Two Groups of Diabetic KK-CA’ Mice Specifically Bred for High and Low Sensitivity to Exogenous Acetylcholine and β₁-Adrenergic Stimulation: Interaction of Higenamine and Aconitine on Pulse Rate

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Diabetic KK-CA’ mice were specifically bred for high and low sensitivity to the addition of exogenous acetylcholine (ACh). The sensitivity to ACh was measured by the change in pulse rate 2 min after the administration of ACh (10 mg/kg, s.c.). The two groups of mice, with high and low sensitivity to ACh, were specially selected and mated sequentially until the 12th filial generation. Although higenamine (100 μg/kg, i.p.), a β₁-adrenergic agonist (a compound derived from aconite), had no effect per se, it inhibited aconitine (another compound derived from aconite extract)-induced bradycardia within 30 s of administration in ACh-low sensitive mice but not in ACh-high sensitive mice. The effects of aconitine and higenamine alone did not differ between these two groups of mice. This demonstrates that the high muscarinic and high β₁-adrenergic sensitive mice may be stratified into two groups based upon an antagonistic interaction between higenamine and aconitine.

Key words high, low acetylcholine-sensitivity; aconitine; high, low β₁-adrenergic-sensitivity; diabetic KK-CA’ mice

KK-CA’ mice, animal models for spontaneous diabetes mellitus, have been used in pharmacological assays due to their display of diabetic symptoms resembling human type II diabetes (NIDDM). Diabetic peripheral neuropathies are a group of heterogeneous syndromes with considerable morbidity. The neuropathy is caused by the abnormality of glucose metabolism in a hyperglycemic state. Involvement of the autonomic nervous system in diabetes with clinical abnormalities including several systems is well recognized, although the assessment of damage has been largely confined to the cardiovascular system. Cardiac dysfunction and hypertension occur in the experimental animals as well as in humans with diabetes mellitus. We have previously investigated the abnormal increase in basal pulse rate (PR) and basal blood pressure (BP) during the diabetic state of both streptozotocin (bolus injection)-mice and KK-CA’ mice. The increases can be explained by diminished acetylcholine (ACh) release. High cardiac sensitivity to the ACh of the PR response can be induced in the diabetic state more readily than in the non-diabetic state. This suggests that the diabetic state also influences the sensitivity of PR to exogenous ACh, although the diabetic state is affected by autonomic nervous system regulation. Agents improving diabetic complications may be effective depending on the state with autonomic (parasympathetic and sympathetic) neuropathy. Therefore, models for parasympathetic and sympathetic neuropathy are required. We stratified the diabetic KK-CA’ mice by sensitivity to ACh on PR response as models with autonomic neuropathy.

We previously reported that aconitine, a component of processed aconite root, induced bradycardia in mice and that higenamine, another component, inhibited the bradycardia. The effect of aconite root is demonstrated to consist of an interaction between aconitine and higenamine. In the present study, ACh-high and low sensitive models from diabetic KK-CA’ mice were produced to investigate the contribution of autonomic dysfunction to diabetic symptoms, and the interaction of aconitine and higenamine on PR in the two stratified groups of mice was tested.

MATERIALS AND METHODS

Animals Genetically obese diabetic male KK-CA’ mice and male ddY mice were used. KK-CA’ mice were inbred in our laboratory by mating male KK-CA’ mice (genotype; A’aBBC) with female KK-C mice (aaBBC). Mice were maintained under a constant temperature (23±1 °C), fed the usual laboratory diet (CA-1, Japan Clea) and tap water ad libitum, with light from 8 a.m. to 6 p.m.

Blood samples were obtained at 10 a.m. from the orbital vein plexus with a capillary glass pipette. Blood glucose levels in the non-fasting state were measured by a glucose oxidase method using a glucose analyzer (Beckman, typeII, CA, U.S.A.).

Measurement of PR and BP PR and systolic BP were determined simultaneously with a tail artery-cuff using a photoelectric sensor plethysmograph (PS-200, Riken-Kaihatsu, Tokyo, Japan) at a constant temperature (37±1 °C), while animals were fully conscious. The pulse detector of the plethysmograph was attached to the tail proximal to the occluding cuff. The PR response was continuously measured by a tachometer which was triggered by the detector. The pressure inside the cuff was expressed as the systolic BP. After monitoring the PR and BP every 5 min for 30 min, 0.9% NaCl (saline) or ACh (10 mg/kg) was administered subcutaneously (s.c.) and then the PR and BP were measured every 2 min for a duration of 10 min. The changes in PR and BP were expressed as the % change from the basal level, and were normalized by subtracting the % change in PR and BP after saline administration. The ACh sensitivity was determined every 2 weeks during the period from 8 to 20 weeks of age.

Production of ACh-High and -Low Sensitive Mice Male KK-CA’ mice with more than a 10% fall in PR responses

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to ACh and over 300 mg/dl blood glucose level (type I in Fig. 1) were selected and mated with female KK-C mice, which yielded the ACh-high sensitive mice group.

Male KK-CA2 mice with more than a 10% rise in PR responses to ACh and over 300 mg/dl blood glucose level (type IV in Fig. 1) were selected and mated with female KK-C mice, which yielded the ACh-low sensitive mice group.

From the F1 generation, type I male mice in the high-ACh sensitive group and type IV male mice in the ACh-low sensitive group were mated with female KK-C mice belonging to the same groups, respectively.

**Materials** Acetylcholine chloride (Daichi, Tokyo), aconitine (Sigma, U.S.A.), carbachol (Tokyo-Kasei, Tokyo), (±)-isoproterenol (Wako, Osaka) and higenamine chloride (a gift from the late Prof. H. Hikino, Tokohoku University) were used. These drugs were dissolved in saline, and administered intraperitoneally (i.p.), except for ACh (s.c.).

**Statistical Analysis** Significant differences between the mean PR and BP values of the high and low ACh-sensitive mice after the administration of ACh were evaluated by analysis of variance (ANOVA) and then the Tukey multiple range test. The difference was considered to be significant when the p value was smaller than 0.05.

**RESULTS**

**Stratification of Four Types of KK-CA2 Mice Grouped by Their Sensitivity to Exogenous ACh Addition** The sensitivity to ACh in the mice was determined by the PR response 2 min after the administration of ACh (10 mg/kg, s.c.), because at this time point, a maximum decrease in BP response was also obtained simultaneously.

The sensitivity of the PR response to ACh was influenced by hyperglycemia and age. Some of the mice changed from high to low or from low to high sensitivity when they became hyperglycemic. The mice were stratified into 4 groups, type I: ACh-high sensitive in both the prediabetic and diabetic state, type II: ACh-low sensitive in the prediabetic state and ACh-high sensitive in the diabetic state, type III: ACh-high sensitive in the prediabetic state and ACh-low sensitive in the diabetic state, and type IV: ACh-low sensitive in both the prediabetic and diabetic state.

The percentage of mice falling into the type II group was the highest (60%) for all KK-CA2 mice in the primary (P) generation. A large percentage (86%) of mice in this generation demonstrated ACh-high sensitivity in the diabetic state. In the ACh-high sensitive mice group, 76% of the mice were type I in the first filial (F1) generation. In subsequent generations, type I mice continued to be produced until they reached 80% of the ACh-high sensitive group of mice.

While most of the diabetic mice indicated ACh-high sensitivity in the P generation, only a few mice (10%) indicated ACh-low sensitivity in both the prediabetic state and the diabetic state (type IV). In the ACh-low sensitivity mice group, on the other hand, the percentage of type IV mice increased in subsequent generations until they made up over 80% of the mice after the F2 generation (Fig. 1).

The histogram of the PR response to ACh in the diabetic state is shown in Fig. 2 at 14—16 weeks of age in KK-CA2 mice with hyperglycemia. The PR response to ACh had a slight negative chronotropy in the primary generation (−5.7 ± 1.6%). The distribution curves were shifted to the

![Diagram](image)

**Fig. 1. The Proportion of ACh (10 mg/kg, s.c.)-High Sensitive Mice (Right) and -Low Sensitive KK-CA2 Mice (Left) in PR Responses in Each Generation from the Primary (P) to Filial (F12) Generation**

ACh-high and -low sensitive mice were selected from the mouse groups belonging to the slash and shadow columns in each generation, and were mated, respectively. The mice were stratified into 4 groups: type I (ACh-high sensitive in both the prediabetic and diabetic state), type II (ACh-low sensitive in the prediabetic state and ACh-high sensitive in the diabetic state), type III (ACh-high sensitive in the prediabetic state and ACh-low sensitive in the diabetic state), and type IV (ACh-low sensitive in both the prediabetic and diabetic state).
left in the ACh-high sensitive mice group and to the right in the ACh-low sensitive mice group in advanced filial generations. The mean values of the PR responses to ACh in the F₄ (F₁₂) generation fell $24.4 \pm 2.8\%$ ($13.1 \pm 2.55$) in the ACh-high sensitive mice group, and rose $29.4 \pm 5.0\%$ ($22.8 \pm 5.0$) in the ACh-low sensitive mice group. The PR response to ACh (10 mg/kg, s.c.) was significantly different between the ACh-high and -low sensitive groups in F₁ and advanced filial generations.

Body weight, blood glucose, basal PR and basal BP were not different between the ACh-high and -low sensitive groups (Table 1).

In the normal ddY mice, ACh increased PR at low doses (<3 mg/kg, s.c.), decreased PR at high doses (>30 mg/kg, s.c.), and at a dose of 10 mg/kg the chronotropic response was $-0.9 \pm 5.2\%$. The dose–response curves for PR to exogenous ACh addition in ACh-high and -low sensitive mice showed significant differences at a dose of 10 mg/kg (Fig. 3). The left and right curves belonged to ACh-high and -low sensitive mice, respectively.

Bradycardial Effects of Aconitine on PR in ACh-High and -Low Sensitive Mice

ACh-high sensitive mice with a blood glucose level of more than 300 mg/dl showed more than a 10% fall in PR after ACh administration (10 mg/kg, s.c.), and ACh-low sensitive mice with a blood glucose level of more than 300 mg/dl demonstrated more than a 10% increase in PR after ACh administration (10 mg/kg, s.c.). Both were used to investigate the effect of aconitine and higenamine. The mean PR and BP responses of these mice to ACh addition were $27.9 \pm 5.8\%$ and a $39.7 \pm 5.0\%$ fall in the ACh-high sensitive mice group, and a $27.1 \pm 5.6\%$ rise and a $36.2 \pm 2.7\%$ fall in the ACh-low sensitive mice group, respectively.

Aconitine (100 µg/kg, i.p.) was administered to both ACh-high and -low sensitive mice. In the ACh-high sensitive mice group, aconitine decreased the PR to half the basal value within 15–20 min after administration in 10 out of 18 mice. In the ACh-low sensitive mice group, the occurrence ratio of bradycardia was 8 out of 20 mice.

Table 1. The Data of ACh-High and -Low Sensitive Mice (P, F₄ and F₁₂) in a Diabetic and Prediabetic State

<table>
<thead>
<tr>
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<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
<th>Basal PR (beats/min)</th>
<th>Basal BP (mmHg)</th>
<th>PR response to ACh (%)</th>
<th>BP response to ACh (%)</th>
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<tr>
<td>P</td>
<td>36.6 ± 0.6</td>
<td>345 ± 11</td>
<td>637 ± 16</td>
<td>122 ± 1</td>
<td>-5.7 ± 1.6</td>
<td>-40.9 ± 0.9</td>
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<tr>
<td>F₄</td>
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<tr>
<td>ACh-high sensitive group</td>
<td>36.8 ± 0.6</td>
<td>309 ± 14</td>
<td>637 ± 12</td>
<td>124 ± 2</td>
<td>-24.4 ± 2.9</td>
<td>-39.4 ± 1.6</td>
</tr>
<tr>
<td>ACh-low sensitive group</td>
<td>33.5 ± 0.7</td>
<td>306 ± 14</td>
<td>581 ± 20</td>
<td>121 ± 3</td>
<td>29.4 ± 5.0</td>
<td>-40.5 ± 1.2</td>
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<tr>
<td>F₁₂</td>
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<tr>
<td>ACh-high sensitive group</td>
<td>34.2 ± 0.6</td>
<td>419 ± 21</td>
<td>581 ± 11</td>
<td>118 ± 2</td>
<td>-13.1 ± 2.5</td>
<td>-42.4 ± 2.4</td>
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<tr>
<td>ACh-low sensitive group</td>
<td>34.5 ± 0.4</td>
<td>420 ± 13</td>
<td>589 ± 8</td>
<td>118 ± 1</td>
<td>22.8 ± 5.0</td>
<td>-40.8 ± 1.8</td>
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*Significant at $p < 0.01$.

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<tr>
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<th>3</th>
<th>10</th>
<th>30</th>
<th>% change in PR response</th>
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<tr>
<td>PR</td>
<td></td>
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<td>BP</td>
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Fig. 2. Distribution of % Change in PR Responses to ACh (10 mg/kg, s.c.) Administration at the Age of 14–16 Weeks in Each Generation from the Primary (P) to Filial (F₁, F₂, F₄, F₁₂) Generation of Diabetic KK-CAV Mice

The PR responses to ACh (10 mg/kg, s.c.) were significantly different between the ACh-high (left) and -low (right) sensitive groups in F₁, F₂, F₄ and advanced generations.

Fig. 3. The Dose-Response Curves of ACh for PR Responses in ACh-High Sensitive (▲) and ACh-Low Sensitive (▼) KK-CAV Mice in the F₁₂ Generation

Each point represents the mean ± S.E.M. of 10 mice. A significant difference ($p < 0.01$) was observed at the dose of 10 mg/kg between both groups.
Fig. 4. The Time Course of Bradycardial Effects of Aconitine (100 μg/kg, i.p.) on ACh-High (Left) and ACh-Low (Right) Sensitive KK-CA^y Mice in the F_4 Generation

Values represent the response in each mouse. The occurrence ratios of bradycardia were 10 out of 18 mice in ACh-high sensitive mice and 8 out of 20 mice in ACh-low sensitive mice.

Fig. 5. Inhibition by Higenamine (100 μg/kg, i.p.) on Aconitine (100 μg/kg, i.p.)-Induced PR Response in ACh-Low (Right) Sensitive, but not in ACh-High (Left) Sensitive, KK-CA^y Mice in the F_4 Generation

Higenamine was administered 30 min after the administration of aconitine when the aconitine-induced bradycardia had become stabilized. Values represent the responses of each mouse. No inhibition of aconitine-induced BP lowering by higenamine was observed in either type of mice.

(Fig. 4). The sensitivity to aconitine did not differ between the two groups in the F_4 generation.

Inhibitory Effects of Higenamine on Aconitine-Induced Bradycardia in ACh-Low Sensitive Mice but not in ACh-High Sensitive Mice The inhibitory effects of higenamine on aconitine-induced bradycardia were tested in ACh-high and -low sensitive mice in the F_4 generation. Higenamine (100 μg/kg, i.p.), which had no effect alone, was administered 30 min after the administration of aconitine (100 μg/kg, i.p.). In ACh-high sensitive mice, higenamine did not inhibit aconitine-induced bradycardia (the inhibitory ratio was 0/9), but inhibited bradycardia for all mice observed (9/9) in the ACh-low sensitive mice (Fig. 5). Higenamine increased PR above the basal level, but this rise was followed by bradycardia 20 min after the administration of higenamine. In neither type of mice di
Higenamine affect aconitine-induced lowering of BP.

The administration of higenamine (300 μg/kg and 1 mg/kg, i.p.) and isoproterenol (3 and 10 μg/kg, s.c.) alone dose-dependently increased PR but did not change BP, and the PR and BP response did not differ significantly between ACh-high and -low sensitive mice (data not shown). The PR and BP responses by combining carbachol (0.6 mg/kg, i.p.; negative chronotropic and isotropic) and isoproterenol (1—10 μg/kg, s.c.; positive chronotropic) did not differ significantly between ACh-high and -low sensitive mice (data not shown).

In isolated right atrial preparations, the concentration—frequency curves of higenamine (bath-applied) completely overlapped in both the ACh-high and -low sensitive mice at the Fg generation. In the same preparations, the concentration—frequency curve of ACh (bath-applied) in ACh-high sensitive mice tended to be more sensitive than that in ACh-low sensitive mice (data not shown).

DISCUSSION

A major complication in chronic diabetes mellitus is autonomic neuropathy. Autonomic neuropathy is a common and severe complication of diabetes mellitus that leads to vagal denervation, and sensorimotor and reflex dysfunction of the cardiovascular, urogenital, and gastrointestinal systems. The parasympathetic control of the heart causes several cardiac disturbances in the diabetic state. Furthermore parasympathetic tone is reduced in diabetic KK-CA/> mice.

BP is dose-dependently decreased by ACh, whereas PR is increased at low doses of ACh but decreased at high doses in control mice. The increase in PR by low doses of ACh is indirectly caused by a vago-sympathetic reflex, the baroreceptor reflex. The decrease in PR by high doses of ACh is caused by direct action on the sino-atrial node. Diabetic mice demonstrating a positive chronotropic response were defined as ACh-low sensitive, and mice demonstrating a negative chronotropic response were defined as ACh-high sensitive to a dose of ACh (10 mg/kg). The maximal BP responses were measured simultaneously, but did not differ between the two groups in the Fg generation. Both the PR and BP responses to carbachol did not differ significantly between ACh-high and -low sensitive mice. These results suggest that the ACh sensitivity in the whole animal may reflect partly the sensitivity of the sino-atrial node.

In the F generation of diabetic KK-CA/> mice, 86% of the diabetic mice were hypersensitive to ACh, whereas 14% of the mice were low sensitive to ACh. This data is in agreement with another report of supersensitivity to ACh in the diabetic state. The ACh-high and -low sensitive male KK-CA/> mice were selected and mated with female KK-C mice. ACh-high and -low sensitive groups of mice were obtained separately in advanced filial generations. A closed colony strain was established beyond the 20th filial generation. We further advanced the generation to establish the ACh-high and -low sensitive strains of mice. The present study is an intermediate step.

Other models of imbalanced autonomic function have been produced by a different method in our laboratory.

Repeated cold stress-treatment decreased, and adrenalectomy increased, the sensitivity of mice to ACh. This suggests that these conditions are caused by a continuous high or low level of ACh, respectively. ACh-high and -low sensitive KK-CA/> mice may therefore be related to continual differences in endogenous ACh concentration.

The stimulation of the muscarinic receptor in the ventricular myocardium has little effect on the force of ventricular contraction, but produces a pronounced negative isotropic effect when the stimulation is applied in the presence of sympathetic stimulation. This enhancement of muscarinic inhibition can be explained by accentuated antagonism, which is defined by Levy. This dual function of muscarinic action is concerned with the regulation of cyclic AMP levels in the myocardial cells. Muscarinic receptor stimulation inhibits adenylate cyclase activity through an inhibitory GTP-binding protein (Gi), whereas β₁-adrenergic receptor activation stimulates adenylate cyclase through a stimulatory GTP-binding protein (Gs). Isolated right atria from streptozotocin-diabetic rats are hypersensitive to the negative chronotropic effects of ACh and other muscarinic agonists, while the density and the affinity of muscarinic receptor populations are decreased. Right atria from streptozotocin-diabetic rats are supersensitive to the negative chronotropic effects of muscarinic agonists but have decreased muscarinic receptors and acetylcholinesterase activity. Muscarinic supersensitivity in the diabetic state is related to a facilitated turnover of the receptor-G protein cycle. Insulin treatment completely prevents the development of these changes.

We have reported that higenamine, a β₁-adrenergic agent, inhibits aconitine-induced bradycardia in conscious mice. In the present study, higenamine inhibited aconitine-induced bradycardia in ACh-low sensitive mice, but not in ACh-high sensitive mice. Effects of aconitine or higenamine alone on PR and BP response did not differ between these mice. The high sensitivity to ACh was accompanied by low sensitivity to higenamine, and vice versa, but this was not caused only by β₁-adrenergic sensitivity. The PR and BP response to isoproterenol did not differ significantly between ACh-high and -low sensitive mice. The mode of inhibitory action of higenamine can be interpreted by an interaction between muscarinic and adrenergic function. A difference in sensitivity was observed in the aconitine—higenamine interaction but not in the carbachol—isoproterenol interaction between ACh-high and -low sensitive mice. Therefore, the above results suggest that the aconitine—higenamine interaction in extract may depend on the imbalance between the cholinergic and adrenergic nervous systems and central nervous system. ACh-high and -low sensitive mice provide useful models for autonomic imbalance.

In conclusion, two groups of diabetic mice were stratified as an animal model for autonomic dysfunction by a high muscarinic and high β₁-adrenergic sensitivity based on an antagonistic interaction between aconitine and higenamine.

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REFERENCES