Insulinotropic activity of the imidazoline derivative RX871024 in the diabetic GK rat

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Insulinotropic activity of the imidazoline derivative RX871024 in the diabetic GK rat. Am J Physiol Endocrinol Metab 282: E117–E124, 2002. 10.1152/ajpendo.000031.2001.—The insulinotropic activity of the imidazoline derivative RX871024 was compared in pancreatic islets from nondiabetic Wistar rats and spontaneously diabetic Goto-Kakizaki (GK) rats. RX871024 significantly stimulated insulin secretion in islets from both animal groups. The insulinotropic activity of RX871024 was higher than that of the sulfonylurea glibenclamide. This difference was more pronounced in islets from GK rats compared with Wistar rat islets. More importantly, RX871024 substantially improved glucose sensitivity in diabetic β-cells, whereas glibenclamide stimulated insulin secretion about twofold over a broad range of glucose concentrations. RX871024 induced a faster increase in cytosolic free Ca2+ concentration and faster inhibition of ATP-dependent K+ (KATP) channel activity in GK rat islets compared with Wistar rat islets. RX871024 also induced a more pronounced increase in diacylglycerol concentration in GK rat islets. These data support the idea that imidazoline compounds can form the basis for the development of novel drugs for treatment of type 2 diabetes, which can restore glucose sensitivity in diabetic β-cells.

 MATERIALS AND METHODS

Materials. The imidazoline compound RX871024 was obtained from Reckitt & Colman Pharmaceuticals (Kingston Upon Hull, UK). Fura 2-AM was from Molecular Probes, Eugene, OR. Diacylglycerol (DAG) kinase was from Calbiochem-Novabiochem (San Diego, CA). Glibenclamide and all other reagents were obtained from Sigma Chemical (St. Louis, MO).

Isolation and incubation of islets of Langerhans. Islets were isolated from 2- to 3-mo-old male nondiabetic Wistar and diabetic GK rats (Karolinska Hospital colony) by collagenase digestion as described before (13). Islets were then maintained overnight in RPMI 1640 culture medium (Life Technologies, Paisley, UK) supplemented with 11 mM glucose, 10% fetal calf serum, 2 mM glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. In experiments with single cells, islets were dispersed by vigorous shaking in Ca2+-free medium. The cells were then plated onto coverslips. All experiments with islets and cells from Wistar and GK rats were run in the presence of 11 mM glucose. The insulinotropic effect of RX871024 on insulin release was demonstrated to be due to both inhibition of the ATP-dependent K+ (KATP) channel and a direct effect on exocytosis (4, 15).

The spontaneously diabetic nonobese Goto-Kakizaki (GK) rat displays many of the characteristics of type 2 diabetes in humans (7). Insulin response to glucose in pancreatic islets from GK rats is severely impaired, whereas the response to nonnutrient stimuli is preserved or exaggerated (1, 2).

The aim of this study was to further explore the insulinotropic effect of RX871024 and to compare this effect with that of the sulfonylurea glibenclamide in pancreatic islets from GK rats.
in parallel in triplicate or quadruplicate from at least three different rats.

Measurements of insulin release in intact pancreatic islets were performed in Krebs-Ringer bicarbonate (KRB) buffer containing (in mM): 115 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 20 NaHCO₃, and 16 HEPES, pH 7.4, supplemented with 2 mg/ml bovine serum albumin (BSA) as described previously (15). Measurements of insulin release in electropermeabilized islets were performed according to a procedure described before (4, 15). Under these conditions, a buffer containing (in mM): 140 potassium glutamate, 5 NaCl, 1 MgCl₂, 10 EGTA, 25 HEPES, 2 ATP, and 0.25 mg/ml BSA, pH 7.0, was used. An ATP-regenerating system consisting of 2 mM creatine phosphate and 10 U/ml creatine phosphokinase was added to the system. The actual free Ca²⁺ concentrations ([Ca²⁺]ᵢ) in the buffer were adjusted using a Ca²⁺-selective electrode.

Measurements of cytosolic free [Ca²⁺]ᵢ. [Ca²⁺]ᵢ were measured with the Ca²⁺-sensitive fluorescent probe fura 2-AM in single rat pancreatic β-cells, as previously described (15, 18).

Ion channel recordings. The activity of KATP channels was studied in inside-out patches, and the activity of voltage-dependent Ca²⁺ channels was examined in the whole cell configuration of the patch-clamp technique, as previously described (15). The volume of the perfusion chamber was 300 µl, with the perfusion rate 4 ml/min.

Glucose metabolism measurements. Glucose oxidation and glucose utilization in rat islets were analyzed according to procedures previously described (19).

Measurements of DAG concentration. The procedure for DAG measurements was adopted from Kanoh et al. (9). One hundred islets were incubated in KRB buffer with test compounds at 37°C for 10 min. Incubation was terminated by taking out the medium and adding 0.2 ml of 0.2% SDS and 0.75 ml of chloroform-methanol (1:2). The cellular lipid fraction was extracted at 4°C for 1 h. The water fraction and organic fraction were separated. The organic fraction was washed with methanol and 0.2 M NaCl and dried. Extracted

Fig. 1. Concentration-response curves for effects of the imidazoline RX871024 on insulin secretion in pancreatic islets from nondiabetic Wistar and diabetic Goto-Kakizaki (GK) rats in the presence of 3 (○ Wistar, ■ GK) and 15 mM (○ Wistar, ■ GK) glucose. Data are means ± SE for 16 observations from 3 independent islet preparations. Insulin secretion expressed either in absolute values (A) or as a percentage of insulin secretion at the corresponding glucose concentration and without the imidazoline derivative RX871024 (B). *P < 0.05, **P < 0.01, ***P < 0.001 vs. insulin secretion at the corresponding glucose concentration and without RX871024. † †P < 0.05, † † †P < 0.01 vs. insulin secretion at 15 mM glucose and corresponding RX871024 concentration in Wistar islets.

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Lipids were dissolved in a buffer containing (in mM): 100 Tris-HCl, 2.5 sodium deoxycholate, and 1.25 dithiothreitol, pH 7.4. Twenty microliters of lipid solution and 20 μl of 100 mM Tris-HCl, 1 mM EGTA, and 2 μg DAG kinase, pH 7.4, were mixed. Reaction of DAG conversion to phosphatidic acid was initiated by addition of 1.6 mM [γ-32P]ATP (5,000 cpm/nmol). Samples were incubated at 29°C for 30 min. Then, a chloroform-methanol (1:2) mixture was added, and water and organic phases were separated by centrifugation. The water layer was discarded, and the organic layer was washed with 1% perchloric acid. The organic phase was dried, dissolved in chloroform, and applied on thin-layer chromatography (TLC; Silica gel 60 plates, Merck, Darmstadt, Germany), using chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5) as a developing solvent. The TLC plate with separated lipids was submitted to autoradiography with the use of Bio-Imaging Analyzer BAS 1000 (Fuji, Japan).

Statistical analysis. The difference of means was estimated with a t-test or analyses of variance (ANOVA), with P values corrected by the Bonferroni method using Statistica for Windows (version 5.0, StatSoft, Tulsa, OK).

RESULTS

Effects of RX871024 and glibenclamide on insulin release in batch-incubated GK and Wistar rat islets. In GK rat islets, insulin release in response to 15 mM glucose was significantly decreased compared with that in nondiabetic Wistar rat islets (Fig. 1A). Addition of RX871024 stimulated insulin release in both animals, the effect being particularly pronounced at elevated glucose concentration. At 3 mM glucose, 10 μM and higher concentrations of RX871024 significantly increased insulin release. At 15 mM glucose, a significant increase in insulin release was observed already at 1 μM RX871024 (Fig. 1A). At 15 mM glucose, the relative stimulation at any particular concentration of RX871024 was higher in GK rat islets compared with nondiabetic islets (Fig. 1B). At 50 μM RX871024, insulin release in GK rats in the presence of 15 mM glucose reached the level of 59% of the response in correspond-

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Table 1. Time parameters for [Ca^{2+}]_i response development in GK and Wistar rat pancreatic β-cells

<table>
<thead>
<tr>
<th>Glucose, mM</th>
<th>RX871024</th>
<th>t_{in} (ms)</th>
<th>t_{max} (ms)</th>
<th>n</th>
<th>RX871024</th>
<th>t_{in} (ms)</th>
<th>t_{max} (ms)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>10 μM</td>
<td>200 ± 25*</td>
<td>149 ± 9 †</td>
<td>11</td>
<td>321 ± 43</td>
<td>130 ± 11</td>
<td>234 ± 21</td>
<td>10</td>
</tr>
<tr>
<td>50 μM RX871024</td>
<td>96 ± 10*</td>
<td>102 ± 10</td>
<td>9</td>
<td>7</td>
<td>74 ± 6</td>
<td>61 ± 5</td>
<td>118 ± 12</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>10 μM</td>
<td>158 ± 18*</td>
<td>206 ± 15 ‡</td>
<td>17</td>
<td>116 ± 9</td>
<td>145 ± 8</td>
<td>139 ± 19</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>50 μM RX871024</td>
<td>27 ± 10†</td>
<td>88 ± 20*</td>
<td>7</td>
<td>74 ± 6</td>
<td>61 ± 5</td>
<td>118 ± 12</td>
<td>7</td>
</tr>
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Data are means ± SE; n = 7–20 observations from 4 independent cell preparations. [Ca^{2+}]_i, Intracellular Ca^{2+} concentration; GK, Goto-Kakizaki rats; RX871024, an imidazoline derivative; t_{in}, lag time for [Ca^{2+}]_i response; t_{max}, time of development of maximal [Ca^{2+}]_i response. In the case of 3 mM glucose + 10 μM RX871024, only t_{in}, when oscillations started, was estimated. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. the same parameter in Wistar rats.
Fig. 4. Comparison of RX871024-induced changes in \([Ca^{2+}]_i\) in single Wistar (A and C) and GK (B and D) rat pancreatic \(\beta\)-cells at basal glucose concentration (3 mM). Time of additions of RX871024 is shown by arrows. Representative traces out of 7–20 from 4 independent cell preparations.

Fig. 5. Single ATP-dependent \(K^+\) (\(K_{ATP}\)) channel recordings in \(\beta\)-cells isolated from GK and Wistar rat islets. Recordings of freshly isolated inside-out patches from Wistar (A) and GK (B) \(\beta\)-cells exposed to 50 \(\mu\)M RX871024 and channel open probability (\(P_{\text{open}}\)) analysis of the corresponding recordings from Wistar (C) and GK (D) \(\beta\)-cells. \(P_{\text{open}}\) was assessed during 1-s frames. E: mean current after addition of RX871024; F: latency between addition of the compound and channel inhibition. Single-channel kinetics in both cell types was not altered in the presence of RX (data not shown). Representative traces out of 5 from 3 independent cell preparations.
Insulin release in response to glucose was decreased in isolated islets from GK rats over a broad range of glucose concentrations (Fig. 2). At maximal effective concentrations of RX871024 (50 μM) and glibenclamide (2 μM), both compounds significantly increased insulin release at all glucose concentrations in Wistar and GK rats. However, RX871024 produced a larger potentiation of insulin release at all glucose concentrations in both Wistar and GK rat islets (Fig. 2).

Influence of 15 mM glucose on [Ca^{2+}]_i in pancreatic β-cells from GK and Wistar rats. In GK rat β-cells, the increase in [Ca^{2+}]_i, after addition of 15 mM glucose, was slower compared with that in Wistar rat β-cells (Fig. 3). One of the reasons was a delayed lag time for [Ca^{2+}]_i response (t_{in}) (Table 1).

Effects of RX871024 on [Ca^{2+}]_i response in pancreatic β-cells from GK and Wistar rats. Addition of RX871024 increased [Ca^{2+}]_i in β-cells from GK and Wistar rats. At basal glucose concentration (3 mM), an oscillatory pattern of Ca^{2+} response was observed with 10 μM RX871024 (Fig. 4, A and B). At 50 μM RX871024, the increase in [Ca^{2+}]_i, was higher, and oscillations were rarely observed (Fig. 4, C and D). The lag time for the [Ca^{2+}]_i responses in GK animals was significantly shorter than in nondiabetics at both concentrations of RX871024 and decreased with the elevation of RX871024 concentration from 10 to 50 μM (Table 1). In the presence of 15 mM glucose, [Ca^{2+}]_i responses to RX871024 were faster than those at basal glucose concentration in both GK and Wistar rats (Fig. 3). Despite the pronounced differences in kinetics of [Ca^{2+}]_i responses to RX871024 between the two groups of animals (Table 1), there were no significant differences in the amplitude of [Ca^{2+}]_i responses between GK and Wistar rat β-cells. The increases in [Ca^{2+}]_i, in GK rat β-cells were 114 ± 30% (n = 8), 70 ± 26% (n = 7), and 90 ± 20% (n = 9) of corresponding responses in Wistar β-cells at 50 μM RX871024 and 3.3 mM glucose (n = 10), at 10 μM RX871024 and 15 mM glucose (n = 11), and at 50 μM RX871024 and 15 mM glucose (n = 7), respectively.

Effects of RX871024 on K_{ATP} channel activity in pancreatic β-cells from GK and Wistar rats. To examine whether the aforementioned decreased lag time for [Ca^{2+}]_i responses to RX871024 in GK rat β-cells compared with Wistar β-cells was due to a more efficient regulation by the compound of the K_{ATP} channel, patch-clamp experiments (inside-out configuration) were performed (Fig. 5, A and B). Inhibitions of K_{ATP} channel by RX871024 tend to develop faster in GK rat β-cells compared with nondiabetic β-cells (Fig. 5F, P = 0.11), the results being in line with the effect of the compound on [Ca^{2+}]_i. No difference in amplitude of RX871024-induced inhibition or single-channel kinetics between two cell types has been observed (Fig. 5, C-E).

Activity of voltage-gated Ca^{2+} channels was recorded in β-cells from GK and Wistar rats by use of the
whole-cell configuration. RX871024 did not show any effect on L- and T-type voltage-gated Ca\(^{2+}\) channels in \(\beta\)-cells from either animal model (data not shown).

Effects of RX871024 on insulin release in perifused GK and Wistar rat islets. To evaluate whether RX871024 affects glucose metabolism in GK and Wistar rats, the effect of the compound on glucose oxidation and glucose utilization was studied (Fig. 8). The obtained data confirmed our previous studies showing that the rates of glucose oxidation and glucose utilization are higher in GK rat islets (11). However, neither 10 nor 50 \(\mu\)M RX871024 influenced glucose oxidation or glucose utilization in the two groups of animals.

Effects of RX871024 on DAG concentration in GK and Wistar rat islets. DAG is an example of a second messenger that plays an important role in the process of insulin secretion in the pancreatic \(\beta\)-cell (10, 12). Hence, effects of RX871024 on DAG formation were examined in pancreatic islets from nondiabetic and diabetic rats. At 3 mM glucose, 50 \(\mu\)M RX871024 induced a 30% increase in DAG concentration in Wistar rat islets (Fig. 9). Basal DAG concentration in GK islets was 50% higher than in Wistar rat islets. RX871024 at 50 \(\mu\)M produced a nearly 100% DAG increase over basal level in GK rat islets. The increase in DAG concentration produced in GK rat islets was significantly higher than that in Wistar rat islets.

DISCUSSION

The aim of this study was to evaluate the effect of the imidazoline compound RX871024 on insulin release in pancreatic islets from GK rats, an animal model of type I diabetes, which was similar to that described previously (4, 15). RX871024 stimulated insulin release in electropermeabilized islets from GK rats too (Fig. 7). There were no significant differences in the effects of RX871024 between the two groups of animals.

Effects of RX871024 on glucose oxidation and glucose utilization in islets from GK and Wistar rats. To examine whether RX871024 affects glucose metabolism in GK and Wistar rats, the effect of the compound on glucose oxidation and glucose utilization was studied (Fig. 8). The obtained data confirmed our previous studies showing that the rates of glucose oxidation and glucose utilization are higher in GK rat islets (11). However, neither 10 nor 50 \(\mu\)M RX871024 influenced glucose oxidation or glucose utilization in the two groups of animals.
2 diabetes, which demonstrates a severe impairment in insulin release in response to glucose both in vivo and in vitro (1, 7, 11). Effects of RX871024 were compared with those of the classic sulfonylurea compound glibenclamide. We have confirmed our previous findings obtained in the islets from normoglycemic Wistar rats, namely that the imidazoline compound RX871024 was more effective in promoting islet insulin release compared with the sulfonylurea glibenclamide (4). In islets from diabetic GK rats, RX871024 was also a more potent insulinotropic agent than glibenclamide.

An important finding of this study was that, in GK rat islets, RX871024 induced a progressive rise in stimulation of insulin release in response to glucose when the concentration of glucose was increased from 3 to 15 mM. What, then, can constitute the mechanisms behind the pronounced effect of RX871024 on glucose-induced insulin release in GK rats? We addressed this question by studying effects of RX871024 on [Ca^{2+}], insulin exocytosis, glucose metabolism, and DAG concentration in rat pancreatic islets.

The [Ca^{2+}]i response to 15 mM glucose was delayed in GK rat islets compared with nondiabetic rat islets. This observation is in line with our previous study demonstrating a delayed [Ca^{2+}]i response to high glucose in GK rat islets compared with that in Wistar rat islets (16). However, comparison of [Ca^{2+}]i responses to RX871024 in pancreatic β-cells from GK and Wistar rats has shown a faster [Ca^{2+}]i response to RX871024 stimulation in diabetic compared with nondiabetic β-cells. The faster [Ca^{2+}]i response to RX871024 in GK rat β-cells might be explained by the faster blockade of KATP channel activity with RX871024 in diabetic rats. However, we have not observed significant changes in the dynamics of RX871024-induced insulin release in islet perfusion experiments between the two groups of animals.

Comparative studies of the effects of RX871024 on insulin release in electropermeabilized GK and Wistar rat islets under Ca^{2+}-clamped conditions and in the presence of high ATP concentration showed no significant differences between the two groups of animals. Hence, under these conditions, the ability of RX871024 to activate the insulin exocytotic machinery was the same in GK and Wistar rats. Likewise, RX871024 did not affect glucose metabolism in islets from GK or Wistar rats. This indicates that the higher potentiation of glucose-induced insulin secretion by RX871024 in GK rat islets cannot be attributed to a direct effect of RX871024 on glucose metabolism.

We have, however, found a significantly increased islet DAG level in GK rats under basal conditions. These data are similar to a previous observation that muscles from GK rats have also increased levels of DAG (3). The increased level of DAG in GK rat islets may probably be explained by increased de novo synthesis of DAG from glucose under conditions of persistent hyperglycemia, as has been reported from other tissues (14). It remains to be identified whether increased DAG levels can be responsible for the deterioration in β-cell functions in diabetic animals as has been suggested for other tissues where elevated DAG levels may be involved in adverse effects of hyperglycemia (14).

In GK rat islets, the magnitude of the DAG response to RX871024 was almost threefold higher compared with that in nondiabetic islets. The increased DAG response in GK rat islets to RX871024 may shed light on the mechanism by which RX871024 exerts this effect. Thus it may be speculated that RX871024 works as a positive modulator of DAG de novo synthesis. In this case, increased flux of glucose to DAG in GK rats and positive modulation of this pathway with RX871024 would give a synergistic increase in DAG. Alternatively, RX871024 may inhibit DAG conversion/degradation processes in the cell. Higher DAG synthesis and lower DAG conversion would give a high rise in DAG concentration in diabetic islets when treated with RX871024.

In conclusion, we have confirmed that the imidazoline compound RX871024 exhibits glucose-dependent insulinotropic activity in normal rats (15). In diabetic GK rat islets, RX871024 elicits pronounced insulinotropic effects and restores glucose sensitivity. The restoration of glucose sensitivity seen in GK rat islets could, at least in part, be accounted for by the higher RX871024-induced DAG elevation. These observations favor the idea that this class of imidazoline compounds may form the basis of a novel principle for treatment of type 2 diabetes.

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