

# ADP mediates inhibition of insulin secretion by activation of P2Y<sub>13</sub> receptors in mice

S. Amisten · S. Meidute-Abaraviciene · C. Tan ·  
B. Olde · I. Lundquist · A. Salehi · D. Erlinge

Received: 22 March 2010 / Accepted: 19 April 2010 / Published online: 6 June 2010  
© Springer-Verlag 2010

## Abstract

**Aims/hypotheses** To investigate the effects of extracellular purines on insulin secretion from mouse pancreatic islets.

**Methods** Mouse islets and beta cells were isolated and examined with mRNA real-time quantification, cAMP quantification and insulin and glucagon secretion. ATP release was measured in MIN6c4 cells. Insulin and glucagon secretion were measured in vivo after glucose injection.

**Results** Enzymatic removal of extracellular ATP at low glucose levels increased the secretion of both insulin and glucagon, while at high glucose levels insulin secretion was reduced and glucagon secretion was stimulated, indicating an autocrine effect of purines. In MIN6c4 cells it was shown that glucose does induce release of ATP into the extracellular space. Quantitative real-time PCR demonstrated the expression of the ADP receptors P2Y<sub>1</sub> and P2Y<sub>13</sub> in both intact mouse pancreatic islets and isolated beta cells. The stable ADP analogue 2-MeSADP had no effect on insulin secretion. However, co-incubation with the P2Y<sub>1</sub> antagonist MRS2179 inhibited insulin secretion, while co-incubation with the P2Y<sub>13</sub> antagonist MRS2211 stimulated insulin secretion, indicating that ADP acting via P2Y<sub>1</sub> stimulates insulin secretion, while signalling via P2Y<sub>13</sub>

inhibits the secretion of insulin. P2Y<sub>13</sub> antagonism through MRS2211 per se increased the secretion of both insulin and glucagon at intermediate (8.3 mmol/l) and high (20 mmol/l) glucose levels, confirming an autocrine role for ADP. Administration of MRS2211 during glucose injection in vivo resulted in both increased secretion of insulin and reduced glucose levels.

**Conclusions/interpretation** In conclusion, ADP acting on the P2Y<sub>13</sub> receptors inhibits insulin release. An antagonist to P2Y<sub>13</sub> increases insulin release and could be evaluated for the treatment of diabetes.

**Keywords** ADP · ATP · Beta cell · Glucose · Insulin · P2 receptors · P2Y<sub>1</sub> · P2Y<sub>13</sub> · P2Y<sub>1</sub> · P2Y<sub>13</sub>

## Introduction

High glucose levels stimulate the release of ATP into the extracellular space from several tissues and cell types such as endothelial cells, blood vessels, mesangial cells, macrophages and beta cells [1–5]. The net pharmacological effects of extracellular ATP are difficult to predict since ATP by itself can stimulate P2Y<sub>2</sub>, P2Y<sub>4</sub> (in rodents), P2Y<sub>11</sub> and P2X<sub>1</sub>–P2X<sub>7</sub> receptors present on the cell surface [6]. Furthermore, ATP is rapidly degraded by ectonucleotidases to ADP [7], which can act on P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub> receptors. ADP is then further degraded by ecto-5'-nucleotidase to adenosine, which in turn can activate four different adenosine receptors [8].

A large number of studies indicate that extracellular ATP and ADP have a key role in regulating insulin secretion by purinoreceptors [9–13]. Insulin-secretory granules contain 3.5 mmol/l ATP and 5 mmol/l ADP [14], and with a novel biosensor it has been demonstrated

**Electronic supplementary material** The online version of this article (doi:10.1007/s00125-010-1807-8) contains supplementary material, which is available to authorised users.

S. Amisten · C. Tan · B. Olde · D. Erlinge (✉)  
Department of Cardiology, Lund University,  
Skane University Hospital,  
221 85 Lund, Sweden  
e-mail: david.erlinge@med.lu.se

S. Meidute-Abaraviciene · I. Lundquist · A. Salehi  
Department of Clinical Science, Lund University,  
Skåne University Hospital,  
Malmö, Sweden

that glucose stimulation releases ATP from a single pancreatic beta cell to a local extracellular ATP concentration exceeding 25  $\mu\text{mol/l}$  [4].

ADP has been shown to increase insulin release via P2Y receptors *in vitro*, *in vivo* and in human beta cells [9–11]. Beta cells are activated by ADP and can also propagate this signal via intermittent release of ATP [15]. However, ADP has also been shown to decrease insulin release [12, 13]. These discrepancies are probably in part due to involvement of several different purinoreceptors [13, 15–22]. Therefore, we wanted to examine whether endogenous release of purines may regulate insulin secretion and to determine the P2Y receptors involved. To do so we quantified the mRNA levels for all the purinergic P2Y receptors, and based on these findings we did pharmacological studies on insulin and glucagon secretion in isolated mouse islets. We found an unanticipated role for ADP acting on P2Y<sub>13</sub> receptors as an autocrine inhibitor of insulin release both *in vitro* and *in vivo*.

## Methods

**Ethics** The study conforms to the Guide for the Care and Use of Laboratory Animals, US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee of Lund University, Sweden.

**Animals** Female mice of the NMRI strain (B&K Universal, Sollentuna, Sweden), weighing 25–30 g were used for *in vitro* and *in vivo* studies. They were fed a standard pellet diet (B&K Universal) and tap water *ad libitum*.

**Isolation of islets and beta cells** Collagenase (CLS 4) was obtained from Worthington Biochemicals, Freehold, NJ, USA. Bovine serum albumin was from ICN Biochemicals, High Wycombe, UK. IBMX (isobutylmethylxanthine), an inhibitor of phosphodiesterase, and all other drugs and chemicals were from Sigma Chemicals, St Louis, MO, USA or Merck AG, Darmstadt, Germany. Radioimmunoassay kits for insulin and glucagon determination were obtained from Diagnostika (Falkenberg, Sweden) and from Eurodiagnostica (Malmö, Sweden).

Pancreatic islets were isolated by retrograde injection of a collagenase solution via the bile–pancreatic duct. Islets were then collected under a stereomicroscope at room temperature. Purified islets that were to be used for total RNA extraction were collected directly in 1,000  $\mu\text{l}$  TRIzol (Invitrogen, Paisley, UK) and stored at  $-80^{\circ}\text{C}$ .

Beta cells were purified using a method previously used for enterochromaffin-like-cell purification [23] with some modifications. Freshly isolated mouse pancreatic islets

(5,000 islets) were dispersed using trypsin digestion (0.9 mg/ml, Boehringer Mannheim, Mannheim, Germany) and omission of calcium in the presence of EDTA. After dispersion, the beta cell preparations were further purified by repeated counterflow elutriation using first a standard chamber and then a Sanderson chamber (Beckman, Palo Alto, CA, USA). The enriched cells from the standard chamber were collected at 25 ml/min at 380–560 g. They were purified further in a Sanderson chamber and collected at 18 ml/min and 800 g. This cell preparation consisted of about 80% beta cells. The beta cell preparation was then subjected to density gradient centrifugation. A stock solution of 60% iodixanol (Optiprep, Nycomed Pharma, Oslo, Norway), containing 1.2 mmol/l  $\text{MgCl}_2$ , 15 mmol/l HEPES at pH 7.4 and 10 mg/ml BSA, was diluted to 15% and 10.8%, respectively, with medium consisting of (mmol/l): 140 NaCl, 1.2  $\text{MgSO}_4$ , 1  $\text{CaCl}_2$ , 15 HEPES at pH 7.4, 11 glucose, 0.5 DL-dithiothreitol and 10 mg/ml BSA. In a 15 ml centrifuge tube, 5 ml of the 15% iodixanol solution was overlaid with 5 ml 10.8% iodixanol. Enriched beta cells ( $1 \times 10^6$  cells/ml) were layered on the two layers of iodixanol and centrifuged (Beckman, Spinchron R centrifuge). A slow acceleration period (400 rpm) was followed by 5 min centrifugation during which the speed reached 1,000 rpm. Deceleration lasted 5 min. The cells in the light density fraction above the 10.8% cushion were collected. The yield of cells in this fraction was  $1 \pm 0.15 \times 10^6$  cells (mean  $\pm$  SEM,  $n=10$ ).

The purity of each beta cell preparation was assessed by RIA measurement of insulin, glucagon, somatostatin and pancreatic polypeptide per mg protein [24]. At least 10 samples (tubes) of the cells were examined for each hormone. The final cell preparation consisted of around  $95 \pm 8\%$  beta cells. The beta cells were then collected in 1,000  $\mu\text{l}$  TRIzol (Invitrogen) and stored at  $-80^{\circ}\text{C}$ .

**RNA purification, cDNA synthesis and quantitative real-time PCR** Mouse islet and beta cell total RNA was isolated using a modified TRIzol method as described elsewhere [25]: after phase separation, the upper aqueous phase was incubated at  $-20^{\circ}\text{C}$  overnight with 10  $\mu\text{g}$  glycogen (Invitrogen) and centrifuged for 15 min at 12,000 g,  $4^{\circ}\text{C}$ . After addition of ethanol, the pellet was centrifuged for 10 min at 7,500 g,  $4^{\circ}\text{C}$ . Each individual RNA pellet was dissolved in RNase-free water and converted into cDNA using TaqMan reverse transcription (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

All quantitative real-time PCR primers were designed using the VectorNTI software (Invitrogen, Informax, UK), using RefSeq mRNA sequences as templates. All PCR primers were designed to generate products of 199–201 base pairs (see [Electronic supplementary material](#)).

Light Cycler quantitative real-time PCR (Roche, Basel, Switzerland) was used to determine P2Y receptor expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to the manufacturer's instructions as described elsewhere [25].

*In vitro quantification of ATP release* ATP release was measured using an adaptation of the luciferase method previously described by Taylor et al. [26]. Briefly, MIN6c4 cells were grown in DMEM, 25 mmol/l glucose containing 15% FBS (Invitrogen). A total of 35,000 cells were plated in 35 mm Petri dishes and were allowed to grow for 24 h. The medium was then replaced with 1 ml serum-free DMEM containing 5.6 mmol/l glucose and the cells were cultivated for another 24 h. Subsequently, 1 ml 2× ATP SL reagent (BioThema, Handen, Sweden), in serum-free DMEM, 5.6 mmol/l glucose, was added and the Petri dish was incubated for 30 min at room temperature. After the baseline luciferase activity in the Petri dish had been recorded for 1 min, in a GloMax 20/20 Luminometer (Promega Corporation, Madison, WI, USA), 400 µl of the medium was removed. The test substances were added to the removed medium and returned to the Petri dish, where the luminescence was recorded for another 10 min.

*Insulin and glucagon secretion from islets in vitro* The freshly isolated islets were pre-incubated for 30 min at 37°C in KRB, pH 7.4, supplemented with 10 mmol/l *N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.1% BSA and 4 mmol/l glucose before addition of the agents to be tested. Each incubation vial contained 12 islets in 1.0 ml buffer solution and was gassed with 95% O<sub>2</sub>–5% CO<sub>2</sub> to obtain constant pH and oxygenation. After pre-incubation, the medium was changed and supplemented with various receptor agonists and antagonists. The islets were then incubated for 1 h at 37°C in a metabolic shaker (30 cycles per min). Aliquots of the medium were then removed for the assay of insulin and glucagon.

*Measurement of cAMP levels* MIN6c4 cells, at 4×10<sup>5</sup> cells/well, were cultured for 3 days. After being washed with OptiMEM and treated with 25 µmol/l Rolipram, the cells were further incubated with the test compounds for 20 min. Incubations were terminated by addition of 0.1 mol/l hydrochloric acid. Cell lysates were centrifuged at 1,000 rpm for 10 min at 4°C. cAMP levels in cell extracts were measured by ELISA using the Cyclic AMP Enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's instructions.

*In vivo measurements of insulin and glucagon secretion* Either MRS2211 (100 mg/kg) in physiological saline or

saline only was administered intraperitoneally (i.p.) 10 min prior to i.p. glucose injection of 1.2 g/kg with or without MRS2211 (50 mg/kg) i.p., and blood samples were collected at 0, 3, 10 and 40 min post glucose administration as previously described [27].

*Calculations and statistics* Calculations and statistics were performed using the GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Values are presented as mean ± SEM. Statistical significance was accepted when  $p < 0.05$  (Mann–Whitney's test).

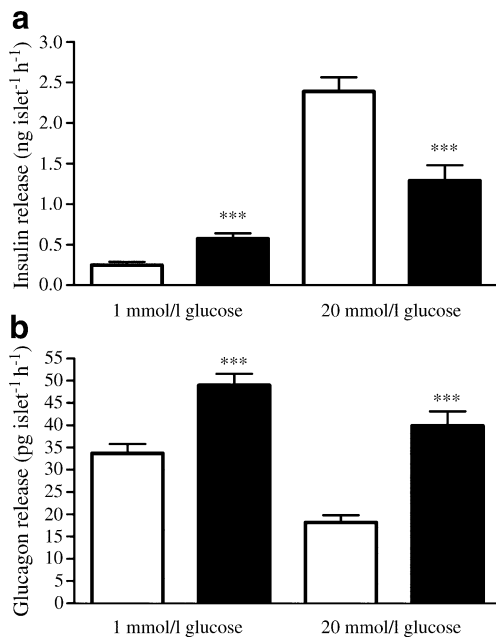
## Results

*Effects of glucose on insulin and glucagon secretion in the presence of apyrase* Apyrase rapidly eliminates ATP and ADP by degradation to AMP [28], which in turn may then be degraded to adenosine by CD73 [7]. Addition of 1 U/ml apyrase (Sigma) to pancreatic islets in the presence of 1 mmol/l glucose stimulated an increase in insulin release from 0.27±0.04 to 0.59±0.06 ng islet<sup>-1</sup> h<sup>-1</sup> ( $p < 0.001$ ). Raising the glucose level to 20 mmol/l resulted, as expected, in increased insulin release to 2.41±0.18 ng islet<sup>-1</sup> h<sup>-1</sup>. In the presence 1 U/ml of apyrase, this increase was attenuated to 1.31±0.19 ng islet<sup>-1</sup> h<sup>-1</sup> ( $p < 0.001$ ) (Fig. 1a).

Addition of 1 U/ml apyrase to pancreatic islets in the presence of 1 mmol/l glucose stimulated an increase in glucagon release from 33.9±2.1 to 49.2±2.5 pg islet<sup>-1</sup> h<sup>-1</sup> ( $p < 0.001$ ). Raising the glucose level to 20 mmol/l resulted in slightly decreased glucagon release to 18.4±1.3 pg islet<sup>-1</sup> h<sup>-1</sup> ( $p < 0.001$ ). In the presence of 1 U/ml apyrase this decrease was counteracted and the glucagon level was increased to 40.1±3.2 pg islet<sup>-1</sup> h<sup>-1</sup> ( $p < 0.001$ ) (Fig. 1b).

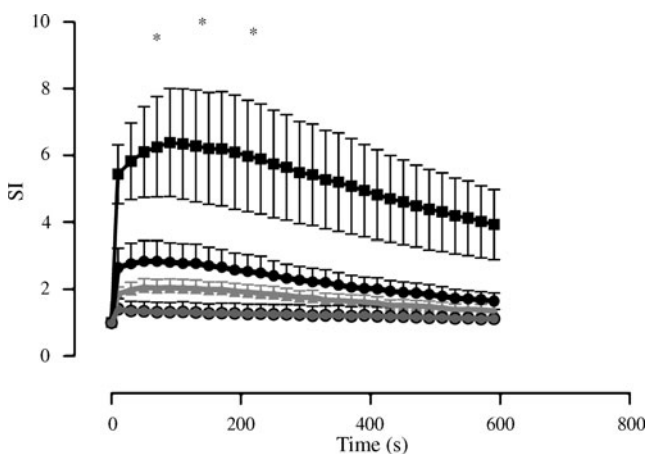
*ATP release from MIN6c4 cells* Release of ATP into the extracellular space was significantly elevated (4.8 times,  $p < 0.05$ ) in the presence of glucose (16.6 mmol/l). The ATP release was unaffected by equimolar concentrations of mannitol but was inhibited by the calcium-channel blocker nifedipine (Fig. 2) [29].

*P2Y receptor expression in mouse islets and beta cells* Real-time PCR revealed that, among the purine-sensitive P2Y receptors, the highest mRNA levels were found for the ADP receptors P2Y<sub>1</sub> and P2Y<sub>13</sub> in mouse pancreatic islets (6.7±0.6×10<sup>-3</sup> and 1.3±0.6×10<sup>-3</sup> respectively) and mouse beta cells (4.0±3.0×10<sup>-3</sup> and 1.8±0.3×10<sup>-3</sup> respectively) (ratio of GAPDH mRNA expression, Fig. 3). No expression of the ADP receptor P2Y<sub>12</sub> could be detected.



**Fig. 1** Effect of the ATP and ADP-degrading enzyme apyrase (1 U/ml) on insulin and glucagon secretion in mouse islets at different glucose concentrations. Data are expressed as mean  $\pm$  SEM,  $n=10$ , \*\*\* $p<0.001$  between control and apyrase for each glucose concentration. Black columns, apyrase; white columns, control

**Effects of ADP on insulin and glucagon secretion** At 10  $\mu\text{mol/l}$ , the stable ADP analogue 2-MeSADP, which activates both P2Y<sub>1</sub> and P2Y<sub>13</sub> receptors [30], did not have any effect per se on insulin secretion from mouse pancreatic islets at 8.3 mmol/l glucose (Fig. 4). However, after pre-treatment with the selective P2Y<sub>1</sub> receptor antagonist MRS2179 [31], 10  $\mu\text{mol/l}$  of 2-MeSADP resulted in a marked decrease in insulin secretion ( $p<0.001$ ), indicating



**Fig. 2** ATP release from MIN6c4 cells into the extracellular space. The addition of 16.6 mmol/l glucose, but not mannitol, resulted in a significant increase ( $p<0.05$ ) in ATP release that was completely abolished by the addition of the Ca<sup>2+</sup>-channel blocker nifedipine (20  $\mu\text{mol/l}$ ). Black squares, glucose; black circles, mannitol; grey triangles, nifedipine; grey circles, PBS (control). SI, signal intensity

an insulin inhibitory effect via the P2Y<sub>13</sub> receptor. In contrast, pre-treatment with the selective P2Y<sub>13</sub> receptor antagonist MRS2211 [32] resulted in a marked increase in insulin secretion ( $p<0.001$ ), indicating an insulin-stimulating effect via the P2Y<sub>1</sub> receptor.

To test whether endogenously released ADP regulates insulin release via P2Y<sub>13</sub> receptors, we added the P2Y<sub>13</sub> receptor antagonist MRS2211 (10  $\mu\text{mol/l}$ ). Interestingly, MRS2211 per se increased insulin secretion at 8.3 ( $p<0.001$ ) and 20 mmol/l glucose ( $p<0.01$ , Fig. 5a).

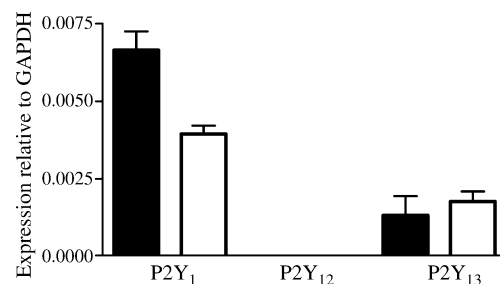
A similar increase in glucagon secretion was seen with 10  $\mu\text{mol/l}$  MRS2211 per se both at 8.3 ( $p<0.001$ ) and 20 mmol/l glucose ( $p<0.01$ , Fig. 5b).

**Effect of P2Y<sub>13</sub> on MIN6c4 cell cAMP level** We studied the mechanism underlying the stimulatory effect of the P2Y<sub>13</sub> agonist on insulin secretion. We examined the effect of 2-MeSADP on cAMP production. We found that 10 nmol/l of 2-MeSADP inhibited the cAMP level by 45% ( $p<0.0001$ , Fig. 4b), while addition of the P2Y<sub>13</sub> antagonist, MRS2211, reversed this effect of 2-MeSADP on cAMP levels ( $p<0.0001$ ).

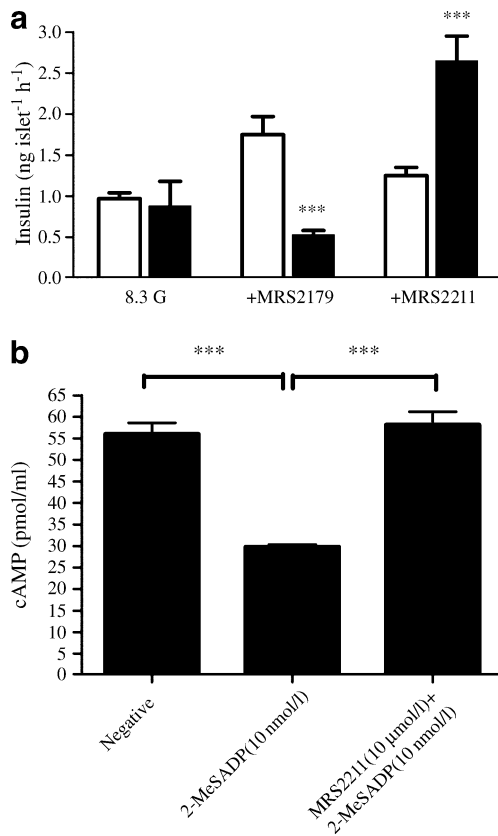
**In vivo measurements of insulin and glucagon secretion** Administration of the P2Y<sub>13</sub> antagonist MRS2211 in vivo resulted in a significant increase of insulin and a diminished suppression of glucagon secretion following an i.p. injection of glucose (Fig. 6a, c). MRS2211 in vivo also resulted in a significant decrease of glucose following the i.p. injection of glucose (Fig. 6b).

## Discussion

In this study we found evidence for glucose-stimulated endogenous release of insulin-regulating purines. In MIN6 cells, we observed that ATP release is induced by glucose, and that it can be blocked by the Ca<sup>2+</sup> channel blocker nifedipine. Using real-time PCR, we identified high mRNA levels of the ADP receptors P2Y<sub>1</sub> and P2Y<sub>13</sub>, but very low



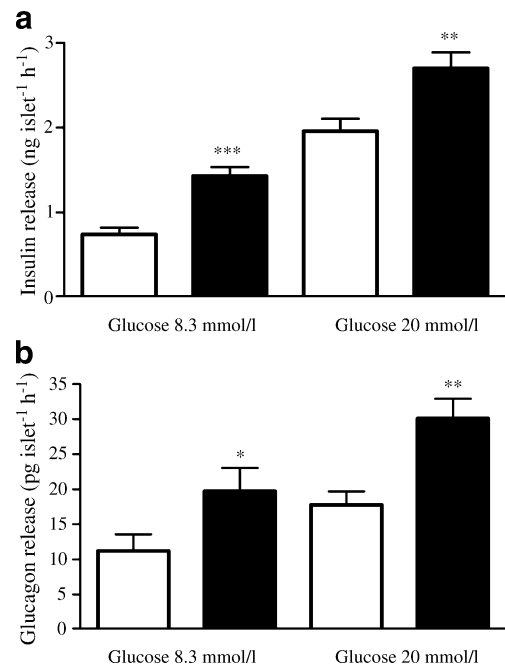
**Fig. 3** Expression of mouse ADP receptors P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub> in pancreatic islets and beta cells from the mouse ( $n=3$ ). Only trace amounts of P2Y<sub>12</sub> were detected. Black columns, whole islets; white columns, isolated beta cells



**Fig. 4** **a** Effect of the stable ADP analogue 2-MeSADP on insulin secretion in mouse islets at 8.3 mmol/l glucose. 2-MeSADP had a limited effect per se. In the presence of the P2Y<sub>1</sub> antagonist MRS2179, 2-MeSADP reduced insulin secretion. In the presence of the P2Y<sub>13</sub> antagonist MRS2211, insulin secretion was increased. Data are expressed as mean ± SEM, *n*=9, \*\*\**p*<0.001. Black columns, 2-MeSADP; white columns, control. **b** Effect of 2-MeSADP on cAMP production in MIN6c4 cells. MIN6c4 cells were treated with 2-MeSADP (10 nmol/l) in the absence or presence of MRS2211 (10 μmol/l). Cells were treated with the compounds for 20 min, in the presence of 25 μmol/l Rolipram, and were extracted with 200 μl of 0.1 mol/l hydrochloric acid and analysed for cAMP. Data are expressed as mean ± SEM, *n*=5, \*\*\**p*<0.001

levels of the ADP receptor P2Y<sub>12</sub>, in mouse islets and beta cells. ADP was found to have a dual effect on beta cells, stimulating insulin release via P2Y<sub>1</sub> receptors and inhibiting insulin release via P2Y<sub>13</sub> receptors. We discovered that glucose stimulated ATP release and that the ATP metabolite ADP activated P2Y<sub>13</sub> receptors, exerting an autocrine inhibitory effect on insulin secretion. The P2Y<sub>13</sub> antagonist MRS2211 was shown to increase glucose-stimulated insulin release in vitro. In vivo administration of the P2Y<sub>13</sub> antagonist MRS2211 resulted in increased release of insulin and lowered glucose levels, suggesting that P2Y<sub>13</sub> antagonism might be of therapeutic value for the treatment of type 2 diabetes.

Purinergic nucleotides (ATP and ADP) have been found to serve as autocrine regulators of insulin release [15, 16, 18, 20, 22]. We confirmed that nucleotides are important



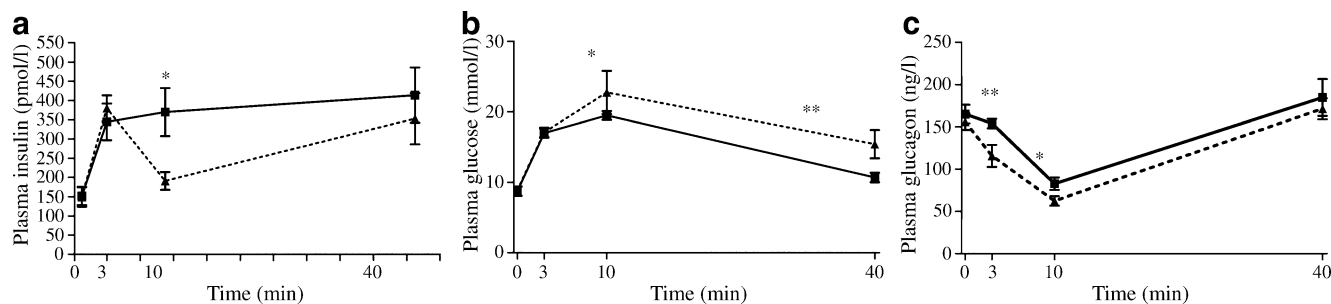
**Fig. 5** Effect of the P2Y<sub>13</sub> antagonist 10 μmol/l MRS2211 on (a) insulin and (b) glucagon secretion in mouse islets at different glucose concentrations. Data are expressed as mean ± SEM, *n*=9, \**p*=0.05, \*\**p*<0.01, \*\*\**p*<0.001. Black columns, MRS2211; white columns, control

regulators of insulin and hormone release by the addition of the enzyme apyrase, which removes ATP and ADP by degradation [30, 33]. At low glucose concentrations, the addition of apyrase led to increased insulin secretion, possibly due to reduced ADP-mediated P2Y<sub>13</sub> activation. However, apyrase together with ecto-5'-nucleotidases also generates adenosine, which inhibits insulin release via A<sub>1</sub> receptors [34]. At high glucose levels, we found inhibition of insulin secretion by apyrase, and this could possibly be explained by enzyme-mediated generation of adenosine. On the other hand, apyrase had a stimulating effect on glucagon release at both low and high glucose concentrations. This effect could be explained by removal of extracellular ATP, which has previously been demonstrated to inhibit glucagon release by interfering with the oscillatory Ca<sup>2+</sup> signalling of the alpha cell [35].

It has been suggested that ATP and insulin are co-secreted from pancreatic beta cells [36]. Using MIN6 cells, we found that glucose significantly increases the release of ATP into the extracellular space, and that this release can be blocked by the Ca<sup>2+</sup> channel blocker nifedipine, leading to the inhibition of Ca<sup>2+</sup>-mediated insulin exocytosis [37].

P2Y receptor research is hampered by the shortage of specific receptor antagonists. mRNA quantification is therefore a useful complement to investigate the importance of the many subtypes. In mouse beta cells and also in whole





**Fig. 6** Effect of the P2Y<sub>13</sub> antagonist MRS2211 or saline on (a) insulin, (b) glucose and (c) glucagon secretion in vivo. MRS2211 in vivo resulted in a significant increase in insulin and

decrease of glucose following the i.p. injection of glucose (a, b). Data are expressed as mean  $\pm$  SEM,  $n=10$ , \* $p<0.05$ , \*\* $p<0.01$ . Black squares, MRS2211; black triangles, saline

islets we found a very clear and consistent pattern, with high expression levels of P2Y<sub>1</sub> and P2Y<sub>13</sub> receptors, but extremely low or undetectable levels of P2Y<sub>2</sub>, P2Y<sub>4</sub> [38] or P2Y<sub>12</sub> receptors (P2Y<sub>11</sub> is absent from the mouse genome). Therefore, we conclude that no pharmacologically relevant P2Y receptors for ATP are present in mouse beta cells or islets. As a consequence, extracellularly released ATP can only stimulate islet P2Y receptors after conversion into ADP and can then act on P2Y<sub>1</sub> or P2Y<sub>13</sub> receptors. ATP could still stimulate ionotropic P2X receptors on beta cells, but activation of P2X receptors has been shown to mediate only small and transient increases in insulin secretion at low glucose concentrations [39].

For the pharmacological experiments we used the stable ADP analogue 2-MeSADP to limit conversion of ADP into adenosine, which is known to inhibit insulin secretion [34]. 2-MeSADP alone did not have any measurable effect on insulin secretion at 8.3 mmol/l glucose. However, when the P2Y<sub>1</sub> receptor was blocked by the specific P2Y<sub>1</sub> antagonist MRS2179, a pronounced decrease in insulin secretion was observed, revealing an inhibitory effect of ADP via the P2Y<sub>13</sub> receptor. In contrast, when the P2Y<sub>13</sub> receptor was blocked by MRS2211 during 2-MeSADP stimulation, a marked increase in insulin secretion was seen. This indicates that ADP exerts opposing regulatory effects on insulin secretion via different receptor subtypes. ADP stimulates insulin secretion from beta cells via the G $\alpha_q$ -coupled P2Y<sub>1</sub> receptor and inhibits insulin release via the G $\alpha_i$ -coupled P2Y<sub>13</sub> receptor [30]. This finding may explain the sometimes contradictory results from previous studies [9–13] where ADP has been shown to both increase [9–11] and decrease [12, 13] insulin release via P2Y receptors. ADP acting on P2Y<sub>1</sub> receptors has been reported to increase pulsatility of insulin release, but not Ca<sup>2+</sup> rhythmicity in intact pancreas [16]. At the same time, selective P2Y<sub>1</sub> receptor agonists have been shown to stimulate insulin release, as ADP stimulates Ca<sup>2+</sup> transients via P2Y<sub>1</sub> receptors [40]. The stimulatory effect of ADP via P2Y<sub>1</sub> receptors was confirmed in our study. It is possible that the inhibitory effect of ADP via P2Y<sub>13</sub> receptors

dominated the experimental settings in which ADP decreased insulin release [12, 13].

However, the effects also depend on the presence of other cell types in the model. In studies using intact pancreas, P2Y<sub>1</sub> receptors on endothelial cells could indirectly regulate insulin secretion via effects on blood flow or even via release of ATP from endothelial cells. It becomes even more complex in whole animal studies. The P2Y<sub>1</sub> receptor knockout mouse exhibits both increased plasma insulin and plasma glucose [41]. The animals display increased body weight and a tendency towards glucose intolerance. To explain these findings it is speculated that P2Y<sub>1</sub> receptors in the hypothalamus, liver and fibroblasts may be involved. In contrast to the P2Y<sub>1</sub> receptor, the P2Y<sub>13</sub> receptor has a more limited expression, mainly in haematopoietic cells [42], and now also in pancreatic alpha and beta cells.

In our in vivo experiments, insulin increased acutely in response to glucose, as expected, and this early response was unaffected by the P2Y<sub>13</sub> antagonist MRS2211. However, the second phase (after 10 min), during which insulin is normally attenuated, was markedly affected by MRS2211 and insulin remained on the same level as in the acute phase. A possible explanation is that ATP is co-released with insulin during degranulation. After degradation to ADP, the ADP molecule binds to the P2Y<sub>13</sub> receptor on beta cells and turns off insulin release. In the presence of a P2Y<sub>13</sub> antagonist this negative feedback system is disrupted and insulin levels remain high.

For glucagon the in vivo experiments demonstrated a diminished suppression of glucagon secretion following an injection of glucose at both 3 and 10 min. The effects are probably mediated by a direct effect of MRS2211 on alpha cells, because secondary effects via increased insulin release would have decreased glucagon levels, instead of increasing them as we observed. Furthermore, these results are supported by the previously described influence of apyrase on isolated islets, and taken together they indicate an inhibitory effect either of ATP, or of its degradation products, on glucagon secretion. Although it is presently

not known whether pancreatic alpha cells express P2Y<sub>13</sub> receptors, it has been demonstrated that Gα<sub>i</sub> activation inhibits exocytosis of glucagon [43]. The well-described glucagon release-inhibiting peptide somatostatin, for example, is mediated by Gα<sub>i</sub>-coupled receptors [44]. Consequently, it is likely that inhibition of P2Y<sub>13</sub> receptors on alpha cells would result in increased glucagon release.

The mechanism of action for the Gα<sub>i</sub>-coupled P2Y<sub>13</sub> receptor is to decrease intracellular levels of the second messenger cAMP, and ADP has been shown to increase cAMP in pancreatic beta cells [45]. We found that an ADP analogue reduced cAMP levels and blocking the P2Y<sub>13</sub> receptor with MRS2211 restored cAMP levels in beta cells. cAMP has been shown to improve insulin release and to protect against apoptosis and cell death [24]. Interestingly, the adenosine A<sub>1</sub> receptor is also Gα<sub>i</sub>-coupled and also decreases cAMP levels. Caffeine, which blocks the adenosine A<sub>1</sub> receptor, thus protects against the development of diabetes [46]. Based on their similar effects on cAMP, it is to be expected that a P2Y<sub>13</sub> antagonist would have a similar protective effect against diabetes.

In conclusion, we have found a new role for extracellular ADP, whereby ADP acts on the P2Y<sub>13</sub> receptor in an autocrine inhibitory pathway regulating insulin secretion in the mouse. Furthermore, we have shown that by means of a P2Y<sub>13</sub> antagonist (MRS2211) it is possible to improve insulin secretion both in vitro and in vivo.

**Acknowledgements** We would especially like to thank B.-M. Nilsson and S. Svensson for excellent technical assistance. The study has been supported by the Swedish Scientific Research Council, the Swedish Heart and Lung Foundation, the Vascular Wall program, Lund University, Lund University Hospital funds and the Söderberg Foundation.

**Duality of interest** The authors declare that there is no duality of interest associated with this manuscript. D. Erlinge is a holder of the Lars Werkö distinguished research fellowship from the Swedish Heart and Lung Foundation.

## References

- Parodi J, Flores C, Aguayo C, Rudolph MI, Casanello P, Sobrevia L (2002) Inhibition of nitrobenzylthioinosine-sensitive adenosine transport by elevated D-glucose involves activation of P2Y<sub>2</sub> purinoceptors in human umbilical vein endothelial cells. *Circ Res* 90:570–577
- Nilsson J, Nilsson LM, Chen YW, Molkentin JD, Erlinge D, Gomez MF (2006) High glucose activates nuclear factor of activated T cells in native vascular smooth muscle. *Arterioscler Thromb Vasc Biol* 26:794–800
- Solini A, Iacobini C, Ricci C et al (2005) Purinergic modulation of mesangial extracellular matrix production: role in diabetic and other glomerular diseases. *Kidney Int* 67:875–885
- Hazama A, Hayashi S, Okada Y (1998) Cell surface measurements of ATP release from single pancreatic beta cells using a novel biosensor technique. *Pflugers Arch* 437:31–35
- Chen YJ, Hsu KW, Chen YL (2006) Acute glucose overload potentiates nitric oxide production in lipopolysaccharide-stimulated macrophages: the role of purinergic receptor activation. *Cell Biol Int* 30:817–822
- Burnstock G (2006) Purinergic signalling. *Br J Pharmacol* 147 (Suppl 1):S172–S181
- Alvarado-Castillo C, Harden TK, Boyer JL (2005) Regulation of P2Y<sub>1</sub> receptor-mediated signaling by the ectonucleoside triphosphate diphosphohydrolase isozymes NTPDase1 and NTPDase2. *Mol Pharmacol* 67:114–122
- Fredholm BB (2007) Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ* 14:1315–1323
- Loubatieres-Mariani MM, Chapal J, Lignon F, Valette G (1979) Structural specificity of nucleotides for insulin secretory action from the isolated perfused rat pancreas. *Eur J Pharmacol* 59:277–286
- Bertrand G, Chapal J, Puech R, Loubatieres-Mariani MM (1991) Adenosine-5'-O-(2-thiodiphosphate) is a potent agonist at P<sub>2</sub> purinoceptors mediating insulin secretion from perfused rat pancreas. *Br J Pharmacol* 102:627–630
- Fernandez-Alvarez J, Hillaire-Buys D, Loubatieres-Mariani MM, Gomis R, Petit P (2001) P<sub>2</sub> receptor agonists stimulate insulin release from human pancreatic islets. *Pancreas* 22:69–71
- Petit P, Bertrand G, Schmeer W, Henquin JC (1989) Effects of extracellular adenine nucleotides on the electrical, ionic and secretory events in mouse pancreatic beta-cells. *Br J Pharmacol* 98:875–882
- Poulsen CR, Bokvist K, Olsen HL et al (1999) Multiple sites of purinergic control of insulin secretion in mouse pancreatic beta-cells. *Diabetes* 48:2171–2181
- Hutton JC, Penn EJ, Peshavaria M (1983) Low-molecular-weight constituents of isolated insulin-secretory granules. Bivalent cations, adenine nucleotides and inorganic phosphate. *Biochem J* 210:297–305
- Hellman B, Dansk H, Grapengiesser E (2004) Pancreatic beta-cells communicate via intermittent release of ATP. *Am J Physiol Endocrinol Metab* 286:E759–E765
- Salehi A, Qader SS, Grapengiesser E, Hellman B (2005) Inhibition of purinoceptors amplifies glucose-stimulated insulin release with removal of its pulsatility. *Diabetes* 54:2126–2131
- Grapengiesser E, Dansk H, Hellman B (2005) External ATP triggers Ca<sup>2+</sup> signals suited for synchronization of pancreatic beta-cells. *J Endocrinol* 185:69–79
- Grapengiesser E, Dansk H, Hellman B (2004) Pulses of external ATP aid to the synchronization of pancreatic beta-cells by generating premature Ca(2+) oscillations. *Biochem Pharmacol* 68:667–674
- Farret A, Filhol R, Linck N et al (2006) P2Y receptor mediated modulation of insulin release by a novel generation of 2-substituted-5'-O-(1-boranotriphosphate)-adenosine analogues. *Pharm Res* 23:2665–2671
- Blachier F, Malaisse WJ (1988) Effect of exogenous ATP upon inositol phosphate production, cationic fluxes and insulin release in pancreatic islet cells. *Biochim Biophys Acta* 970:222–229
- Hillaire-Buys D, Chapal J, Bertrand G, Petit P, Loubatieres-Mariani MM (1994) Purinergic receptors on insulin-secreting cells. *Fundam Clin Pharmacol* 8:117–127
- Squires PE, James RF, London NJ, Dunne MJ (1994) ATP-induced intracellular Ca<sup>2+</sup> signals in isolated human insulin-secreting cells. *Pflugers Arch* 427:181–183
- Lindstrom E, Bjorkquist M, Boketoft A et al (1997) Release of histamine and pancreastatin from isolated rat stomach ECL cells. *Inflamm Res* 46(Suppl 1):S109–S110
- Qader SS, Jimenez-Feltstrom J, Ekelund M, Lundquist I, Salehi A (2007) Expression of islet inducible nitric oxide synthase and inhibition of glucose-stimulated insulin release after long-term

- lipid infusion in the rat is counteracted by PACAP27. *Am J Physiol Endocrinol Metab* 292:E1447–E1455
25. Amisten S, Braun OO, Bengtsson A, Erlinge D (2008) Gene expression profiling for the identification of G-protein coupled receptors in human platelets. *Thromb Res* 122:47–57
  26. Taylor AL, Kudlow BA, Marrs KL, Gruenert DC, Guggino WB, Schwiebert EM (1998) Bioluminescence detection of ATP release mechanisms in epithelia. *Am J Physiol* 275:C1391–C1406
  27. Salehi A, Chen D, Hakanson R, Nordin G, Lundquist I (1999) Gastrectomy induces impaired insulin and glucagon secretion: evidence for a gastro-insular axis in mice. *J Physiol* 514:579–591
  28. Ralevic V, Burnstock G (1991) Roles of P<sub>2</sub>-purinoceptors in the cardiovascular system. *Circulation* 84:1–14
  29. Vater W, Kroneberg G, Hoffmeister F et al (1972) Pharmacology of 4-(2'-nitrophenyl)-2, 6-dimethyl-1, 4-dihydropyridine-3, 5-dicarboxylic acid dimethyl ester (Nifedipine, BAY a 1040). *Arzneimittelforschung* 22:1–14 (article in German)
  30. Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413–492
  31. Boyer JL, Mohanram A, Camaioni E, Jacobson KA, Harden TK (1998) Competitive and selective antagonism of P2Y<sub>1</sub> receptors by N<sup>6</sup>-methyl 2'-deoxyadenosine 3', 5'-bisphosphate. *Br J Pharmacol* 124:1–3
  32. Kim YC, Lee JS, Sak K et al (2005) Synthesis of pyridoxal phosphate derivatives with antagonist activity at the P2Y<sub>13</sub> receptor. *Biochem Pharmacol* 70:266–274
  33. Robson S, Sévigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Signalling* 2:409–430
  34. Johansson SM, Salehi A, Sandstrom ME et al (2007) A1 receptor deficiency causes increased insulin and glucagon secretion in mice. *Biochem Pharmacol* 74:1628–1635
  35. Tuduri E, Filiputti E, Carneiro EM, Quesada I (2008) Inhibition of Ca<sup>2+</sup> signaling and glucagon secretion in mouse pancreatic alpha-cells by extracellular ATP and purinergic receptors. *Am J Physiol Endocrinol Metab* 294:E952–E960
  36. Karanaukaite J, Hoppa MB, Braun M, Galvanovskis J, Rorsman P (2009) Quantal ATP release in rat beta-cells by exocytosis of insulin-containing LDCVs. *Pflugers Arch* 458:389–401
  37. Trus M, Corkey RF, Nesher R et al (2007) The L-type voltage-gated Ca<sup>2+</sup> channel is the Ca<sup>2+</sup> sensor protein of stimulus-secretion coupling in pancreatic beta cells. *Biochemistry* 46:14461–14467
  38. Parandeh F, Abaraviciene SM, Amisten S, Erlinge D, Salehi A (2008) Uridine diphosphate (UDP) stimulates insulin secretion by activation of P2Y<sub>6</sub> receptors. *Biochem Biophys Res Commun* 370:499–503
  39. Petit P, Hillaire-Buys D, Manteghetti M, Debrus S, Chapal J, Loubatieres-Mariani MM (1998) Evidence for two different types of P<sub>2</sub> receptors stimulating insulin secretion from pancreatic B cell. *Br J Pharmacol* 125:1368–1374
  40. Fischer B, Chulkin A, Boyer JL et al (1999) 2-thioether 5'-O-(1-thiotriphosphate)adenosine derivatives as new insulin secretagogues acting through P2Y-Receptors. *J Med Chem* 42:3636–3646
  41. Léon C, Freund M, Latchoumanin O et al (2005) The P2Y<sub>1</sub> receptor is involved in the maintenance of glucose homeostasis and in insulin secretion in mice. *Purinergic Signalling* 1:145–151
  42. Communi D, Gonzalez NS, Detheux M et al (2001) Identification of a novel human ADP receptor coupled to G(i). *J Biol Chem* 276:41479–41485
  43. Gromada J, Hoy M, Buschard K, Salehi A, Rorsman P (2001) Somatostatin inhibits exocytosis in rat pancreatic alpha-cells by G (i<sub>2</sub>)-dependent activation of calcineurin and depriming of secretory granules. *J Physiol* 535:519–532
  44. Waeber G, Gomez F, Chaubert P et al (1997) In vivo and in vitro effects of somatostatin and insulin on glucagon release in a human glucagonoma. *Clin Endocrinol (Oxf)* 46:637–642
  45. Chevassus H, Roig A, Belloc C et al (2002) P2Y receptor activation enhances insulin release from pancreatic beta-cells by triggering the cyclic AMP/protein kinase A pathway. *Naunyn Schmiedeberg Arch Pharmacol* 366:464–469
  46. Salazar-Martinez E, Willett WC, Ascherio A et al (2004) Coffee consumption and risk for type 2 diabetes mellitus. *Ann Intern Med* 140:1–8