COMPOUNDS BLOCKING ATP-dependent K⁺ channels (K\(_{ATP}\)) in pancreatic β-cells stimulate insulin release by causing membrane depolarization with subsequent elevation of cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(]_i\)). Sulfonylureas have been used for several decades in the treatment of diabetes and have been regarded as selective blockers of K\(_{ATP}\) channels. The sulfonylurea receptor (SUR) has recently been cloned and appears to be not the channel itself but a protein regulating the conductance of the K\(_{ATP}\) channel (1). SUR belongs to a large family of ATP-binding cassette (ABC) proteins (12). We have shown that the sulfonylureas glibenclamide and tolbutamide stimulate insulin secretion in permeabilized and voltage-clamped cells, suggesting that sulfonylurea can promote insulin secretion by two mechanisms, namely closure of K\(_{ATP}\) channels and a direct stimulatory effect on exocytosis (7, 8).

Another group of compounds containing imidazoline and closing K\(_{ATP}\) channels has attracted interest after the observation that α\(_2\)-antagonists with imidazoline structure stimulate insulin release by specific sites that are distinct from α\(_2\)-adrenoceptors (6, 16, 22). Imidazoline binding sites mediating stimulation of insulin secretion in pancreatic β-cells are also different from imidazoline binding sites of I\(_A\) and I\(_D\) types (5, 15, 21). Stimulation of insulin release by the imidazoline RX-871024 cannot be explained solely by closure of K\(_{ATP}\) channels. Our previous study (25) demonstrated that this imidazoline derivative was able to increase insulin release by directly affecting the exocytotic machinery, without parallel changes in membrane potential and [Ca\(^{2+}\)\(]_i\).

Thus RX-871024 represents a new class of compounds with insulinotropic effects. In the evaluation of the suitability of using this compound as a candidate in the treatment of diabetes, it is important to compare effects of RX-871024 with those of sulfonylurea on various aspects of the β-cell stimulus-secreting coupling. The present study was therefore undertaken to clarify signaling pathways and possible interactions between the sulfonylurea glibenclamide and the imidazoline RX-871024 in the regulation of the insulin secretory process.

MATERIALS AND METHODS

Materials. The imidazoline compound RX-871024 was obtained from Reckitt and Colman, and glibenclamide as well as diazoxide was from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Perfusion of isolated pancreas. Pancreas was isolated from Wistar rats (170–230 g) as described earlier (14) and placed in a perfusion chamber. Perfusion medium entered the pancreas through a cannula inserted in the aorta. A Krebs-Ringer bicarbonate (KRB) buffer containing (in mM) 115 NaCl, 4.7 KCl, 2.4 CaCl\(_2\), 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 20 NaHCO\(_3\), 10 HEPES, and 0.2% albumin, at pH 7.4 and supplemented with 3.3 mM glucose was used as perfusion medium. The flow rate of perfusion was 2.8 ml/min. Perfusate samples were collected, frozen, and used for radioimmunoassay.

Isolation and incubation of islets of Langerhans. Pancreatic islets were isolated from Wistar rats by collagenase digestion as previously described (17) and were maintained overnight in RPMI 1640 culture medium (Flow Laboratories) containing 11 mM glucose and supplemented with 10%
(vol/vol) fetal calf serum, 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin.

For measurements of insulin release in intact islets, static incubations were used. Islets were preincubated in KRB supplemented with 3.3 mM glucose for 30 min at 37°C. Batches of three islets were then incubated for 60 min at 37°C in KRB with appropriate test substances. The incubation was stopped by chilling the samples on ice. The incubation medium was removed and stored at −20°C until its insulin content was analyzed by radioimmunoassay.

Insulin release in electroporpermeabilized islets was studied as previously described (13). Briefly, islets were washed in a permeabilization buffer containing (in mM) 140 potassium glutamate, 5 NaCl, 1 MgCl₂, 10 EGTA, 25 HEPES, and 0.025% albumin. pH was adjusted to 7.0 with KOH. Islets were electroporpermeabilized in this buffer by five pulses of a 3-kV/cm electric field. Electroporpermeabilized islets were incubated in groups of three for 15 min at 37°C in modified permeabilization buffer with an ATP-regenerating system containing 2 mM ATP, 2 mM creatine phosphate, 10 U/ml creatine phosphokinase, and different concentrations of free Ca²⁺. The actual Ca²⁺ concentration in the buffer was adjusted with CaCl₂ using a Ca²⁺-selective electrode (Orion Research) and solutions with standard Ca²⁺ concentrations (World Precision Instruments, New Haven, CT). Insulin content was analyzed by radioimmunoassay.

Measurements of [Ca²⁺]. For [Ca²⁺] measurements, KRB was used. [Ca²⁺] was measured in dispersed cells from Wistar rat pancreatic islets. Cells attached to a coverslip were loaded with 2 µM fura 2-AM for 30 min in KRB containing 3.3 mM glucose. After loading, the coverslip was placed in an open perfusion chamber custom built for microscopic work and maintained at 37°C. Cells were perfused with medium at a flow rate of 0.15 ml/min. Measurements of 340/380 nm fluorescence ratio, reflecting [Ca²⁺], were done as previously described (26). Cells were perfused with test substances for 1 h.

Statistics. Data analysis was carried out using programs Sigma Plot for Windows (version 1.02, Jandel) and InStat (version 1.15, Graph PAD software). All results are expressed as means ± SE for an indicated number of experiments. The statistical significance of differences between means was assessed by Student’s t-test for unpaired data and one-way analysis of variance for multiple comparisons, with P values corrected by Bonferroni method. Areas under curves (AUC) for [Ca²⁺] (AUC<sub>i</sub>) and insulin secretion (AUC<sub>Ins</sub>), corresponding to net increases in [Ca²⁺] and insulin from basal levels, were evaluated by cutting and weighing plotted traces.

RESULTS

We performed measurements of insulin secretion in batch incubations of isolated rat pancreatic islets for 1 h. At 3.3 mM glucose, stimulation of insulin secretion by RX-871024 was concentration dependent and was observed in the range of 20–50 µM, whereas concentrations <20 µM RX-871024 did not affect insulin secretion (Fig. 1A). Glibenclamide induced insulin release at concentrations of 0.05–5 µM, reaching the maximal level of stimulation at 0.5 µM. The effect of 50 µM imidazoline, the maximal effective concentration (25), on insulin secretion was larger than the effect of the maximal effective concentration of glibenclamide (0.5 µM), at ~500 and 140% of basal secretion for RX-871024 and glibenclamide, respectively (P < 0.001). The combination of glibenclamide and RX-871024 at the maximal concentration of each did not further increase insulin secretion compared with the effect of 50 µM imidazoline alone. Glibenclamide (0.05 and 2 µM), however, potentiated insulin release induced by lower concentrations of RX-871024; for example, 20 µM RX-871024 induced 500 and 120% increases of basal secretion with and without 2 µM glibenclamide, respectively (P < 0.001).

Measurements of insulin secretion were accompanied by measurements of [Ca²⁺] in β-cells from the
same islet preparation. Both RX-871024 and glibenclamide increased \([\text{Ca}^{2+}]\) at a basal glucose concentration of 3.3 mM (Fig. 1B). The effects of compounds were concentration dependent. Ten micromolar RX-871024 induced only a slight net increase (AUC<sub>Ca</sub>) in \([\text{Ca}^{2+}]\), whereas 50 µM of the imidazoline induced the same net increase in \([\text{Ca}^{2+}]\), as 2 µM glibenclamide. The data obtained clearly show a dissociation between the effects of RX-871024 and glibenclamide on insulin secretion and \([\text{Ca}^{2+}]\). A high concentration of RX-871024 was more effective than a high concentration of the sulfonylurea in stimulating insulin release but had a similar efficiency in elevating \([\text{Ca}^{2+}]\), (Fig. 1).

Figure 2 presents the original traces of \([\text{Ca}^{2+}]\), used for calculation of AUC<sub>Ca</sub> in Fig. 1B. Parameters of increases in \([\text{Ca}^{2+}]\), induced by the compounds (lag time and relative amplitude of response) are shown in Table 1. The kinetics of changes in \([\text{Ca}^{2+}]\) were dependent on concentrations of RX-871024 and glibenclamide. The effects appeared faster with increasing concentrations of the compounds. At both low and high concentrations of the compound, the lag time for the increases in \([\text{Ca}^{2+}]\) induced by RX-871024 was delayed compared with the lag time in the presence of both suboptimal and maximal concentrations of glibenclamide (10 µM RX-871024 vs. 0.05 µM glibenclamide and 50 µM RX-871024 vs. 2 µM glibenclamide). Not sustained increases in \([\text{Ca}^{2+}]\), but rather slow oscillations from the basal level were induced by 10 µM RX-871024 and 0.05 µM glibenclamide. A similar sustained increase in \([\text{Ca}^{2+}]\) (Fig. 2) resulted from 2 µM glibenclamide and 50 µM RX-871024.

Interaction between the imidazoline and the sulfonylurea was investigated also in a perfused rat pancreas. For this type of experiment, we chose the concentration of the imidazoline compound (10 µM) that at 3.3 mM glucose had a negligible effect on insulin secretion per se but strongly potentiated glibenclamide-induced insulin secretion in isolated pancreatic islets. In the perfused rat pancreas, 2 µM glibenclamide induced a modest insulin release at 3.3 mM glucose (Fig. 3). Perfusion of pancreas with 10 µM RX-871024 did not influence insulin release at a basal glucose level (3.3 mM). However, when 10 µM RX-871024 was combined with 2 µM glibenclamide, at 3.3 mM glucose, the peak as well as the integral insulin release (AUC<sub>Ins</sub>) was

![Figure 2. Effects of RX (A and B), Glib (C and D), and their combinations (E–H) on fluorescence ratio F<sub>340</sub>/F<sub>380</sub> corresponding to \([\text{Ca}^{2+}]\), in single rat pancreatic β-cells perifused with 3.3 mM Glc. Each trace is representative of 3 experiments.](image1)

![Figure 3. Insulin release in response to 10 µM RX (●), 2 µM Glib (○), and 10 µM RX with 2 µM Glib (●) in rat perfused pancreas. Results are means ± SE for 6 experiments.](image2)

### Table 1. Parameters of increases in \([\text{Ca}^{2+}]\) induced by RX and Glib in single β-cells from rat pancreatic islets

<table>
<thead>
<tr>
<th>Drug, µM</th>
<th>Lag Time of Response, s</th>
<th>Relative Increase, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 RX</td>
<td>505 ± 114†</td>
<td>51 ± 16*</td>
</tr>
<tr>
<td>50 RX</td>
<td>143 ± 18‡</td>
<td>104 ± 14</td>
</tr>
<tr>
<td>0.05 Glib</td>
<td>145 ± 25‡</td>
<td>56 ± 14*</td>
</tr>
<tr>
<td>2 Glib</td>
<td>48 ± 11</td>
<td>100 ± 13</td>
</tr>
<tr>
<td>0.05 Glib, 10 RX</td>
<td>154 ± 19*</td>
<td>93 ± 19</td>
</tr>
<tr>
<td>0.05 Glib, 50 RX</td>
<td>112 ± 24†</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>2 Glib, 10 RX</td>
<td>47 ± 18</td>
<td>111 ± 21</td>
</tr>
<tr>
<td>2 Glib, 50 RX</td>
<td>58 ± 19</td>
<td>100 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3–4. \([\text{Ca}^{2+}]\), cytoplasmic free Ca<sup>2+</sup> concentration; RX, RX-871024; Glib, glibenclamide. *P < 0.05 and †P < 0.01 relative to same parameter for 2 µM Glib. One hundred percent corresponds to Ca<sup>2+</sup> release induced by 2 µM Glib.
RX-871024 had already evoked a net increase in \([\text{Ca}^{2+}]_i\). However, in the presence of high glucose, 10 µM RX-871024 did not further increase insulin secretion stimulated by 50 µM RX-871024 and 16.7 mM glucose. Similar to mide, respectively (7, 8, 25) we have shown that the sulfonylurea glibenclamide and the compound RX-871024 promote insulin release from the \(\beta\)-cell by two mechanisms. One is by blocking K\(_{\text{ATP}}\) channels, resulting in membrane depolarization, activation of voltage-dependent \(\text{Ca}^{2+}\) channels, and an increase in \([\text{Ca}^{2+}]_i\). The other is a more distal effect of the compounds acting directly on the exocytotic machinery and unrelated to changes in the K\(_{\text{ATP}}\) channel activity. The aim of the present investigation was to further clarify possible differences in signaling and sites of interactions between these two potent insulinotropic agents.

Both RX-871024 and glibenclamide stimulated insulin secretion in permeabilized islets to a similar extent in the presence of elevated concentrations of \(\text{Ca}^{2+}\). Notably, even concentrations as low as 2 nM glibenclamide and 500 nM RX-871024 induced significant increases in insulin secretion. The combination of the maximally stimulatory concentration of glibenclamide and different concentrations of RX-871024 did not further increase insulin secretion observed with either 0.5 µM glibenclamide or 10 µM imidazoline alone. RX-871024 and glibenclamide revealed the same glucose dependency under these conditions, stimulating insulin secretion only at glucose concentrations >16 mM (Fig. 6).

Both RX-871024 and glibenclamide stimulated insulin secretion in permeabilized islets at high Glc concentration (16.7 mM). A: concentration-response curves for effects of RX (○), Glib (□), and RX in presence of 2 µM Glib (■) on insulin release for 1-h incubation. Results are means ± SE for at least 12 experiments. **P < 0.01 and ***P < 0.001 relative to insulin release at 16.7 mM Glc. ###P < 0.001 relative to insulin release at 16.7 mM Glc and 2 µM Glib. B: AUC\(_{\text{Ca}}\), 1-h perfusion, for RX, Glib, and their combination. Results are means ± SE for 5 experiments. One hundred percent represents integral response to 16.7 mM Glc and 2 µM Glib. *P < 0.05 relative to \(\text{Ca}^{2+}\) response at 16.7 mM Glc and 2 µM Glib.

**DISCUSSION**

In previous studies (7, 8, 25) we have shown that the sulfonylurea glibenclamide and the compound RX-871024 promote insulin release from the \(\beta\)-cell by two mechanisms. One is by blocking K\(_{\text{ATP}}\) channels, resulting in membrane depolarization, activation of voltage-dependent \(\text{Ca}^{2+}\) channels, and an increase in \([\text{Ca}^{2+}]_i\). The other is a more distal effect of the compounds acting directly on the exocytotic machinery and unrelated to changes in the K\(_{\text{ATP}}\) channel activity. The aim of the present investigation was to further clarify possible differences in signaling and sites of interactions between these two potent insulinotopic agents.

Both sulfonylurea and imidazolines increase \([\text{Ca}^{2+}]_i\), by closure of K\(_{\text{ATP}}\) channels (24, 25), binding to sites located on the intracellular side of the plasma membrane (6, 23). The effect of RX-871024 on \([\text{Ca}^{2+}]_i\) is delayed compared with that of glibenclamide, which is consistent with the study showing slow kinetics for the inhibition of the K\(_{\text{ATP}}\) channel current by the imidazoline compound phenotamine (20). The delay in the development of the imidazoline effect on \([\text{Ca}^{2+}]_i\), could thus be explained by slow kinetics of RX-871024 bind-
ing to the imidazoline site or by a slow rate of permeability through the cell membrane for the compound. RX-871024, as well as other imidazolines, in addition to blocking of KATP channels, inhibits the activity of delayed rectifier K⁺ channels and Ca²⁺-activated K⁺ channels (20, 25), which is not true for sulfonylureas. However, blocking of voltage-dependent K⁺ channels by RX-871024 seems not to contribute to the increase in [Ca²⁺]i produced by the compound, because the magnitude of [Ca²⁺]i increase produced by the two compounds was similar. These data are consistent with the observation that blockade of voltage-dependent K⁺ channels

Fig. 5. Effects of RX and Glib on insulin release and [Ca²⁺]i in isolated pancreatic islets in presence of 55 mM KCl and 250 µM diazoxide. A: concentration-response curves for effects of RX (○), Glib (□), and RX with 2 µM Glib (●) on insulin release in isolated pancreatic islets in presence of 3.3 mM Glc. Results are means ± SE for at least 12 experiments. B: concentration-response curves for effects of RX (○), Glib (□), and RX with 2 µM Glib (●) on insulin release in presence of 20 mM Glc. Results are means ± SE for at least 12 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 relative to insulin release at 20 mM Glc, 55 mM KCl, and 250 µM diazoxide. C: AUCCa, 1-h perifusion, for RX, Glib, and their combination. Results are means ± SE for 3 experiments. One hundred percent represents integral response at 20 mM Glc, 55 mM KCl, 250 µM diazoxide, and 2 µM Glib.

Fig. 6. Effects of 10 µM RX (○) and 0.5 µM Glib (□) on insulin release in isolated pancreatic islets in presence of 55 mM KCl and 250 µM diazoxide (●) and different Glc concentrations. Results are means ± SE for at least 12 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 for insulin release stimulated by RX relative to insulin release at 55 mM KCl, 250 µM diazoxide, and corresponding Glc concentration. #P < 0.05 and ##P < 0.01 for insulin release stimulated by Glib relative to insulin release at 55 mM KCl, 250 µM diazoxide, and corresponding Glc concentration.

Fig. 7. Stimulation of insulin release by 2 µM Glib and 20 µM RX in electropерmeabilized pancreatic islets. Results are means ± SE for 8 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 relative to insulin secretion at corresponding concentration of Ca²⁺; #P < 0.05 and ###P < 0.001 relative to insulin secretion at 50 nM Ca²⁺.
does not lead to a significant increase in insulin secretion (11).

The effects of low concentrations (5–10 µM) of imidazoline on insulin secretion were strongly dependent on the ambient glucose concentration. The compound did not initiate insulin secretion at 3.3 mM glucose but potentiated insulin secretion induced by 16.7 mM glucose. This glucose dependency is important when considering possible use of the compound in diabetic patients. RX-871024 is thereby not likely to cause hypoglycemia. From a mechanistic point of view, one of the reasons for the glucose dependency of the RX-871024 effect on insulin release can be the demand for an increased energy state of the cell. The requirement for a high energy state of the cell (high ATP/ADP ratio) for RX-871024 was obvious in experiments under Ca\textsuperscript{2+}-clamped conditions. The observed stimulatory effects of glibenclamide on insulin secretion under Ca\textsuperscript{2+}-clamped conditions in rat pancreatic islets are in agreement with our previous data on sulfonylurea effects in mouse pancreatic β-cells and in insulin-producing cell lines and clearly reflect a direct effect of sulfonylurea on the exocytotic machinery (7, 8). The fact that Gardabarrado et al. (9) claim that they cannot repeat these experiments in islets depolarized with high concentrations of KCl may simply reflect their different experimental design. Nevertheless, Gardabarrado et al. obtained a direct stimulatory effect of glibenclamide on insulin secretion in permeabilized cells, suggesting that the mechanism is also operating in their hands but that the experimental protocol used may mask this effect.

The binding site for glibenclamide is SUR, a member of the ABC superfamily (1). Other members of the ABC superfamily, such as cystic fibrosis transmembrane conductance regulator (CFTR), in addition to regulating channel activity, also affect vesicle transport (3). It might be suggested that glibenclamide, in analogy to CFTR, can regulate both K\textsubscript{ATP} channels and exocytosis through SUR. The observation that SUR is abundant in insulin secretory granules may serve as indirect evidence for the interaction between SUR and proteins involved in vesicle trafficking (18). The binding site for RX-871024 stimulating insulin secretion has not been characterized. Imidazolines do not displace glibenclamide from the membrane fraction, suggesting that these two groups of compounds do not have a common binding site (4). However, the imidazolines may regulate K\textsubscript{ATP} channel activity as well as exocytosis by binding either to SUR at a site distinct from the sulfonylurea binding site, to a protein associated with this receptor, or to a protein activating a pathway that affects SUR. This idea is supported by three observations. First, sulfonylurea and RX-871024 produced similar inhibitions of K\textsubscript{ATP} channel activity. Second, they directly induced exocytosis in a similar manner. Finally, the imidazoline RX-801080 blocked the effect of efaroxan and glibenclamide on insulin secretion (4). The fact that SUR has binding sites for ATP and/or ADP as well as phosphorylation sites for protein kinases A and C may explain the glucose dependency of the effects of sulfonylurea and RX-871024 on insulin secretion as well as their modulation by protein kinase inhibitors (8, 25).

Glibenclamide potentiated insulin secretion induced by low doses of RX-871024 in both perfused pancreas and isolated islets but did not affect insulin secretion induced by the maximal effective concentration of RX-871024. A similar mechanism probably explains the enhancing effect of the imidazoline compound efaroxan on glibenclamide-induced insulin release in rats (2). The concentration of glibenclamide applied in the present study completely blocks K\textsubscript{ATP} channel activity (27), and combination of the sulfonylurea and RX-871024 did not lead to a further increase in [Ca\textsuperscript{2+}], compared with that of glibenclamide alone. Hence, potentiation of glibenclamide-induced insulin release by RX-871024 cannot be explained by additive effects of both compounds on K\textsubscript{ATP} channel activity. This suggests the existence of a signal transduction pathway activated by RX-871024 but not by the sulfonylurea. The finding that the maximal insulinotropic effect of RX-871024 was stronger than that of glibenclamide (Figs. 2 and 4), whereas the increase in [Ca\textsuperscript{2+}] (AUC\textsubscript{Ca}) produced by these two compounds was indistinguishable, supports this notion. This more potent stimulatory effect of RX-871024 on insulin secretion disappeared when islets were either depolarized with high concentration of KCl or electropermeabilized, probably due to changes in several cell characteristics under these conditions.

In conclusion, two important and novel observations are reported in this study. 1) RX-871024 and glibenclamide stimulate insulin secretion under Ca\textsuperscript{2+}-clamped conditions through a similar mechanism, and 2) RX-871024 possesses higher insulinotropic activity than glibenclamide, which implies the existence of an RX-871024-specific pathway for stimulation of insulin secretion.

A. M. Efendic and S. V. Zaitsev contributed equally to the manuscript.

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Address for reprint requests: S. Efendic, Dept. of Molecular Medicine, Karolinska Institute, Karolinska Hospital, S-171 76 Stockholm, Sweden.

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