPONSES

Atria from STZ-D rats were supersensitive to the negative chronotropic effects of acetylcholine and bethanechol chloride (Fig. 1). EC_{50} values for the slowing of beat rates by acetylcholine and bethanechol chloride in right atria isolated from STZ-D rats (14 ± 4 and 4.5 ± 0.6 μM, respectively) were significantly lower than the corresponding values obtained in atria from age-matched control rats (205 ± 25 and 11 ± 1 μM, respectively). Daily administration of insulin to STZ-D rats completely prevented the development of this

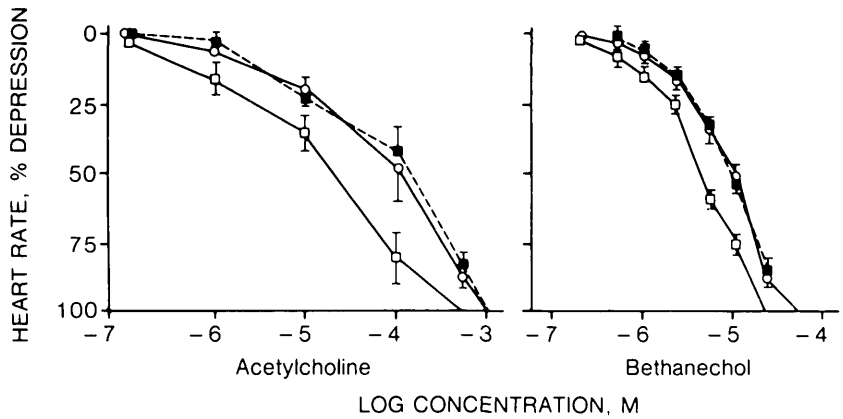

FIG 1. Influence of cholinergic agonists on chronotropic responses of right atria from streptozocin-induced diabetic (STZ-D; □), age-matched control (○), and insulin-treated STZ-D (■) rats. Influence of acetylcholine and bethanechol chloride on firing rate of spontaneously beating right atria was determined. Each *point* and *bar* represents mean ± SE of 10–12 experiments. Resting beat rates were 135 ± 6, 97 ± 4, and 129 ± 8 beats/min in atria from control, STZ-D, and insulin-treated STZ-D rats, respectively.

supersensitivity (Fig. 1); i.e., there were no differences between the EC_{50} value for each agonist in atria from insulin-treated STZ-D and age-matched control rats. The negative chronotropic responses to acetylcholine and bethanechol chloride were inhibited by 1 μM atropine, indicating the muscarinic nature of the responses.

ATRIAL MUSCARINIC RECEPTORS

Antagonist binding. The binding of [^{3}H]MS to muscarinic receptors in right atria from control, STZ-D, and insulin-treated STZ-D rats is summarized in Fig. 2 and Table 1. In each tissue, binding was well described by a single-receptor population model. The number of [^{3}H]MS binding sites was lower in atria from diabetic rats (309 vs. 179 fmol/mg protein). In diabetic rats treated with insulin, receptor density was restored to 268 fmol/mg protein. [^{3}H]MS binding affinity was the same in atria from all three groups of rats (K_d values ranged from 0.19 to 0.26 nM).

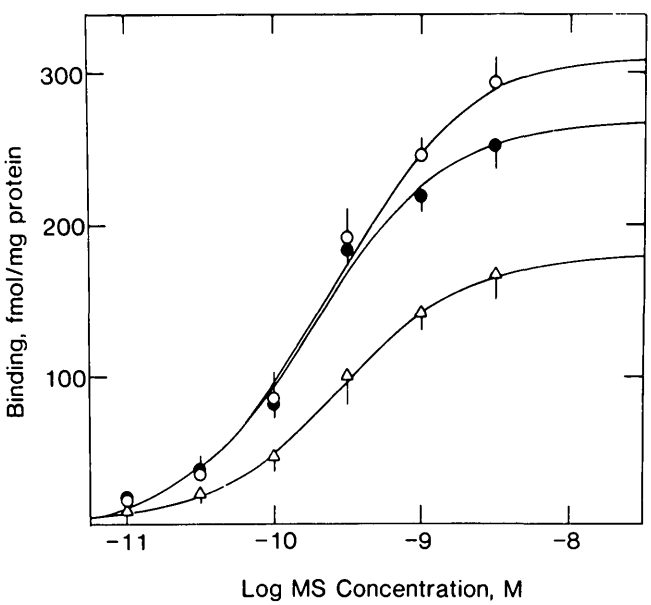

FIG. 2. Influence of streptozocin-induced diabetes (STZ-D) and insulin on antagonist binding to atrial muscarinic receptors. Binding of [^{3}H]-*N*-methylscopolamine to muscarinic receptors in right atria from control (○), STZ-D (△), and insulin-treated STZ-D (●) rats was determined. *Lines* are drawn according to nonlinear regression analysis with *single-receptor population model, which revealed parameters listed in* Table 1. Each *point* and *bar* represents mean ± SD of 5 experiments.

Agonist binding. Carbachol binding was best described by a two-receptor population model (Fig. 3; Table 2). In atria from control rats, 60% of the receptors displayed high-affinity binding (K_h = 0.11 μM); the remaining receptors had a much lower affinity (K_l = 26 μM). Carbachol affinity was lower in atria from STZ-D rats. This was reflected in a decrease in the proportion of receptors displaying high-affinity binding from 60 to 45%; dissociation constants associated with both the high- and low-affinity binding components were unchanged. Insulin treatment of STZ-D rats prevented the decrease in the high-affinity binding component; again, individual binding constants were not different from those of control or untreated STZ-D rats.

Guanine nucleotides lower agonist binding affinity for muscarinic (and other) receptors (14,15). This effect is believed to reflect receptor dissociation from G proteins (16,17). In the presence of 10 μM 5′-guanylylimidodiphosphate (Gpp(NH)p), a stable analogue of GTP, the fraction of atrial receptors from control tissues displaying high-affinity binding was decreased from 60 to 40% (Fig. 4; Table 2); high- and low-affinity binding dissociation constants did not change. In contrast, the high-affinity binding component was completely eliminated in atria from STZ-D rats; binding isotherms were best described by a model incorporating a single population of low-affinity (K_d = 15 μM) binding sites. The influence of Gpp(NH)p on agonist binding to atria from insulin-treated STZ-D rats, on the other hand, was indistinguishable from the influence of Gpp(NH)p on binding to receptors in control atria: the high-affinity binding component was reduced from 59 to 36% with no change in the high- and low-affinity binding constants (Fig. 4; Table 2).

TABLE 1
[^{3}H]-*N*-methylscopolamine ([^{3}H]MS) binding to muscarinic receptors in right atria from control, streptozocin-induced diabetic (STZ-D), and insulin-treated STZ-D rats

Binding parameter	Control	STZ-D	Insulin treated
B_{max} (fmol/mg protein)	309 ± 36	179 ± 13*	268 ± 42
K_d (nM)	0.23 ± 0.08	0.26 ± 0.05	0.19 ± 0.09

Values are means ±SD. n = 5. B_{max}, concentration of binding sites. K_d, [^{3}H]MS dissociation constant. Insulin-treated values did not differ significantly from control values for either parameter.
*P < .01 vs. control or insulin-treated STZ-D rats.

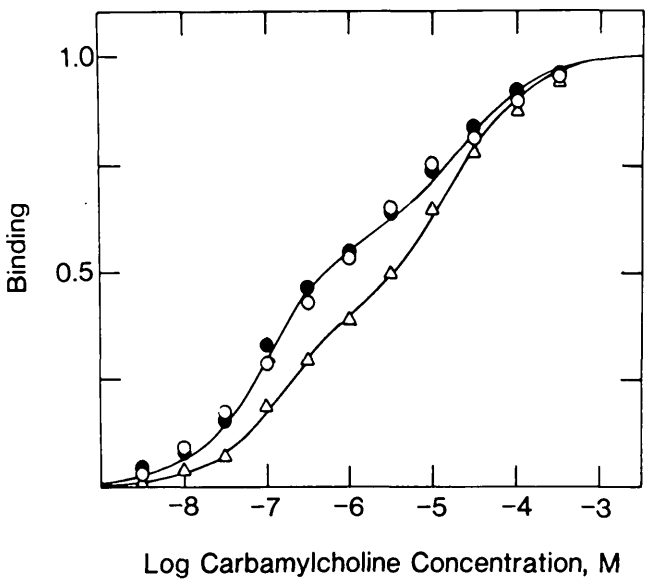

FIG. 3. Influence of streptozocin-induced diabetes (STZ-D) and insulin on agonist binding to atrial muscarinic receptors. Carbachol binding to muscarinic receptors in right atria from control (○), STZ-D (△), and insulin-treated STZ-D (●) rats was determined. *Lines* are drawn according to nonlinear regression fits of data to model incorporating 2 populations of independent binding sites, which revealed parameters listed in Table 2. *Line* for insulin-treated atria is very close to control *line* and has been omitted for clarity. Each *point* represents mean of 5 experiments, which varied by <20%.

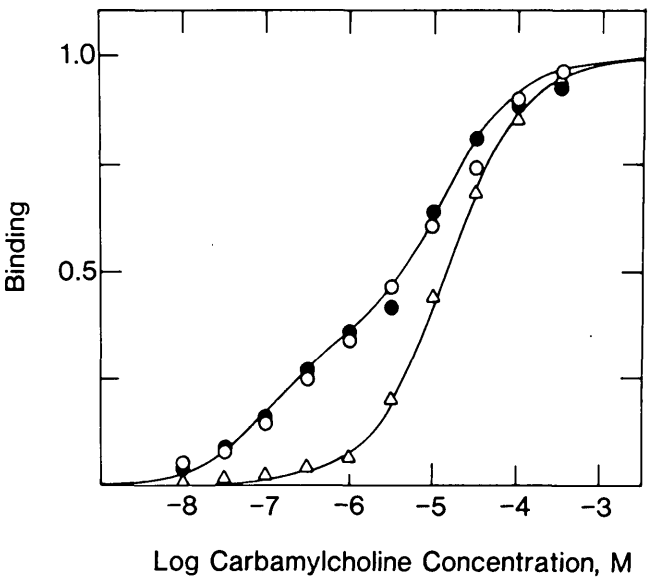

FIG. 4. Influence of streptozocin-induced diabetes (STZ-D) and insulin on guanine nucleotide sensitivity of atrial muscarinic receptors. Influence of 10 μM 5′-guanylylimidodiphosphate on carbachol binding to muscarinic receptors in right atria from control (○), STZ-D (△), and insulin-treated STZ-D (●) rats was determined. *Lines* are drawn according to nonlinear fit of data to models incorporating 1 (STZ-D) or 2 (control) populations of binding sites, which revealed binding parameters listed in Table 2. *Line* for insulin-treated group has been omitted for clarity. Each *point* represents mean of 5 determinations, which varied by <20%.

ACETYLCHOLINESTERASE ACTIVITY

Acetylcholinesterase activity was decreased 45% in right atria from STZ-D rats compared with age-matched control rats. Daily insulin treatment prevented the decrease in acetylcholinesterase activity. Control activity was 5.48 ± 0.83 vs. 3.03 ± 0.64 and 5.54 ± 0.63 nmol · mg^{-1} protein · min^{-1} in right atria from STZ-D and insulin-treated STZ-D rats, respectively.

DISCUSSION

A common feature in both clinical and experimental forms of diabetes mellitus is an impaired relationship between insulin- and endogenous hormone–receptor mechanisms (e.g., glucagon, catecholamines, growth hormone), which contribute to long-term complications (18). However, the consequences of diabetes for muscarinic mechanisms are poorly understood. This study indicates that the coupling of right atrial muscarinic receptors to G proteins is altered in short-term (8–10 wk) STZ-D rats. Furthermore, insulin treatment prevented the development of *1*) an increased sensitivity to the chronotropic effects of muscarinic agonists (7,8), *2*) a decrease in acetylcholinesterase content, and *3*) changes in muscarinic receptor–binding properties and coupling to G proteins observed in STZ-D rats.

The number of [^{3}H]MS binding sites in atria was decreased 42% 8–10 wk after STZ injection. We earlier reported a 30% decrease in [^{3}H]-3-quinuclidinyl benzilate ([^{3}H]QNB) binding to atrial receptors from STZ-D rats (7). In neither study was the affinity of the tritiated antagonist changed. Lee and El-Fakahany (19) have shown that the tertiary muscarinic probe

TABLE 2
Carbamylcholine binding to muscarinic receptors in right atria from control, streptozocin-induced diabetic (STZ-D), and insulin-treated STZ-D rats

	Control	STZ-D	Insulin treated
Gpp(NH)p, 0 μm			
B_h	0.60 ± 0.05	0.45 ± 0.05*	0.59 ± 0.05
K_h (μM)	0.11 ± 0.05	0.15 ± 0.08	0.09 ± 0.03
K_l (μM)	26 ± 13	20 ± 7	18 ± 5
Gpp(NH)p, 10 μm			
B_h	0.40 ± 0.12		0.36 ± 0.07
K_h (μM)	0.19 ± 0.11		0.14 ± 0.07
K_l (μM)	26 ± 13	14 ± 3	16 ± 6

Values are means ± SD. n = 5–6. Gpp(NH)p, 5′ guanylylimidodiphosphate; B_h, fraction of receptors displaying high-affinity carbamylcholine binding; K_h and K_l, dissociation constants associated with carbamylcholine binding to high- and low-affinity sites, respectively. Carbamylcholine binding data were fitted by nonlinear regression analysis to a model incorporating one or two independent binding sites. Binding to diabetic atria in the presence of 10 μM Gpp(NH)p was best described by a single-receptor model.
*P < .01 vs. control and insulin-treated STZ-D rats.

[^{3}H]QNB recognizes 35% more muscarinic binding sites than the quaternary ligand [^{3}H]MS. It is possible that [^{3}H]QNB can also bind to muscarinic sites sequestered within the hydrophobic membrane matrix, whereas [^{3}H]MS recognizes only the more readily accessible surface sites. The greater decrease in [^{3}H]MS compared with [^{3}H]QNB binding sites suggests that exposed muscarinic binding sites (presumably the functionally active sites) are more susceptible to downregulation in STZ-D.

The question of what is the most meaningful basis on which to compare receptor densities in atria from normal and diabetic animals is problematical. We have expressed this density on the basis of unit of membrane protein. However, note that 8–10 wk STZ-D rats weigh considerably less than control rats and have smaller hearts. The wet weights of paired atria from these two groups were the same (112 ± 12 and 119 ± 35 mg for control and STZ-D rats, respectively; n = 21), although the ventricles from STZ-D rats were considerably smaller (870 ± 150 vs. 1134 ± 194 mg). However, the fraction of atrial wet weight contributed by particulate protein was lower in STZ-D rats (10.1 ± 1.2 vs. 12.4 ± 2.2%). Thus, the apparent density of muscarinic receptors was lower in atria from STZ-D animals whether this density was expressed on the basis of membrane protein (179 vs. 309 fmol/mg), wet weight (18.1 vs. 38.3 fmol/mg), or total tissue (atrium) content (2.15 vs. 4.29 pmol).

The relationship between tissue sensitivity to muscarinic agonists and the number of muscarinic receptors is incompletely understood. In STZ-D, there is a dissociation in the two measurements insofar as sensitivity is increased (Fig. 1), although receptor density is decreased (Fig. 2; Table 1). A decrease in muscarinic receptor level (downregulation) is usually associated with chronic receptor activation, as may be produced by administration of receptor agonists or acetylcholinesterase inhibitors (20,21). In this study, atrial acetylcholinesterase activity was depressed in STZ-D, raising the possibility that increased concentrations of acetylcholine contributed to the decrease in muscarinic receptor content. However, agonists and acetylcholinesterase inhibitors produce a depression of cellular responsiveness to muscarinic agonists (tachyphylaxis or desensitization) and not a supersensitivity, as is observed in STZ-D. Thus, the downregulation of muscarinic receptors in STZ-D appears to reflect an increased state of sensitivity of atrial cells to muscarinic agonists rather than a simple change in the availability of acetylcholine. It would appear that receptor downregulation can be caused by increased internal (cellular responsiveness) and external stimulation.

However, the basis of this increased cellular sensitivity is not clear. Activation of muscarinic receptors precipitates a wide variety of biochemical events, including activation of phospholipase C, inhibition of adenylate cyclase, and stimulation of K^+ channels. Presumably, a functional alteration in any of these systems (or in a subsequently activated system) could contribute to cellular supersensitivity. G proteins mediate many of these processes, serving to link the receptor to various effector mechanisms (16,17). In this study, information concerning receptor–G protein interactions was obtained from measurement of agonist-binding–state conformation and sensitivity of high-affinity agonist binding to a guanine nucleotide.

Agonist binding leads to a transient coupling of the receptor to a G protein, which in turn induces the release of GDP from the G protein (17). Subsequent GTP binding leads to a dissociation of G protein subunits and separation of receptor and G protein. G protein subunits then modulate various cellular processes. Subsequent to GTP hydrolysis by the α-subunit, the G proteins reassemble and are free to reassociate with the receptor (17,22). Muscarinic receptors in vitro are heterogeneous with respect to agonist-binding affinity; interconvertible high- and low-affinity receptor populations can be distinguished (12,23,24). Guanine nucleotides lower agonist-binding affinity (14,15), which led to the suggestion that high-affinity agonist binding involves receptor–G protein complexes, whereas low-affinity binding involves uncoupled receptors. This has been confirmed in reconstitution experiments with purified muscarinic receptor and G proteins: high-affinity, GTP-sensitive agonist binding is obtained only on regeneration of the receptor–G protein complex (25). The distribution of muscarinic receptors between states of high and low affinity for agonists determined by in vitro binding assays follows regular developmental, tissue, and regional patterns and is affected in a predictable manner by drugs and certain chemical and physical treatments. This suggests that agonist affinity state (and hence, receptor sensitivity to guanine nucleotides), as determined in ligand-binding studies, provides meaningful information concerning the state of receptor–G protein coupling. In atria from STZ-D rats, the fraction of receptors displaying high-affinity agonist binding decreased from 60 to 44%, and this decrease is prevented by insulin treatment. This indicates a lower degree of receptor–G protein coupling in atria from STZ-D rats. However, guanine nucleotides completely convert receptors from STZ-D rats to a low-affinity state. In contrast, only 33% of the high-affinity receptors in atria from control rats (or from insulin-treated STZ-D rats) are converted to a low-affinity state by a guanine nucleotide.

The functional significance of these in vitro measurements is not certain. Coupled and uncoupled (high- and low-affinity) receptors participate in the interdependent G protein, receptor, and effector cycles (16). Although a greater proportion of muscarinic receptor in membranes isolated from control atria are in the high-affinity coupled state, the receptor–G protein complex must bind guanine nucleotide and dissociate to affect biochemical processes. In the latter regard, receptors in atria from STZ-D rats are clearly more active. It is possible that the lower proportion of high-affinity binding in diabetic atria detected in the in vitro binding assay reflects this greater tendency to bind guanine nucleotide and dissociate. These findings raise the possibility that muscarinic supersensitivity in diabetes is related to a facilitated turnover in the receptor–G protein cycle.

In this regard, note that the number of muscarinic receptors in the low-affinity form measured in vitro in the presence of Gpp(NH)p is not different in atria from control, STZ-D, and insulin-treated STZ-D rats (179, 185, and 172 fmol/mg protein, respectively). Is it possible that the number of "activable" muscarinic receptors (i.e., the number of low-affinity receptors available for interactions with G proteins) is actually the same in all three groups of animals? Evaluation of this possibility awaits a more thorough appreciation of the functional significance of high- and low-affinity receptor

states (e.g., does the guanine nucleotide–sensitive population of receptors measured in vitro actually reflect the pool of active or available receptors, and what is the significance of high-affinity, guanine nucleotide–insensitive muscarinic receptors?).

Our findings with respect to agonist binding are consistent with our earlier findings insofar as agonist affinity was greatly decreased in atria from STZ-D rats (8). However, our earlier analyses ascribed this change to a decrease in binding affinities of both the high- and low-affinity receptor populations and, in the case of left atria, a decrease in the proportion of receptors displaying high-affinity agonist binding (a similar decrease in right atria was not significant). This more extensive study clearly ascribes the decrease in agonist affinity to a decrease in the proportion of receptors in the high-affinity state. The reasons for this discrepancy are not entirely clear, although two factors supporting the validity of these results are our use of a lower concentration of [^{3}H]MS (0.1 vs. 0.3 nM) in the competition experiments and the use of a lower receptor concentration in all binding assays (4.6 vs. 15.5 pM) through use of a lower tissue content (75 vs. 100 μg) and greater assay volume (5 vs. 2 ml). Both of these conditions permit more reliable estimates of binding constants in experiments involving competition between an unlabeled ligand and a radiolabeled probe.

In summary, our findings demonstrate that the concurrent development of changes in atrial muscarinic sensitivity and receptor-binding properties in STZ-D can be completely prevented by insulin therapy. The nature of the alterations of muscarinic receptors suggests that an alteration of functional muscarinic receptor–G protein coupling contributes to the altered physiological responsiveness of the tissue.

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