Inhibition of Purinoceptors Amplifies Glucose-Stimulated Insulin Release With Removal of its Pulsatality

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External ATP has been proposed to be an autocrine regulator of glucose-stimulated insulin secretion and responsible for the synchronization of the Ca\(^{2+}\) rhythm in the \(\beta\)-cells required for a pulsatile release of insulin from the pancreas. The importance of external ATP for glucose-stimulated insulin release was evaluated in rats with the aid of 2-deoxy-N-methyladenosine-3,5-bisphosphate (MRS 2179), an inhibitor of the purinoceptors known to affect the Ca\(^{2+}\) signaling in \(\beta\)-cells. The concentration of cytoplasmic Ca\(^{2+}\) was measured in single \(\beta\)-cells and small aggregates with ratiometric fura-2 technique and the release of insulin recorded from isolated islets and the perfused pancreas. Addition of 1 \mu\text{mol/l} ATP induced premature cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) oscillations similar to those found in \(\beta\)-cells exposed to 20 mmol/l glucose. In most experiments, the presence of 10 \mu\text{mol/l} MRS 2179 did not remove the glucose-induced [Ca\(^{2+}\)]\(_{i}\) rhythmicity in single \(\beta\)-cells or the synchronization seen in coupled cells. Nevertheless, the same concentration of MRS 2179 promptly interrupted the pulsatility (frequency 0.22 ± 0.01/min) of insulin secretion, raising the total amounts released from the pancreas. Prolonged exposure of islets to 1 and 10 \mu\text{mol/l} MRS 2179 enhanced insulin secretion at 20 mmol/l glucose 33\% (\(P < 0.05\)) and 63\% (\(P < 0.01\)), respectively, without affecting the release at 3 mmol/l glucose. The results support the idea that neural ATP signals entrain the islets into a common rhythm resulting in pulsatile release of insulin and that glucose stimulation of the secretory activity is counteracted by accumulation of inhibitory ATP around the \(\beta\)-cells. Diabetes 54:2126–2131, 2005

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FIG. 1. Premature oscillations of [Ca\textsuperscript{2+}], in isolated rat β-cells induced by a pulse addition (60 s) of 1 μmol/l ATP to a superfusion medium containing 20 mmol/l glucose. The dotted line indicates the beginning of the exposure to ATP. Each mark on the ordinate indicates 0 nmol/l for the trace above and/or the 600-nmol/l level for the trace below. Representative for five experiments.

Chemicals and solutions. Reagents of analytical grade and deionized water were used. Roche Diagnostics (Mannheim, Germany) supplied collagenase, BSA, and HEPES. ATP, glucagon, and methoxyverapamil were obtained from Sigma Chemical (St. Louis, MO). The acetoxymethyl ester of fura-2 was purchased from Molecular Probes (Eugene, OR), and MRS 2179 was a product of Tocris Cookson (Bristol, U.K.). The studies were performed at 37°C with a basal medium of Krebs ringer bicarbonate buffer, supplemented with 10 mmol/l HEPES, and gassed with 5% CO\textsubscript{2} to pH 7.4 (18). Medium concentrations of insulin were measured in duplicate with radioimmunoassay (10), and plasma levels of glucose were determined with the glucose oxidase technique.

Isolation of islets and preparation of β-cells. Islets were isolated with the aid of collagenase. When not taken for studies of insulin release, the islets were used for preparation of single cells or small aggregates (<10 cells) by shaking in a Ca\textsuperscript{2+}-deficient medium. After suspension in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 30 μg/ml gentamicin, the cells were allowed to attach to circular coverslips during 2–5 days culture at 37°C in an atmosphere of 5% CO\textsubscript{2} in humidified air. The selection of islets for analyses was based on their large size and low nuclear–cytoplasmic volume ratio compared with the islet cells producing glucagon and somatostatin (20).

Measurements of cytoplasmic Ca\textsuperscript{2+} in isolated β-cells. The cells were loaded with 0.5–1 μmol/l fura-2 acetoxymethyl ester, and [Ca\textsuperscript{2+}], was measured during superfusion at 37°C using an inverted microscope equipped for epifluorescence fluorometry (10). Fura-2 was excited with the aid of a monochromator (Cairn Optoscan; Faversham, Kent, U.K.), and images were collected at 510 nm with an intensified CCD camera (Extended Isis-M; Phototonic Science, Robertsbridge, U.K.). Pairs of 340- and 380-nm images, consisting of 10 accumulated video frames, were captured, followed by a delay resulting in measuring cycles of 2 s. The specimens were only illuminated during the image capture, and the excitation light was minimized by means of neutral density filters. Ratio frames were calculated after background subtraction, and [Ca\textsuperscript{2+}], was estimated as previously described (21,22) using the MetaFluor program (Universal Imaging, Downingtown, PA).

Measurements of insulin release from isolated islets. Freshly isolated islets were preincubated for 30 min in basal medium supplemented with 0.1% BSA and 3 mmol/l glucose. Each vial contained 25 islets in 1.0 ml medium. After preincubation, the medium was replaced and the islets exposed to test agents. Aliquots of medium were taken for analyses both after 12 and 45 min of incubation.

Measurements of insulin release from the perfused pancreas. Pancreas perfusion was performed by modifying the procedure of Grodsky and Fanska (23) to allow contribution of blood from the donor. Unrecycled medium supplemented with 2% BSA (wt/vol) was infused at a rate of 0.4 ml/min. After 5 min of equilibration, effluent from the portal vein was collected as 30-s portions using heparinized vials, centrifuged, and the plasma stored at −20°C.

RESULTS

Individual β-cells responded to 20 mmol/l glucose with oscillations of [Ca\textsuperscript{2+}], varying considerably in frequency (0.1–1/min) and shape (Figs. 1 and 2). However, when situated in aggregates, the β-cells were well synchronized (Fig. 3). Addition of 1 μmol/l ATP elicited premature [Ca\textsuperscript{2+}], oscillations similar to the ordinary ones (Fig. 1). The glucose-induced [Ca\textsuperscript{2+}], rhythmicity usually persisted (30 of 48 cells) in the presence of 10 μmol/l MRS 2179. In responding cells, the oscillations tended to be transformed into sustained elevation of [Ca\textsuperscript{2+}], (Fig. 2). The exposure to MRS 2179 did not affect the synchronization of the [Ca\textsuperscript{2+}], rhythmicity seen in coupled β-cells (Fig. 3).

Increase of the glucose concentration from 3 to 20 mmol/l resulted in distinct pulses (frequency 0.22 ± 0.01/min; n = 5) of insulin release from the perfused pancreas.
Initial rise of the insulin concentration from 114 ± 22 to 1,087 ± 62 pmol/l (n = 5) was followed by cyclic variations with lower peak values. The effects of MRS 2179 on glucose-stimulated insulin release from the pancreas are shown in Fig. 5. At a concentration of 1 μmol/l, MRS 2179 affected neither the amplitude nor the frequency of the oscillations (Fig. 5A). However, exposure to 10 μmol/l MRS 2179 was accompanied by decreased variations of the release (Table 1) and loss of significant insulin pulses verified by cluster analysis (Fig. 5B). The latter effect was due to interruption of the downstroke of the oscillations with resulting increase (P < 0.001) of average insulin release (Table 1).

Further evidence that inhibition of purinoceptors results in an enhanced insulin release from β-cells stimulated with glucose was obtained in experiments with isolated islets. As shown in Table 2, neither 1 nor 10 μmol/l MRS 2179 modified the release of insulin during an initial 12-min period. Subsequent exposure to MRS 2179 did not affect the insulin secretion at 3 mmol/l glucose but increased the amounts released at 20 mmol/l glucose with 33% (1 μmol/l MRS 2179) and 63% (10 μmol/l MRS 2179), respectively.

**DISCUSSION**

Divergent opinions have been expressed whether external ATP has stimulatory or inhibitory effects on insulin release. The controversy is not only due to species differences in the types of purinoceptors expressed in the β-cells. In the rat, the addition of ATP to a medium containing 8.3 mmol/l glucose has been reported to stimulate insulin release from both isolated islets (11) and the perfused pancreas (25). However, other studies have indicated a dual action of ATP on rat islets exposed to 8.3 mmol/l glucose with inhibition of insulin release when the ATP concentration exceeds 1 μmol/l (17). The present data confirm the existence of an inhibitory component in the action of external ATP, demonstrating that the purinoceptor antagonist MRS 2179 augments insulin release at a concentration of glucose (20 mmol/l), which promotes exocytosis.

It is evident from analyses of isolated mouse β-cells that activation of purinoceptors results in prompt depolarization (15), generating premature oscillations based on entry of Ca²⁺ (10). The effect is mimicked by other stimulators of phospholipase A₂ (PLA₂) and disappeared when this enzyme was inhibited. It is known from studies of insulin-secreting HIT cells (26) and ordinary mouse β-cells (15,27) that the products of PLA₂ (arachidonic acid and lyso-phospholipids) have a suppressive effect on ATP-sensitive K⁺ channels. We now observe that ATP generates premature oscillations of [Ca²⁺]ᵢ also in rat β-cells, suggesting that purinoceptor activation of PLA₂ is a general mechanism for depolarization of pancreatic β-cells.

Like in other species, the β-cells from rats have an intrinsic ability to generate slow oscillations of [Ca²⁺]ᵢ (28–32). There is no direct proof for a corresponding rhythmicity of circulating insulin, since the studies performed so far with peripheral blood refer to samples taken at intervals as long as 3 min (33). Analyses of 1-min
portions of the perifusate from rat pancreas revealed insulin pulses of ~6 min (34,35). Increasing the sampling intensity to 30 s, we now demonstrate statistically significant peaks of insulin release with a duration of 4.5 min.

The isolated β-cells differed considerably with regard to shape and frequency of their [Ca^{2+}]_i oscillations. To understand how β-cells in a rat pancreas overcome these differences and generate pulsatile release of insulin into the portal vein, it is important to distinguish between the coordination within and among the islets (10). Within the islet, the β-cells are supposed to interact with mutual entrainment of the [Ca^{2+}]_i oscillations into a common rhythm via both gap junctions and diffusible messengers. Evidence has been provided that β-cells receive and propagate ATP signals, which act as a coupling force for synchronization by temporarily interrupting the electrical activity (2,10). The efficiency of the intraislet coordination of the rat β-cells is illustrated from the observation that pulses of insulin release from different islets have a similar frequency (3). Periodic variations of circulating insulin require that the β-cell oscillations of [Ca^{2+}]_i appear in the same phase in the numerous islets of a pancreas. Previous studies (36) of insulin release from the perfused rat pancreas support the idea that the islets communicate via neurons. Indeed, there are reasons to believe that intermittent release of ATP from nerves entrains the differently phased islets into a common rhythm (10).

The effects of external ATP on rat β-cells are mediated by both unspecific cation channels, referred to as P2X receptors, and G-protein–coupled P2Y receptors (25). Activation of rapidly desensitizing P2X receptors evokes a small and transient increase of basal insulin release at low glucose concentrations (37). However, the P2Y receptors are important modulators of the release of insulin evoked by stimulatory concentrations of glucose (17,37). So far, receptor genes for P2Y_1, P2Y_2, P2Y_4, and P2Y_6 have been detected in rat islets (38), and the P2Y_4 subtype was found in β-cells with immunocytochemistry (39). Addition of 100 μmol/l pyridoxalphosphate-6-azophenyl-2,4′-disulfonic acid, an antagonist of the P2Y_4 and P2Y_6 receptors (7), has been reported to lack effects on the release of insulin from rat islets exposed to 8.3 mmol/l glucose (17). Using the antagonist MRS 2179, which is relatively specific for the P2Y_1 receptors (40,41), we now observe a >60% increase of insulin release in the presence of 20 mmol/l glucose. As an adenosine derivative with two phosphate groups, it is unlikely that MRS 2179 penetrates into the β-cells. Withdrawal of MRS 2179 during superfusion of β-cells from ob/ob mice is known to result in a prompt reversal of its inhibitory effect on [Ca^{2+}]_i, transients (2).

Several reports (11–16) have drawn attention to exocytotic release of ATP as a means for autocrine control of insulin secretion. The present observations make it possible to propose both positive and negative feedback loops for the ATP released from a glucose-stimulated β-cell. It is well established that a metabolically derived signal (ATP-to-ADP ratio) accounts for the slow (2–5 min) periods of depolarization responsible for the Ca^{2+} entry generating the insulin pulses (1,42). Initially, the discharge of ATP can

### TABLE 1
Effects of MRS 2179 on glucose-stimulated insulin release from the perfused pancreas

<table>
<thead>
<tr>
<th>Composition of medium</th>
<th>Insulin release (ng/islet)</th>
<th>Glucose (mmol/l)</th>
<th>MRS 2179 1 μmol/l</th>
<th>MRS 2179 10 μmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (control)</td>
<td>0.26 ± 0.02</td>
<td>0.24 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.25 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 (control)</td>
<td>1.78 ± 0.17</td>
<td>3.10 ± 0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.57 ± 0.20</td>
<td>4.13 ± 0.33*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.62 ± 0.16</td>
<td>5.05 ± 0.50†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE for six batches of islets, each isolated from a single rat. *P < 0.05, †P < 0.01 vs. control values with 20 mmol/l glucose.

### TABLE 2
Effects of MRS 2179 on insulin release from isolated rat islets

<table>
<thead>
<tr>
<th>Composition of medium</th>
<th>Glucose (mmol/l)</th>
<th>Insulin release (ng/islet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>MRS 2179 1 μmol/l</td>
<td>0–12 min</td>
</tr>
<tr>
<td>3 (control)</td>
<td>—</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>20 (control)</td>
<td>—</td>
<td>1.78 ± 0.17</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>1.57 ± 0.20</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>1.62 ± 0.16</td>
</tr>
</tbody>
</table>

Data are means ± SE for five separate experiments. Insulin concentrations in samples of perfusate collected during 5-min periods before and during exposure to MRS 2179 in the presence of 20 mmol/l glucose (see Fig. 5). The later period started 5 min after the addition of the antagonist. Maximal variations refer to differences noted between the highest and lowest level of insulin during each observation period. *P < 0.001 vs. periods without MRS 2179.
be expected to have a positive feedback on insulin release by both augmenting the depolarization via activation of PLA₂ (see above) and mobilizing the Ca²⁺ stored in the endoplasmic reticulum. However, during the oscillatory cycles, there will be an accumulation of inhibitory ATP around the β-cells so that a negative feedback loop will dominate. The presence of negative feedback is consistent with the report that ATP (>1 μmol/l) acts as a G-protein-coupled inhibitor of L-type Ca²⁺ channels in rat β-cells (16). The concentration of ATP reached at the surface of glucose-stimulated rat β-cells has been reported to be >25 μmol/l (14). The concept of accumulation of inhibitory ATP around the β-cells explains why the addition of the purinoceptor antagonist MRS 2179 enhanced insulin release from rat islets exposed to 20 mmol/l glucose.

A prerequisite for pulsatile release of insulin from pancreas is that the β-cells generate oscillations of [Ca²⁺], and that this secretory signal will be entrained into a common rhythm. Within the islets, the synchronization of the β-cell rhythmicity is supposed to occur via both gap junctions (43) and diffusion of ATP (2). Neural activity with intermittent discharge of ATP will then provide the phase shift of the [Ca²⁺] oscillations, coordinating the secretory activity of the different islets in the pancreas (10). The present data reinforce the existing arguments that external ATP has a key role for insulin secretion by demonstrating prompt disappearance of insulin pulses during exposure to a purinoceptor antagonist. The observations that MRS 2179 removes the pulsatility of insulin release into the portal vein with maintenance of β-cell oscillations of [Ca²⁺], and their synchronization in aggregates suggest that the neural coordination of the islets is critically dependent on activation of purinoceptors.

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