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ARTICLE

Autocrine activation of $P2Y_1$ receptors couples Ca^{2+} influx to Ca^{2+} release in human pancreatic beta cells

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Abstract
Aims/hypothesis There is evidence that ATP acts as an autocrine signal in beta cells but the receptors and pathways involved are incompletely understood. Here we investigate the receptor subtype(s) and mechanism(s) mediating the effects of ATP on human beta cells.
Methods We examined the effects of purinergic agonists and antagonists on membrane potential, membrane currents, intracellular Ca^{2+} ($[Ca^{2+}]_i$) and insulin secretion in human beta cells.
Results Extracellular application of ATP evoked small inward currents (3.4±0.7 pA) accompanied by depolarisation of the membrane potential (by 14.4±2.4 mV) and stimulation of electrical activity at 6 mmol/l glucose. ATP increased $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx and evoking Ca^{2+} release via $InsP_3$-receptors in the endoplasmic reticulum (ER). ATP-evoked Ca^{2+} release was sufficient to trigger exocytosis in cells voltage-clamped at −70 mV. All effects of ATP were mimicked by the $P2Y_{(1/12/13)}$ agonist ADP and the $P2Y_1$ agonist MRS-2365, whereas the $P2X_{(1/3)}$ agonist α,β-methyleneadenosine-5-triphosphate only had a small effect. The $P2Y_1$ antagonists MRS-2279 and MRS-2500 hyperpolarised glucose-stimulated beta cells and lowered $[Ca^{2+}]_i$ in the absence of exogenously added ATP and inhibited glucose-induced insulin secretion by 35%. In voltage-clamped cells subjected to action potential-like stimulation, MRS-2279 decreased $[Ca^{2+}]_i$ and exocytosis without affecting Ca^{2+} influx.
Conclusions/interpretation These data demonstrate that ATP acts as a positive autocrine signal in human beta cells by activating $P2Y_1$ receptors, stimulating electrical activity and coupling Ca^{2+} influx to Ca^{2+} release from ER stores.

Keywords ATP · Calcium · Electrophysiology · Exocytosis · Insulin · Islets of Langerhans · Purinergic · Secretion

Abbreviations

α,β-meATP	α,β-Methyleneadenosine-5-triphosphate
ER	Endoplasmic reticulum
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
$AdP2X_2$-GFP	$P2X_2$–green fluorescent protein fusion protein

Introduction

In addition to serving as an energy carrier and intracellular signal, ATP has an important role as an extracellular signal and neurotransmitter. After its release from cells by exocytosis or via non-vesicular pathways, ATP activates two types of purinergic P2 receptors in the plasma membrane. P2X receptors are ligand-gated non-selective cation channels, while P2Y receptors are G-protein coupled. In humans, the P2X and P2Y families comprise 7 and 11 isoforms, respectively [1, 2].

Shara Khan and Richard Yan-Do contributed equally to this study.

Professor M. Braun, who supervised this research, died on 16 November 2013 before publication of this work.

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ATP is present at millimolar concentrations in insulin granules [3, 4] and is released from beta cells upon glucose stimulation [5–7]. There is evidence for the expression of both P2X and P2Y receptors in rat and mouse beta cells, suggesting that ATP acts as an autocrine signal in islets, although it is debatable whether purinergic signalling stimulates or inhibits insulin secretion [8, 9]. Overall, the few studies that have been conducted in human islets suggest a stimulatory role for ATP [10–12]. However, controversy exists regarding the receptor subtypes and signal transduction pathways involved. While one study proposed that ATP acts principally via $P2X_3$ receptors, membrane depolarisation and increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [11], a more recent study suggested a prominent role for $P2Y_1$ receptors and activation of protein kinase C [12]. Involvement of $P2X_7$ has also been proposed [13]. An ATP-evoked, P2X-mediated membrane current in human beta cells has been suggested [14], although the effect of ATP on glucose-induced electrical activity has not been investigated.

We sought to characterise the effects of extracellular ATP on membrane currents and membrane potential in human beta cells. We found that the effects of ATP were mimicked by the P2Y agonist ADP and demonstrated that autocrine activation of $P2Y_1$ receptors plays a significant role in the regulation of electrical activity, $[Ca^{2+}]_i$ and insulin secretion in human beta cells. Autocrine signalling via $P2Y_1$ represents a novel link between Ca^{2+} influx and Ca^{2+} release from intracellular stores.

Methods

Materials MRS-2279, MRS-2365, MRS-2500, α,β-methyleneadenosine-5-triphosphate (α,β-meATP), 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), heparin, thapsigargin and bafilomycin A1 were from R&D Systems (Minneapolis, MN, USA). Fura-2AM and Fura-2 Na^+-salt were from Life Technologies (Burlington, ON, Canada). Nucleotides and other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Islet isolation, culture and transfection Human islets were from the Clinical Islet Laboratory at the University of Alberta or the Alberta Diabetes Institute IsletCore [15, 16]. The study was approved by the local Human Research Ethics Board. Islets were dispersed in Ca^{2+}-free buffer and then plated onto plastic or glass-bottom Petri-dishes (In Vitro Scientific, Sunnyville, CA, USA) and incubated in RPMI-1640 medium containing 7.5 mmol/l glucose for at least 24 h before experiments. For measuring ATP release, cells were infected with an adenovirus encoding a $P2X_2$–green fluorescent protein fusion protein (Ad$P2X_2$-GFP) for 24–48 h [5]. All experiments, except for the assessment of insulin secretion, were carried out using dispersed beta cells.

Immunohistochemistry Paraffin-embedded tissue sections were heated in 10 mmol/l Na^+-citrate (pH 6) for 10 min. Sections were blocked using 20% goat serum and incubated with anti-$P2Y_1$ (1:50 dilution; P6487; Sigma-Aldrich) and anti-insulin antibodies for 1 h, followed by fluorescently labelled secondary antibodies. Images were captured using a Zeiss Apotome inverted microscope (Carl Zeiss Canada, Toronto, ON, Canada). Identification of beta cells by immunocytochemistry after patch-clamp and Ca^{2+} imaging was as described previously [17].

Ca^{2+} imaging Cells were pre-incubated with Fura-2AM (1 μmol/l) for 15 min. Glass-bottom Petri dishes were mounted onto an inverted microscope (Zeiss Axioobserver, Carl Zeiss Canada Ltd.) equipped with an ICCD-camera and a rapid-switching light source (Oligochrome; Till Photonics, Grafelfing, Germany). Fluorophore, excited at 340 and 380 nm (intensity ratio 10:4) and emission detected at 510 nm, was imaged at 0.5 Hz using Life Acquisition software (Till Photonics). Beta cells were identified by immunostaining and fluorescence ratios were calculated using ImageJ (v1.46r; http://imagej.nih.gov/ij/).

Insulin secretion Fifteen size-matched islets (in triplicates) were pre-incubated in 0.5 ml KRB buffer containing 1 mmol/l glucose and 0.1% BSA for 1 h, followed by a 1 h test incubation in KRB with the indicated glucose concentrations and test substances. The supernatant fraction was removed and the insulin concentration was determined using the MSD human insulin kit (Meso-Scale Discovery, Rockville, MD, USA).

Electrophysiology Patch-clamp was performed using an EPC-10 amplifier and Patchmaster software (Heka Electronics, Lambrecht, Germany). Patch-pipettes were pulled from borosilicate glass (resistance 3–8 MΩ; Sutter Instruments, Novato, CA, USA). Solutions for whole-cell and perforated-patch recording are detailed in the electronic supplementary materials (ESM) Methods. Cells were continuously superfused (~1 ml/min) with extracellular solution at ~32°C. Rapid application of ATP was performed using a Fast-Step system (Warner Instruments, Hamden, CT, USA). Beta cells were identified by immunostaining or based on cell size (12.5±0.3 pF; n=189) [17].

PCR analysis Expression of P2Y receptors ($P2RY_{1–14}$) was analysed by RT-PCR in RNA purified from isolated human islets, using a previously described protocol [18]. Primer sequences are detailed in ESM Methods.

Data analysis Data are presented as means ± SEM. The n values represent the number of cells, unless indicated otherwise. Statistical significance was evaluated using

Student's *t* test, or by multiple-comparison ANOVA and Bonferroni post test when comparing multiple groups.

Results

Membrane currents evoked by purinergic receptor agonists Using the whole-cell configuration in cells held at −70 mV in 6 mmol/l glucose, extracellular ATP application evoked a detectable inward current in 25% of human beta cells (15 out of 60). The maximal current amplitude was 24 pA (Fig. 1a). In responding cells, the ATP-activated current averaged 6.7±1.7 pA and was reduced 65±17% by the P2 receptor blocker suramin (100 μmol/l; $p<0.05$, $n=5$).

In perforated-patch whole-cell recordings, ATP-evoked an inward current in all beta cells (1.2–8.6 pA) and averaged 3.4±0.7 pA (Fig. 1b). Similar responses were obtained with the $P2Y_{(1/12/13)}$ agonist ADP (4.2±0.6 pA; 0.4–11.1 pA) and the $P2Y_1$ agonist MRS-2365 (2.4±0.3 pA), but not the $P2X_{(1/3)}$ agonist α,β-meATP (Fig. 1b). The ATP- or ADP-evoked current was inhibited 78±13% by the $P2Y_1$ antagonist MRS-2279 (2–3 μmol/l, $p<0.05$, $n=4$). The ADP-evoked current was inward during voltage ramps from −110 to −60 mV (Fig. 1c, d) and was not inhibited by tolbutamide (4.2±1.1 pA, $n=4$; Fig. 1e). The rapid upstroke in Fig. 1e following application of tolbutamide and ADP is likely to be an artefact. Instead, the current was attenuated (by 90±25%; $p<0.05$, $n=5$) when Na^+ was replaced by the membrane-impermeable cation *N*-methyl-D-glucamine ($NMDG^+$) (Fig. 1f–h).

Effect of purinergic agonists on the membrane potential At 6 mmol/l glucose, isolated beta cells exhibited varying degrees of action potential firing (Fig. 2). Application of ATP (10 μmol/l) depolarised cells (from −53.7±2.2 to −39.3±2.9 mV, Fig. 2a, e) and stimulated or augmented action potential firing. This effect was mimicked by ADP, which depolarised human beta cells from −53.6±2.1 to −38.6±2 mV (Fig. 2b, e). UTP, an agonist at $P2Y_{(2/6/8)}$ receptors, had no effect (data not shown). The $P2Y_1$ agonist MRS-2365 also potently depolarised the cells (from −53.3±4 to −44.2±2.9 mV, Fig. 2c, e), while the $P2X_{(1/3)}$ agonist α,β-meATP depolarised the membrane potential only slightly (from −59.7±4.8 to −57.2±4.1 mV, Fig. 2c, e). The effect of ATP was prevented in four out of five cells by the $P2Y_1$ antagonist MRS-2279 (Fig. 2d). ATP did not directly modulate voltage-gated Ca^{2+} or K^+ currents (ESM Fig. 1).

Effect of purinergic agonists on $[Ca^{2+}]_i$ At 6 mmol/l glucose, ATP induced a biphasic increase in $[Ca^{2+}]_i$ consisting of an initial rapid spike followed by a plateau (Fig. 3a). In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ spike was largely unchanged, whereas the plateau was reduced (Fig. 3b). Pre-treatment of cells with thapsigargin removed the $[Ca^{2+}]_i$ spike without affecting the plateau when extracellular Ca^{2+} was

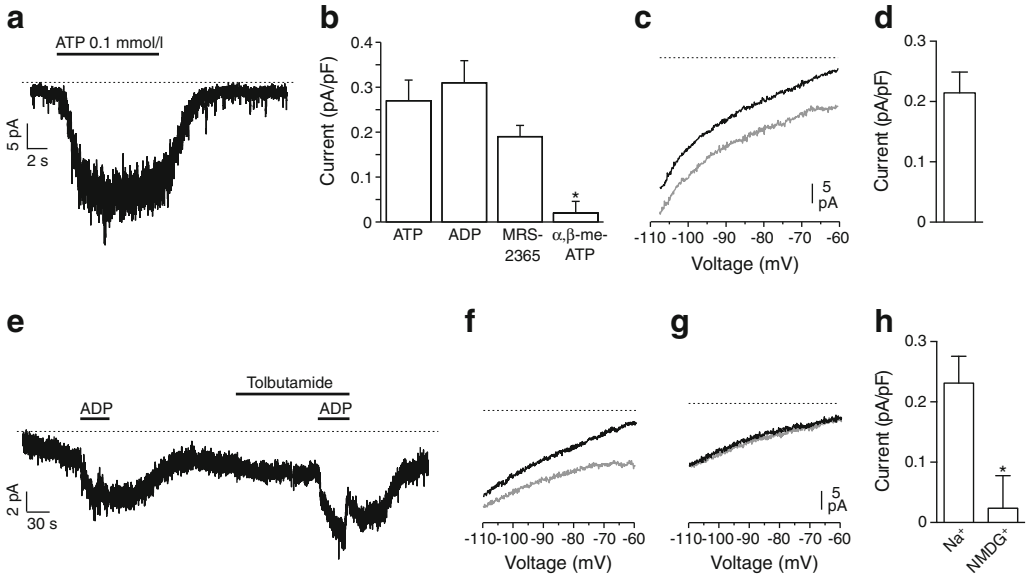

Fig. 1 Effect of purinergic agonists on resting membrane currents. (**a**) Whole-cell membrane current evoked by extracellular application of 0.1 mmol/l ATP. (**b**) Average amplitudes (normalised to cell size) of inward currents evoked by ATP (10 μmol/l, $n=15$ cells), ADP (2 μmol/l, $n=40$ cells), MRS-2365 (0.1 μmol/l, $n=5$ cells, two donors) and α,β-meATP (10 μmol/l, $n=4$ cells). (**c**) Membrane currents evoked by voltage ramps under control conditions (black trace) and after application of 2 μmol/l ADP (grey trace). (**d**) Average ADP-activated inward current measured at −70 mV ($n=21$). (**e**) Membrane current evoked by 2 μmol/l ADP in the absence and presence of 0.2 mmol/l tolbutamide. (**f**, **g**) Control (black traces) and ADP (2 μmol/l) evoked membrane currents (grey traces) during voltage ramps under control conditions (**f**) and after replacement of extracellular Na^+ with $NMDG^+$ (**g**). (**h**) The ADP-activated currents were quantified at −70 mV ($n=5$, two donors). Data is from three to seven donors unless indicated otherwise. *$p<0.05$ compared with ATP or Na^+

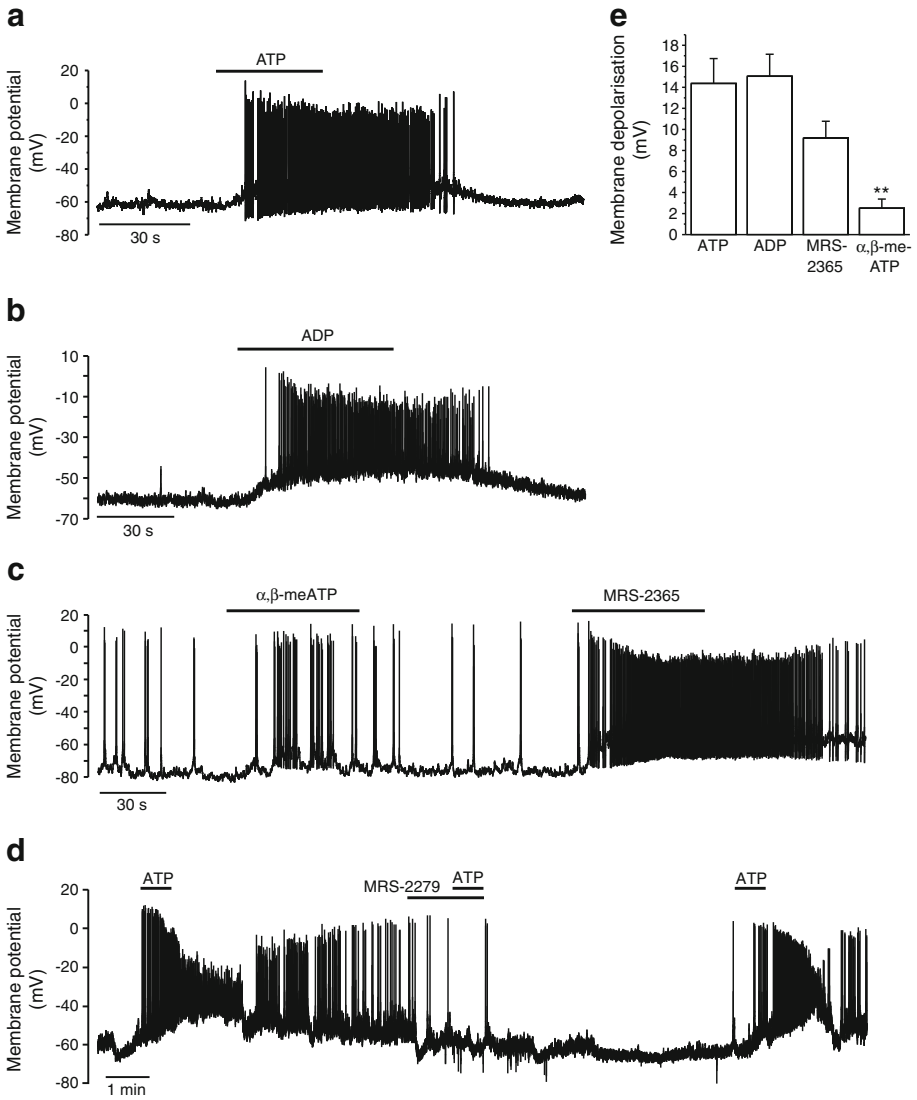

Fig. 2 Effect of purinergic agonists on the membrane potential. Membrane potential recordings from human beta cells by perforated patch. (**a**) ATP (10 μmol/l) was applied as indicated by the bar. (**b**) ADP (1 μmol/l) was added as indicated. (**c**) Effect of α,β-meATP (10 μmol/l) and the agonist MRS-2365 (0.1 μmol/l) in the same cell. (**d**) ATP (10 μmol/l) was applied in the absence or presence of the antagonist MRS-2279 (1 μmol/l) as indicated. (**e**) Average depolarisation evoked by ATP (10 μmol/l, $n=16$), ADP (1–2 μmol/l, $n=11$), MRS-2365 (0.1 μmol/l, $n=11$) and α,β-meATP (10 μmol/l, $n=7$). Data are from four to six donors in each experiment. $^{**}p<0.01$ compared with ATP

present, and completely suppressed the $[Ca^{2+}]_i$ increase under Ca^{2+}-free conditions (Fig. 3c, d, k). In contrast bafilomycin A1, which depletes acidic Ca^{2+} stores, had little effect on the $[Ca^{2+}]_i$ signal (Fig. 3e, k). The effect of ATP on $[Ca^{2+}]_i$ was mimicked by ADP and MRS-2365 (Fig. 3f, h, k). UTP and α,β-meATP evoked only small responses (Fig. 3g, i, k). Beta cells from a donor with type 2 diabetes were observed to have an ATP-sensitive Ca^{2+} response that appeared smaller than the response of healthy beta cells, but did not attain statistical significance and lacked the initial rapid spike (Fig. 3j).

To identify the intracellular Ca^{2+} channel underlying ATP-induced Ca^{2+} release, $[Ca^{2+}]_i$ was measured in cells voltage-clamped at −70 mV and infused with the $InsP_3$ receptor blocker heparin. The $[Ca^{2+}]_i$ increase evoked by extracellular ATP was completely suppressed by heparin (Fig. 4a, b), suggesting that the ATP-evoked $[Ca^{2+}]_i$ spike results from $InsP_3$-dependent endoplasmic reticulum (ER) Ca^{2+} release while the plateau is due to Ca^{2+} influx through plasma membrane channels.

Effect of ATP on exocytosis Exocytosis was elicited by voltage-clamp depolarisations from −70 to 0 mV, which triggers Ca^{2+} influx through voltage-gated Ca^{2+} channels. Exocytosis was potentiated 64% and 32% by extracellular application of ATP and ADP, respectively (Fig. 4c, d). We next monitored the membrane capacitance in cells clamped at −70 mV to prevent opening of voltage-gated Ca^{2+} channels, and in the presence of 5 mmol/l glucose. ATP application alone was sufficient to evoke a clear exocytotic response under these conditions (Fig. 4e). This response was strongly inhibited in cells pretreated with thapsigargin (Fig. 4f).

Expression of P2Y receptors in human beta cells Expression of P2Y receptor isoforms in human islets was analysed by

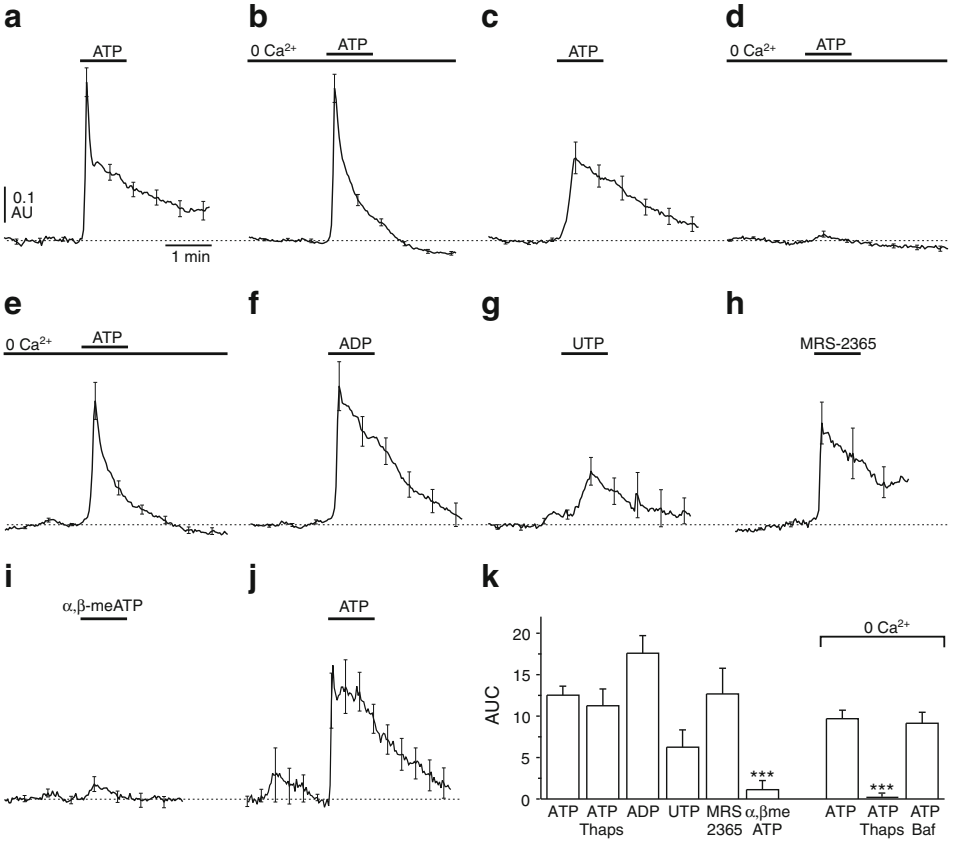

Fig. 3 Effect of purinergic agonists on $[Ca^{2+}]_i$. (**a**–**j**) Averaged $[Ca^{2+}]_i$ responses shown to the same scales (AU, arbitrary units) at 6 mmol/l glucose ($[Ca^{2+}]_i$ spikes of individual beta cells are not apparent in these averaged traces). ATP (10 μmol/l) was applied under the following conditions: (**a**) under control conditions (n=68); (**b**) in the absence of extracellular Ca^{2+} (n=63); (**c**) to cells pretreated with thapsigargin (10 μmol/l, 10 min, n=27); (**d**) to cells pretreated with thapsigargin in the absence of extracellular free Ca^{2+} (n=21, two donors) and (**e**) to cells pretreated with bafilomycin A1 (0.1–2 μmol/l, 10 min) in the absence of extracellular Ca^{2+} (n=27). (**f**) ADP (2 μmol/l) was applied (n=18). (**g**) Effect of UTP (10 μmol/l, n=13). (**h**) Effect of MRS-2365 (0.1 μmol/l, n=9). (**i**) α,β-meATP (10 μmol/l) was added (n=20). (**j**) ATP (10 μmol/l) was added to beta cells from a donor with type 2 diabetes (n=7). (**k**) Bar graphs showing average AUC (baseline-subtracted) during agonist application (Baf, bafilomycin A1; Thaps, thapsigargin). Data are from three to seven donors unless stated otherwise. ***p<0.001 compared with ATP

RT-PCR. Transcripts were identified for $P2Y_1$, $P2Y_2$, $P2Y_{11}$ and $P2Y_{14}$ (Fig. 5a). The expression of $P2Y_1$ in beta cells was confirmed by co-immunostaining of human pancreatic tissue sections with anti-$P2Y_1$ and anti-insulin (Fig. 5b).

Exocytotic release of ATP Rat and mouse beta cells release ATP by Ca^{2+}-dependent exocytosis of insulin granules [5–7, 19, 20]. To examine whether ATP is secreted from human beta cells we overexpressed $P2X_2$ receptors [6, 7] and stimulated exocytosis by infusion of Ca^{2+} (2 μmol/l) via the patch pipette. Resultant transient inward currents were blocked by the P2 antagonist suramin (Fig. 6a; n=4). These events reflect release of ATP from single insulin granules [5, 7]. Beta cells were then incubated in 1 or 10 mmol/l glucose for 1 h with 100 μmol/l diazoxide to prevent K_{ATP}-mediated depolarisation. To avoid artefacts resulting from $P2X_2$ receptor overexpression, transient inward currents were compared with the current produced by 300 μmol/l ATP. Upon infusion of Ca^{2+} (2 μmol/l) the ATP release events were larger (Fig. 6c, p<0.05) and tended to be more frequent (Fig. 6d) following glucose stimulation.

Autocrine activation of $P2Y_1$ receptors potentiates glucose-induced $[Ca^{2+}]_i$ signals, electrical activity and insulin secretion We applied P2 receptor antagonists in the absence of exogenous nucleotides. The $P2Y_1$ antagonists MRS-2279 and MRS-2500, but not the $P2X_{(1/3)}$ blocker TNP-ATP, reduced $[Ca^{2+}]_i$ in beta cells stimulated with 6 mmol/l glucose (Fig. 7a). Both MRS-2279 and MRS-2500 reversibly hyperpolarised the beta cells in the absence of exogenous ATP and inhibited glucose-induced electrical activity (Fig. 7b). The $P2Y_1$ receptor antagonists decreased the membrane potential by 3.5±1.4 mV (from −47.4±2.8 to −50.8±2.2 mV, p<0.05; Fig. 7b). In control experiments, the $P2Y_1$ antagonists had no direct effects on voltage-gated Ca^{2+}, Na^+ or K^+ currents or on K_{ATP} current in human beta cells (ESM Fig. 1). In islets from four donors, MRS-2500 reduced the secretory response to glucose by 35% (Fig. 7c).

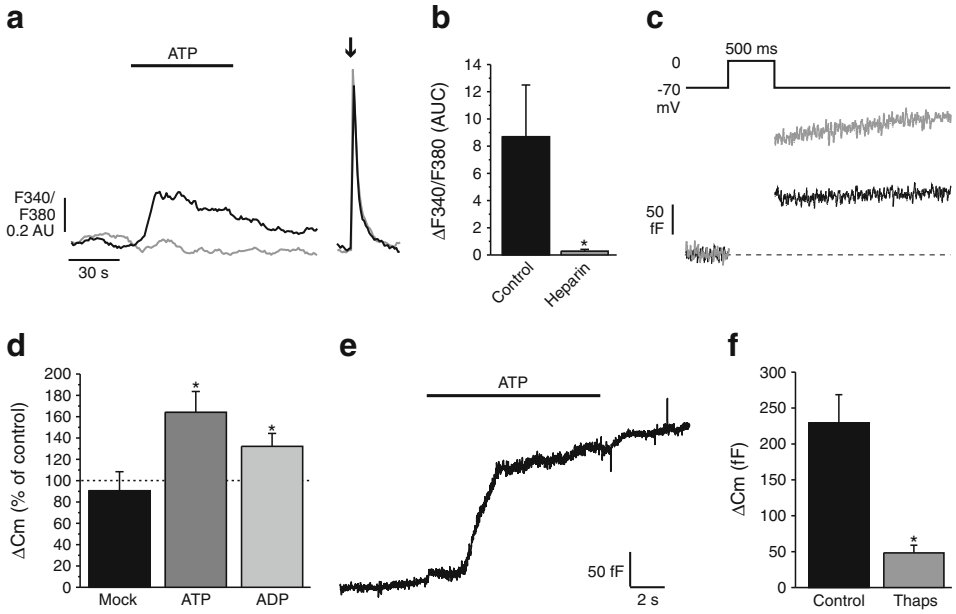

Fig. 4 Effect of ATP on Ca^{2+} release and exocytosis. (**a**) $[Ca^{2+}]_i$ was monitored in voltage-clamped cells, without (control, black trace) or with addition of 0.2 mg/ml heparin (grey trace). ATP was added as indicated. The $[Ca^{2+}]_i$ signal evoked by a 500 ms depolarisation from −70 to 0 mV (arrow) was used as a control. (**b**) Average integrated $[Ca^{2+}]_i$ responses in experiments as described in (**a**) (n=7 and 4). (**c**) Representative traces showing capacitance responses before (black trace) and after addition of ATP (10 μmol/l, grey trace) in the same cell. (**d**) Average changes in exocytotic responses (ΔCm) after mock application (n=5) or application of ATP (10 μmol/l, n=15) or ADP (1 μmol/l, n=9), normalised to control values in the same cells. (**e**) Capacitance response evoked by application of ATP (100 μmol/l) in a cell clamped at −70 mV. (**f**) Average exocytotic responses (ΔCm) evoked by ATP under control conditions (n=18) and in cells pretreated with thapsigargin (Thaps; 10 μmol/l, 10 min; n=6), calculated as the change in average Cm from the 5 s immediately before and immediately after ATP application. Data are from three to four donors. *p<0.05 compared with control

$P2Y_1$ receptors couple Ca^{2+} influx to Ca^{2+} release from stores In voltage-clamped cells $[Ca^{2+}]_i$ was monitored during a series of short depolarisations mimicking glucose-induced electrical activity. In four of nine cells, the capacitance increase was nearly linear and ceased immediately after the end of the stimulation (Fig. 8a) and the $[Ca^{2+}]_i$ signal plateaued after a steep initial rise, returning to baseline with a time constant (τ) of ~2 s. In these cells neither $[Ca^{2+}]_i$ signal nor exocytosis and Ca^{2+} influx were affected by MRS-2279 (Fig. 8a, c–f). In five of nine cells, both exocytotic response and $[Ca^{2+}]_i$ signal displayed a secondary acceleration during the second half of the stimulation (Fig. 8b); also, the $[Ca^{2+}]_i$ signal returned to baseline with a significantly slower τ (~4 s). In these cells MRS-2279 strongly and reversibly inhibited exocytosis, the $[Ca^{2+}]_i$ response and the τ of $[Ca^{2+}]_i$ decline to levels observed in cells lacking secondary acceleration (Fig. 8b–f). The Ca^{2+} influx evoked by the depolarisations was not different between groups of cells (and unaffected by MRS-2279; Fig. 8f).

Discussion

This study provides evidence that ATP acts as a positive autocrine feedback signal in human beta cells, by amplifying

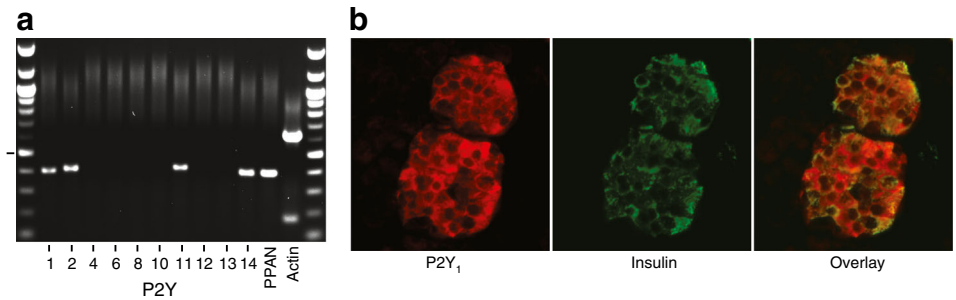

Fig. 5 Expression of P2Y receptors in human islets. (**a**) Expression of P2Y receptor isoforms and the peter pan homologue-P2YR11 transcript (PPAN) were analysed by RT-PCR. The horizontal line indicates the 500 base pair marker. (**b**) A human pancreatic tissue section was co-immunostained with antibodies against the $P2Y_1$ receptor and insulin. Data are representative of results from two donors

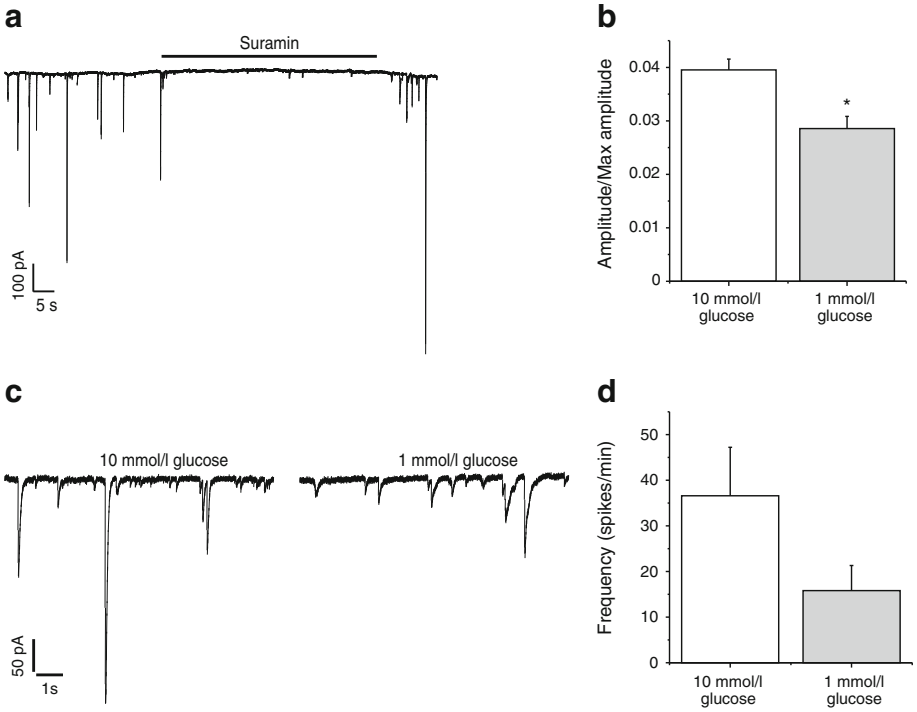

Fig. 6 Exocytotic release of ATP from human beta cells. (**a**) A beta cell infected with AdP2X$_2$-GFP was clamped at −70 mV and infused with solution containing 2 μmol/l free Ca^{2+}. Suramin (100 μmol/l) was added as indicated (n=4). (**b**) Sample trace of ATP transient inward current in human cells in 10 and 1 mmol/l glucose for 1 h (n=11). (**c**) The normalised amplitudes of transient inward currents (n=11). (**d**) The frequency of transient inward currents (n=11). Data are from four donors. *p<0.05 compared with 10 mmol/l glucose. Max, maximum

glucose-induced $[Ca^{2+}]_i$ responses. Several findings support a central role for P2Y$_1$ in this: (1) The effects of ATP were mimicked by ADP (at five- to tenfold lower concentrations), which selectively activates P2Y$_{(1/12/13)}$ (only P2Y$_1$ was detected in human islets) [2]; (2) the selective P2Y$_1$ agonist MRS-2365 increased electrical activity and $[Ca^{2+}]_i$, while the P2X$_{(1/3)}$ agonist α,β-meATP did not; (3) P2Y$_1$ inhibition blocked the ATP-evoked membrane depolarisation; (4) MRS-2500 reduced insulin secretion to a similar extent as the non-specific P2 antagonist suramin [11]. While in

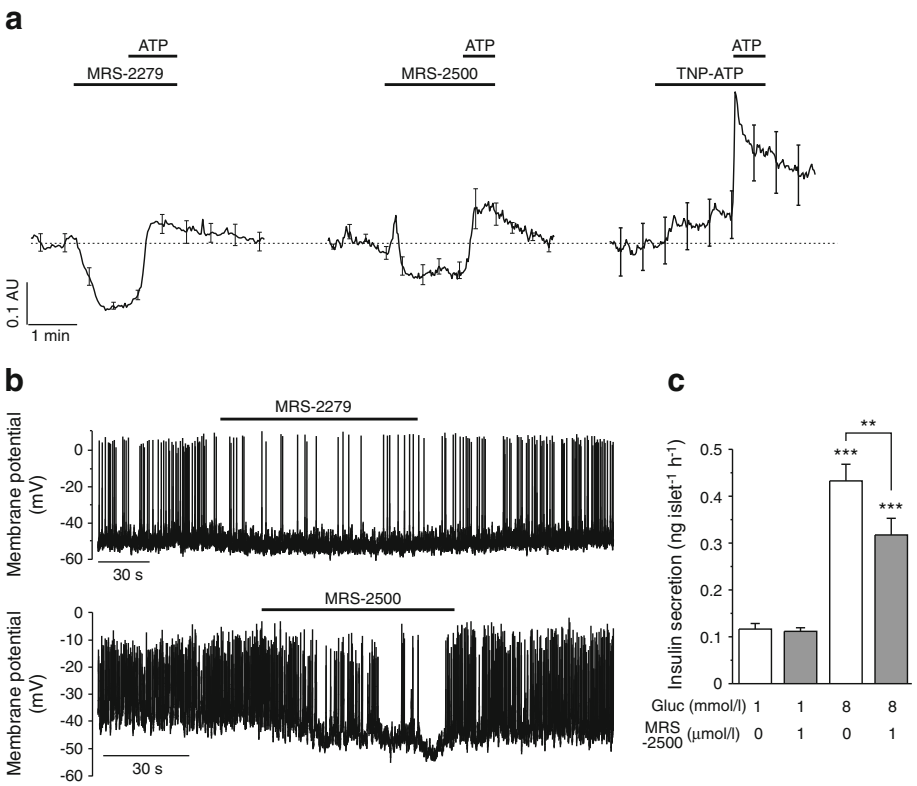

Fig. 7 Effect of P2Y$_1$ antagonists on electrical activity, $[Ca^{2+}]_i$ and insulin secretion. (**a**) Effect of antagonists MRS-2279 (1 μmol/l, n=22), MRS-2500 (1 μmol/l, n=18) and TNP-ATP (1 μmol/l, n=41) on $[Ca^{2+}]_i$ (at 6 mmol/l glucose). (**b**) Effect of MRS-2279 and MRS-2500 (both at 1 μmol/l) on glucose (6 mmol/l)-induced electrical activity (n=8). (**c**) Insulin secretion was measured from isolated human islets at 1 and 8 mmol/l glucose (Gluc) in the absence and presence of MRS-2500 (n=4 donors). Data are from three to six donors. **p<0.01 and ***p<0.001 compared with 1 mmol/l glucose or as indicated

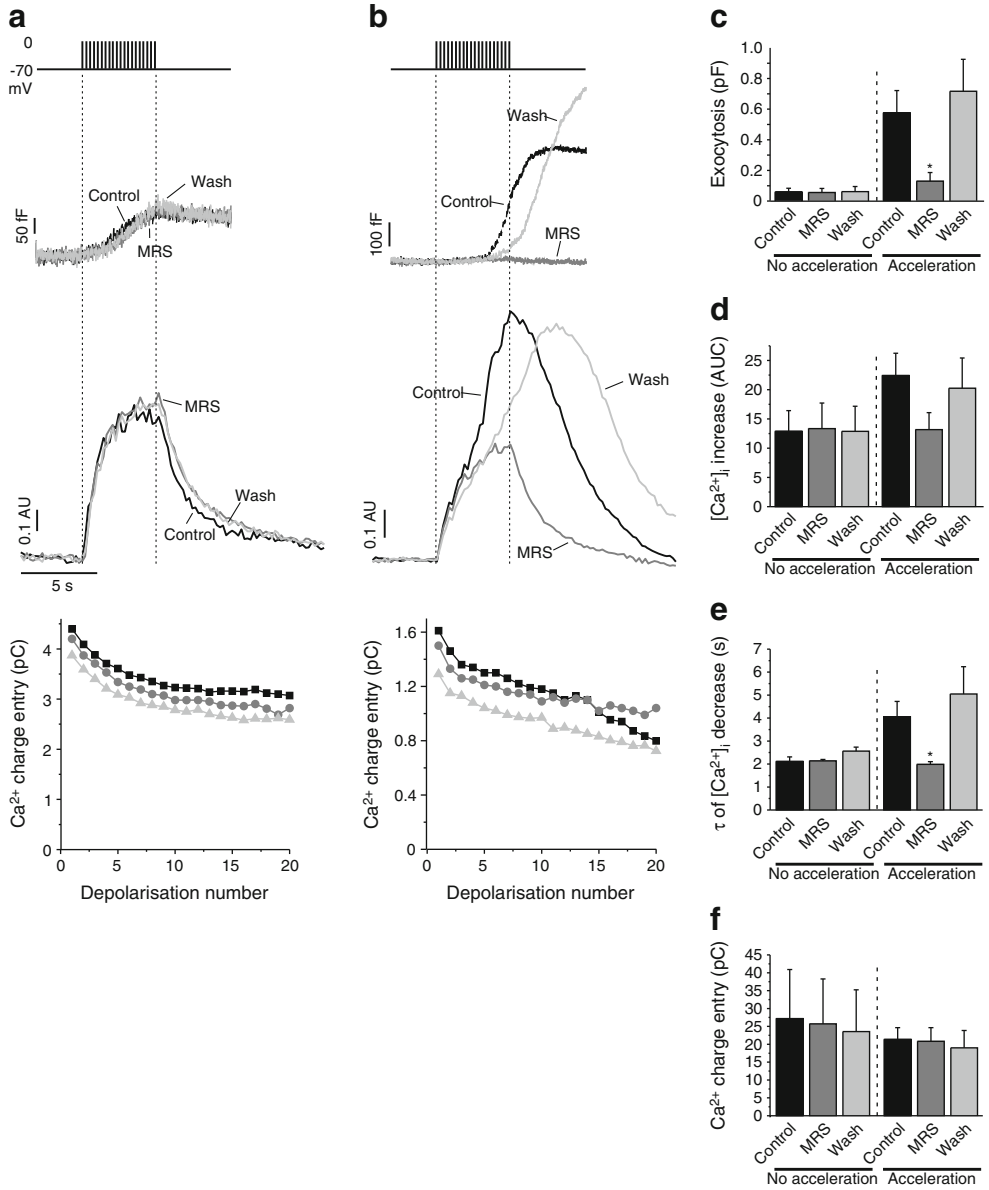

Fig. 8 Contribution of Ca^{2+} release to the autocrine $P2Y_1$-mediated Ca^{2+} signal and exocytosis. (**a**, **b**) Beta cells were stimulated by depolarisations from −70 to 0 mV (20 ms each, at 4 Hz) and membrane capacitance, Ca^{2+} currents and $[Ca^{2+}]_i$ were monitored simultaneously. Tetrodotoxin (0.1 μmol/l) was included to block voltage-gated Na^+ currents. The stimulation was performed under control conditions (black traces, black squares), with 1 μmol/l MRS-2279 (MRS, dark-grey traces, dark-grey circles) and after wash-out of the $P2Y_1$ antagonist (light-grey traces, light-grey triangles) in the same cells. Cells that did not (**a**) or did (**b**) show a secondary acceleration of exocytosis and $[Ca^{2+}]_i$ increase are shown. (**c**) Average exocytotic responses under control conditions, with 1 μmol/l MRS-2279 and after wash-out in cells without (No acceleration, $n=4$) or with (Acceleration, $n=5$) secondary acceleration of exocytosis and $[Ca^{2+}]_i$ increase during the stimulation protocol. (**d**) As for (**c**), showing the integrated $[Ca^{2+}]_i$ increase over baseline (AUC). (**e**) As for (**c**), showing the time constant (τ) of the $[Ca^{2+}]_i$ signal return to baseline after the depolarisation series. (**f**) As for (**c**), showing the integrated Ca^{2+} current evoked by the depolarisations. Data are from two donors. *$p<0.05$ compared with the 'acceleration' control

agreement with the findings of a recent study [12], our findings vary from those of another study suggesting a dominant role for $P2X_3$ [11]. However, the latter study employed pyridoxalphosphate-6-azophenyl-2′,5′-disulfonic acid (iso-PPADS) and oxidised ATP at concentrations that also strongly inhibit $P2Y_1$ [21, 22]. The more selective $P2X_{(1/3)}$ blocker TNP-ATP [23] does not affect $[Ca^{2+}]_i$ (Fig. 7a). While others have also suggested a role for $P2X_7$ [13], human $P2X_7$ has a very low affinity for ATP (half maximal effective concentration [ED_{50}] 0.78 mmol/l) and is insensitive to ADP and AMP [24], making a role for $P2X_7$ unlikely here.

$P2Y_1$ has a ~20-fold lower affinity for ATP than $P2X_3$ [1, 2]. However, it has been reported that insulin granules contain similar concentrations of ATP and ADP [3], suggesting that

both nucleotides play an important role. We demonstrated that human beta cells secrete ATP in response to increased $[Ca^{2+}]_i$, and the magnitude of ATP-release events was increased by glucose. This could result from intragranular ATP accumulation via granule-resident vesicular nucleotide transporter [25]. It should be noted, however, that in intact rodent islets extracellular ATP plays an important role in synchronising the electrical and Ca^{2+} responses among beta cells within and between islets through the induction of Ca^{2+} release from $InsP_3$-sensitive stores [26–28], in addition to stimulating exocytosis.

Some studies conducted in rodents, particularly mice, have found that ATP inhibits insulin secretion [8] and that insulin secretion in islets from mice lacking $P2Y_1$ is elevated [29]. This was attributed to direct inhibitory effects of ATP on exocytosis [30] or voltage-gated Ca^{2+} currents [31]. It has been reported that adenosine, acting on P1 receptors, inhibits insulin secretion from INS-1 cells [32], but this was not confirmed in human islets [11]. We show here that ATP stimulates depolarisation-evoked exocytosis without affecting Ca^{2+} currents in human beta cells. We found no evidence for a negative role of ATP in insulin secretion from human islets, consistent with the potentiation of insulin secretion from human islets following block of extracellular ATP degradation [11, 33].

ATP increased $[Ca^{2+}]_i$ in a biphasic manner, with an initial peak reflecting Ca^{2+} release from stores and a plateau reflecting Ca^{2+} influx. Similar to rat beta cells [34], Ca^{2+} was released via heparin-sensitive $InsP_3$ receptors, but was from thapsigargin-sensitive (ER) rather than bafilomycin-sensitive (acidic) compartments. Our findings differ from those of Jacques-Silva et al, who concluded that Ca^{2+} stores contribute little to the ATP-evoked Ca^{2+} signal in human beta cells [11], but this may be explained by experimental differences: the Ca^{2+} response under Ca^{2+}-free conditions is transient and will appear small (as AUC) when compared with prolonged agonist application in the presence of extracellular Ca^{2+}. Although a recent study suggests that autocrine activation of $P2Y_1$ stimulates diacylglycerol production in rodent and human beta cells [12], we were unable to determine a role for phospholipase C as the inhibitor U-73122 (5–10 μmol/l) also suppressed KCl-evoked Ca^{2+} responses (data not shown).

In mice, $P2Y_1$ receptors depolarise beta cells via inhibition of K_{ATP} channels [30]. In contrast, the ADP-evoked membrane current in human beta cells did not reverse at the K^+ equilibrium potential and was insensitive to tolbutamide. Instead, the current was abolished by removal or replacement of Na^+, indicating a Na^+- or non-selective cation conductance similar to $P2Y_1$-activated currents in neurons [35–37]. This effect was insensitive to thapsigargin (arguing against a store-operated channel) and to Gd^{3+}, a blocker of the Na^+ leak channel NALCN [38]. While the molecular identity of the $P2Y_1$-activated leak channel remains unclear, candidates include members of the transient receptor potential channel family [39].

We now show that blocking $P2Y_1$ receptors also inhibits electrical activity, $[Ca^{2+}]_i$ signalling and insulin secretion in human beta cells in the absence of exogenous ATP (Fig. 7). While $P2Y_1$ blockade inhibits spontaneous $[Ca^{2+}]_i$ transients in mouse beta cells [40], this was in the presence of a Ca^{2+} channel blocker and thus not likely caused by electrical activity and Ca^{2+} influx. We obtained similar results with two different, selective $P2Y_1$ antagonists [41, 42] that lacked non-specific effects on a number of human beta cell ion channels (ESM Fig. 1).

In cells stimulated with action potential-like depolarisations, $P2Y_1$ blockade reduces $[Ca^{2+}]_i$ and exocytosis without affecting Ca^{2+} currents (Fig. 8). Thus the MRS-2279-sensitive component of the $[Ca^{2+}]_i$ increase reflects Ca^{2+} release from stores triggered by autocrine activation of $P2Y_1$ receptors. This, in combination with activation of diacylglycerol and protein kinase C (DAG/PKC) [12], potentiates exocytosis. The Ca^{2+} signal required for insulin secretion is largely generated by Ca^{2+} influx through voltage-gated Ca^{2+} channels [43, 44]; our results are compatible with this because membrane depolarisation and Ca^{2+} influx are necessary to evoke ATP release and initiate the feedback loop. The secondary acceleration of depolarisation-evoked exocytosis, which we suggest reflects ER Ca^{2+} release, is observed in human [17, 45] but not mouse [46] beta cells.

Our data indicate that the contribution of Ca^{2+} release to the glucose-induced Ca^{2+} signal may have been underestimated. We present some limited data suggesting that release of Ca^{2+} from thapsigargin-sensitive stores during ATP stimulation is absent in a donor with type 2 diabetes; this could contribute to impaired secretion. There is evidence that ER stress is involved in the pathogenesis of type 2 diabetes [47] and is associated with reduced SERCA2b [48, 49], the main ER Ca^{2+} pump in beta cells [50]. The resulting lowering of ER Ca^{2+} levels may not only promote apoptosis but also impair beta cell stimulus–secretion coupling.

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Contribution statement SK, RY-D, ED, XW, AB, SC and MB researched data. MB designed the study and wrote the manuscript. All authors reviewed/edited the manuscript and contributed to the discussion. PEM analysed data, reviewed/edited the manuscript and takes full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript. All authors, with the exception of MB, approved the final version of the paper.

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