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ORIGINAL ARTICLE

ATP mediates a negative autocrine signal on stimulus-secretion coupling in mouse pancreatic β-cells

Cita Bauer[1] · Julia Kaiser[1] · Jelena Sikimic[1] · Peter Krippeit-Drews[1] · Martina Düfer[2] · Gisela Drews[1]

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Abstract

Purpose The role of ATP, which is secreted by pancreatic β-cells, is still a matter of debate. It has been postulated that extracellular ATP acts as a positive auto- or paracrine signal in β-cells amplifying insulin secretion. However, there is rising evidence that extracellular ATP may also mediate a negative signal.

Methods We evaluated whether extracellular ATP interferes with the Ca^{2+}-mediated negative feedback mechanism that regulates oscillatory activity of β-cells.

Results To experimentally uncover the Ca^{2+}-induced feedback we applied a high extracellular Ca^{2+} concentration. Under this condition ATP (100 µM) inhibited glucose-evoked oscillations of electrical activity and hyperpolarized the membrane potential. Furthermore, ATP acutely increased the interburst phase of Ca^{2+} oscillations and reduced the current through L-type Ca^{2+} channels. Accordingly, ATP (500 µM) decreased glucose-induced insulin secretion. The ATP effect was not mimicked by AMP, ADP, or adenosine. The use of specific agonists and antagonists and mice deficient of large conductance Ca^{2+}-dependent K^+ channels revealed that P2X, but not P2Y receptors, and Ca^{2+}-dependent K^+ channels are involved in the underlying signaling cascade induced by ATP. The effectiveness of ATP to interfere with parameters of stimulus-secretion coupling is markedly reduced at low extracellular Ca^{2+} concentration.

Conclusion It is suggested that extracellular ATP which is co-secreted with insulin in a pulsatile manner during glucose-stimulated exocytosis provides a negative feedback signal driving β-cell oscillations in co-operation with Ca^{2+} and other signals.

Keywords ATP · P2X receptor · Negative feedback · Calcium · β-cell

Abbreviations

V_m	cell membrane potential
$\Delta\psi$	mitochondrial membrane potential
$[Ca^{2+}]_c$	cytosolic Ca^{2+} concentration
BK-KO	BK channel knock-out

Introduction

ATP is the energy source of the cell, but serves as a signaling molecule, too. ATP enriched in insulin-containing granules via a vesicular nucleotide transporter [1] is thus secreted to the cell surface. The ATP concentration within the granules is around 3.5 mM [2]. ATP is co-released with insulin [3] or secreted without insulin in a process called kiss-and-run exocytosis [4] where only small molecules are discharged. Extracellular ATP is degraded by specific ecto-nucleotidases (for review see [5]) which contribute to the regulation of the extracellular ATP content. ATP exerts numerous important physiological effects in many mammalian cell types including pancreatic islets [6, 7] via activation of purinergic P2 receptors divided in the P2X and P2Y families with seven and eight subtypes, respectively. P2X receptors are ligand-operated cation channels while P2Y receptors belong to the large group of G-protein-coupled receptors. All these subtypes have been identified in the endocrine pancreas (for review see [7]). The effects of

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✉ Gisela Drews
gisela.drews@uni-tuebingen.de

[1] Department of Pharmacology, Institute of Pharmacy, University of Tübingen, Auf der Morgenstelle 8, Tübingen D-72076, Germany

[2] Department of Pharmaceutical and Medicinal Chemistry, University of Münster, Corrensstraße 48, Münster D-48149, Germany

extracellular ATP on β-cell function are numerous, but often controversial depending on the species, cell systems, and experimental conditions. Moreover, activation of different receptor subtypes may induce various and even opposed effects which complicates the understanding of the action of extracellular ATP on β-cell function. Studies with murine β-cells mainly report an inhibitory effect of extracellular ATP on stimulus-secretion coupling (SSC), but the underlying mechanism is far from being clear. Two studies reported that extracellular ATP reduced insulin secretion of isolated mouse islets despite a reduction of K^+ conductance and K_{ATP} current, respectively [8, 9]. Petit et al. [8] assumed that the inhibitory effect of ATP can be attributed to the degradation of ATP to adenosine while Poulsen et al. [9] suggested that it is caused by an interaction with the exocytotic machinery via $P2Y_1$-induced activation of calcineurin. Other groups observed stimulation and inhibition of insulin secretion in dependence of different receptor subtypes [10–12]. Studies with P2Y receptor knockout (KO) mice did not solve the discrepancies since $P2Y_1$ receptor-KO mice show increased glucose-induced insulin secretion while $P2Y_{14}$ receptor-KO mice exhibit decreased secretion [13, 14]. In rats the results of extracellular ATP on β-cell function are less controversial and in contrast to mice a stimulatory effect of ATP is supported by most studies (e.g. [15–17] although participating receptors and involved mechanisms are not entirely clear. IP_3-induced Ca^{2+} mobilization with subsequent activation of Ca^{2+} influx via CRAC channels are mechanisms discussed in this context. ATP-evoked Ca^{2+} release from the endoplasmic reticulum (ER) is observed in mouse, rat, and human β-cells; however, in mouse β-cells this seems not to result in CRAC channel activation [18]. Hellman and colleagues [19–21] suggested that extracellular ATP is involved in the control of the rhythmic activity of β-cells and in the propagation of the oscillations from cell to cell. In the proposed model ATP has a time-dependent dual effect on β-cell function: prompt activation followed by inhibition.

In contrast to other studies, we focused on the influence of the nucleotide on bursting activity of β-cells. In particular, we investigated effects of ATP on β-cell function at conditions with enhanced Ca^{2+}-mediated negative feedback on SSC, especially reflecting the burst phases with action potentials.

Materials and methods

Animals and islet preparation

Islets of Langerhans were isolated from adult C57Bl/6N mice or C57Bl/6N mice with a global knockout of BK channels (BK-KO). The mice were bred in the animal facility of the Department of Pharmacology at the University of Tübingen. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and German laws were followed. Isolation and culture were performed as described previously [22], except, islets of Langerhans were dispersed to single cells or cell clusters by trypsin treatment.

Solutions and chemicals

Recordings of $[Ca^{2+}]_c$ were performed with a bath solution which contained (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES; $CaCl_2$, and glucose as indicated, pH 7.4 adjusted with NaOH. The same bath solution was used for the determination of the mitochondrial membrane potential ($\Delta\psi$) and for measurements of membrane potential (V_m) in the perforated-patch configuration. For this purpose, the pipette solution was composed of (in mM): 10 KCl, 10 NaCl, 70 K_2SO_4, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, 0.27 amphotericin B, pH adjusted to 7.15 with KOH. For perforated-patch measurements of Ca^{2+} currents a bath solution of the following composition was used (mM): 115 NaCl, 1.2 $MgCl_2$, 10 $CaCl_2$, 10 TEA, 10 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. The respective pipette solution contained (in mM): 10 KCl, 10 NaCl, 70 Cs_2SO_4, 7 $MgCl_2$, 10 HEPES, 0.27 amphotericin B, pH adjusted to 7.15 with NaOH. Krebs–Ringer–Hepes solution for insulin secretion was composed of (in mM): 122 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 10 $CaCl_2$, glucose as indicated, 10 HEPES, 0.5 % bovine serum albumin and pH 7.4 adjusted with NaOH.

Adenosine 5′-triposphate (ATP) was obtained from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (Taufkirchen, Germany), the $P2X_{1,3}$-agonist α,β-methyleneadenosine 5′-triphosphate (αβ-MeATP) from Tocris Bioscience (Bristol, United Kingdom). Fura-2/AM was either purchased from Biotrend (Köln, Germany) or Sigma-Aldrich (Schnelldorf, Germany). Rhodamine, RPMI 1640 medium, and penicillin/streptomycin was from Invitrogen (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich or Carl Roth in the purest form available.

Measurements of the mitochondrial membrane potential

$\Delta\Psi$ was measured as rhodamine 123 fluorescence at 480 nm excitation wave length as described in [23]. To evaluate the effects, the values were averaged for 60 s at the end of each interval before solution change.

Measurement of $[Ca^{2+}]_c$

$[Ca^{2+}]_c$ was measured by the fura-2 method as described by Grynkiewicz et al. [24]. Details are described in [22]. In brief, cells were loaded with 5 µM fura-2-AM for 35 min at

37 °C. Fluorescence was excited at 340 and 380 nm, emission was filtered (LP515), and measured by a digital camera. $[Ca^{2+}]_c$ was calculated according to an in vitro calibration with fura2-5K-salt. The area under the curve (AUC) was taken to reveal the effect of ATP or αβ-MeATP on oscillations of the cytosolic Ca^{2+} concentration. The AUC was evaluated in the steady state before the switch to ATP or αβ-MeATP and between min 0 to 5 and 5 to 10 in the presence of the nucleotide. In the experiments with NF-279 the AUC was calculated for 10 min after addition of the drug. In fura-2 measurements with whole islets the fluorescence ratio F340/F380 is given instead of $[Ca^{2+}]_c$.

Patch-clamp measurements

Membrane currents and potentials were recorded with an EPC-9 patch-clamp amplifier using "Patchmaster" software (HEKA, Lambrecht, Germany). For V_m measurements, the plateau potential under control conditions was compared to the maximal hyperpolarization induced by ATP. Where applicable, action potential frequency was determined during a period of 2.5 min before ATP application and separately during the first and second 2.5 min period after addition of ATP. At the high Ca^{2+} concentration of 10 mM the membrane potential oscillated, i.e., burst phases with action potentials changed with silent interburst phases. Under this condition the fraction of plateau phase (FOPP ~ percentage of time with spike activity) was calculated for 2 min before and during min 3 and 5 after drug application. Currents through L-type Ca^{2+} channels were measured using the perforated-patch configuration in the voltage-clamp mode by 50 ms pulses from –70 to 0 mV. The last three currents prior to solution change were used for analysis of the maximum peak current (I_{peak}), the AUC to determine charge movement, and τ to characterize current inactivation.

Insulin secretion

After preparation islets were kept overnight in RPMI 1640 culture medium with 11.1 mM glucose. Details for steady-state incubations are described in [22]. Briefly, insulin secretion under steady-state conditions was measured for 1 h at 37 °C under conditions as indicated. For perifusion experiments 50 islets of Langerhans were perifused continuously with bath solution as described in [23] and test substances as indicated and a sample was taken every 2 min. Levels of insulin were determined by radioimmunoassay (Merck Millipore, Darmstadt, Germany).

Statistics

Each series of experiments was performed with islets of Langerhans or cell clusters from at least three independent preparations unless otherwise indicated. Means ± SEM are given for the indicated number of experiments. Statistical significance of differences was assessed by a Student's *t*-test for paired values. Multiple comparisons were made by ANOVA followed by Student–Newman–Keuls test. *P*-values ≤0.05 were considered significant.

Results

Extracellular ATP affects key parameters of SSC

Cytosolic Ca^{2+} concentration and cell membrane potential

The cell membrane potential (V_m) takes a prominent position within SSC as it connects glucose metabolism to insulin secretion by determining the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). Within the SSC, opening of voltage-dependent L-type Ca^{2+} channels and Ca^{2+} influx represent the decisive trigger for secretion of insulin from storage vesicles. For this reason, alterations of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) are crucial for insulin secretion. The recording in Fig. 1a shows periodic oscillations of $[Ca^{2+}]_c$ in the presence of 10 mM glucose and 2.5 mM Ca^{2+}. Addition of ATP in a concentration of 100 µM interrupted this regular pattern so that the next oscillation appeared later and exhibited a smaller amplitude. To quantify this observation, the AUC of $[Ca^{2+}]_c$ was determined before and after addition of ATP (Fig. 1b). The AUC was reduced from 384 ± 27 nM × 5 min under control conditions to 300 ± 29 nM × 5 min during the first 5 min period in the presence of ATP. The AUC amounted to 353 ± 24 nM × 5 min in the second 5 min period of ATP treatment indicating a transient effect of ATP.

The plasma membrane potential V_m was measured in the perforated-patch configuration under the same conditions. The record (Fig. 1c) and the bar chart (Fig. 1d) show that ATP had no effect on V_m (−37 ± 3 mV under control conditions vs. −39 ± 3 mV in the first 2.5 min and −38 ± 4 mV in the second 2.5 min period in the presence of ATP). Likewise, action potential frequency did not change (Fig. 1c, e). It amounted to 3.3 ± 0.6 Hz before ATP application, to 2.9 ± 0.6 Hz during the first 2.5 min period with ATP ($n = 4$, n.s.), and to 3.4 ± 0.5 Hz during the second 2.5 min period with ATP ($n = 4$, n.s.). Under these conditions characteristic slow waves consisting of electrically silent phases (interbursts) and active phases (bursts with action potentials) are hardly detectable [25]. Bursting activity can be achieved by increasing the extracellular Ca^{2+} concentration (compare Fig. 1c and Fig. 2c). It is known that $[Ca^{2+}]_c$ affects its own entry via opening of K_{ATP} channels, i.e., it exerts an important feedback control on insulin secretion [26, 27]. Increasing the extracellular Ca^{2+}

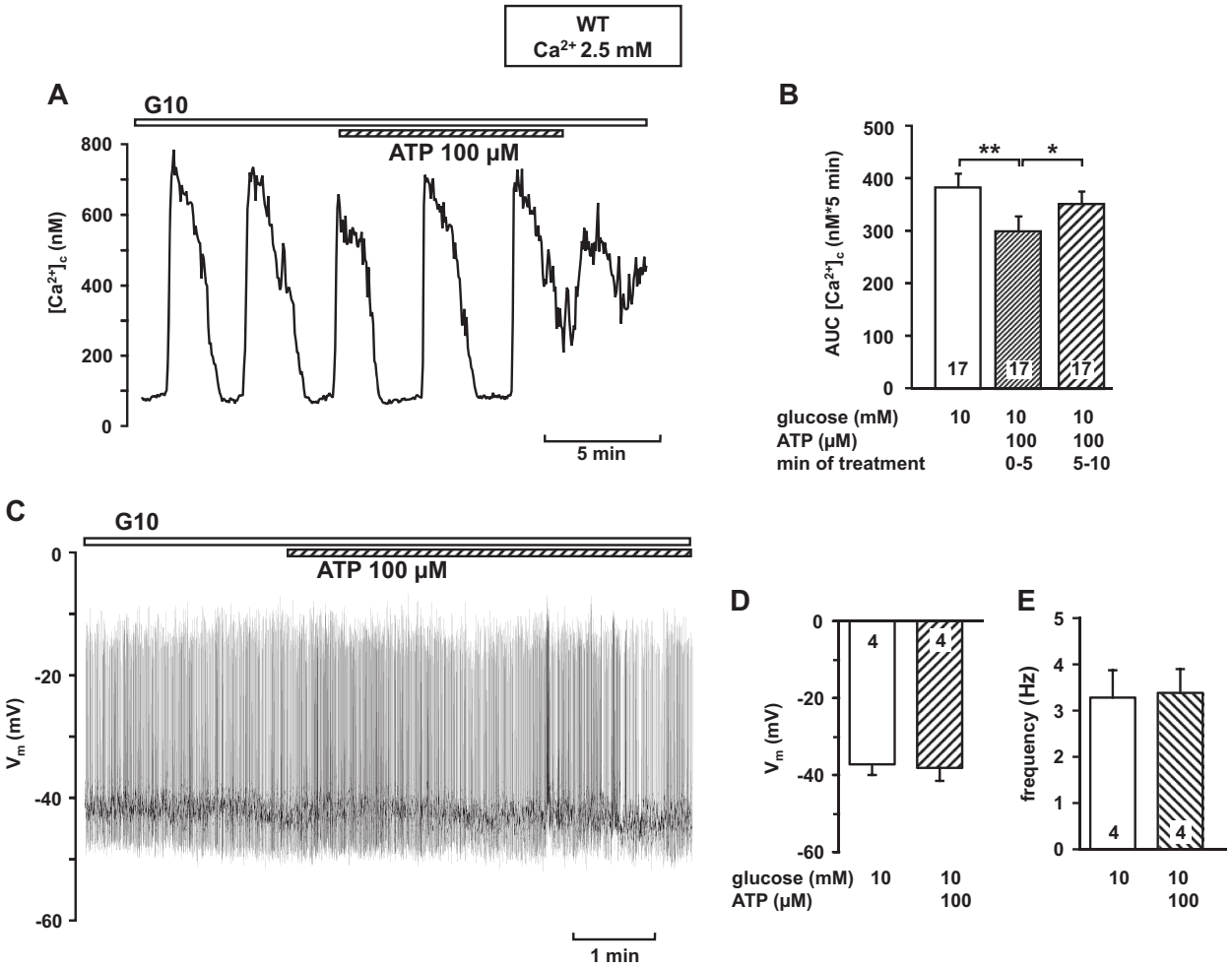

Fig. 1 Extracellular ATP slightly affects V_m and $[Ca^{2+}]_c$ in the presence of 10 mM glucose and 2.5 mM Ca^{2+}. **a** Representative recording presenting regular oscillations of $[Ca^{2+}]_c$. ATP application results in transiently reduced AUC of $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after addition of ATP. **c** ATP does not affect V_m in the presence of 2.5 mM Ca^{2+}. Representative recording showing continuous spike activity in response to 10 mM glucose and 2.5 mM Ca^{2+}, measured in the perforated-patch configuration. **d** Summary of the quantitative analysis of the membrane potential measurements. Values under control condition and after addition of ATP were taken at the plateau potential 2.5 min before solution change. **e** Action potential frequency determined during a period of 2.5 min before ATP application and between min 2.5 and 5 in the presence of ATP. The numbers in the columns indicate the number of experiments with different cell clusters from two to three different preparations. $*P \le 0.05$, $**P \le 0.01$

concentration which enhances the Ca^{2+} gradient and thus Ca^{2+} entry augments this feedback mechanism under experimental conditions. To evaluate whether extracellular ATP interferes with this Ca^{2+}-mediated feedback mechanism, it was amplified in the following experiments by applying 10 mM Ca^{2+} in the presence of 10 mM glucose (G10/Ca10). Treatment with 100 µM ATP strongly reduced the AUC of $[Ca^{2+}]_c$ from 414 ± 26 nM × 5 min under control conditions to 188 ± 19 and 319 ± 34 nM × 5 min, respectively, during the first and second 5 min period of ATP addition (Fig. 2a, b). Under these conditions with 10 mM Ca^{2+} the effect of ATP was much larger in the first 5 min period of ATP addition compared to conditions with 2.5 mM Ca^{2+} and remained significant in the second application period. Furthermore, the interburst phase directly after addition of ATP was markedly longer compared to the mean interburst phase under control condition (247±35 vs. 117±21 s, $n = 20$, $P \le 0.001$). The representative measurement of electrical activity in Fig. 2c shows characteristic slow waves in the presence of G10/Ca10 as a result of the Ca^{2+} feedback described above. Addition of ATP (100 µM) led to a sustained hyperpolarization of V_m from a plateau potential of -44.2 ± 1.4 to -68.0 ± 3.5 mV (Fig. 2d).

Mitochondrial membrane potential

Hyperpolarization of V_m can be caused by opening of K_{ATP} channels due to a reduction in ATP production. The mitochondrial membrane potential ($\Delta\Psi$) is directly linked to

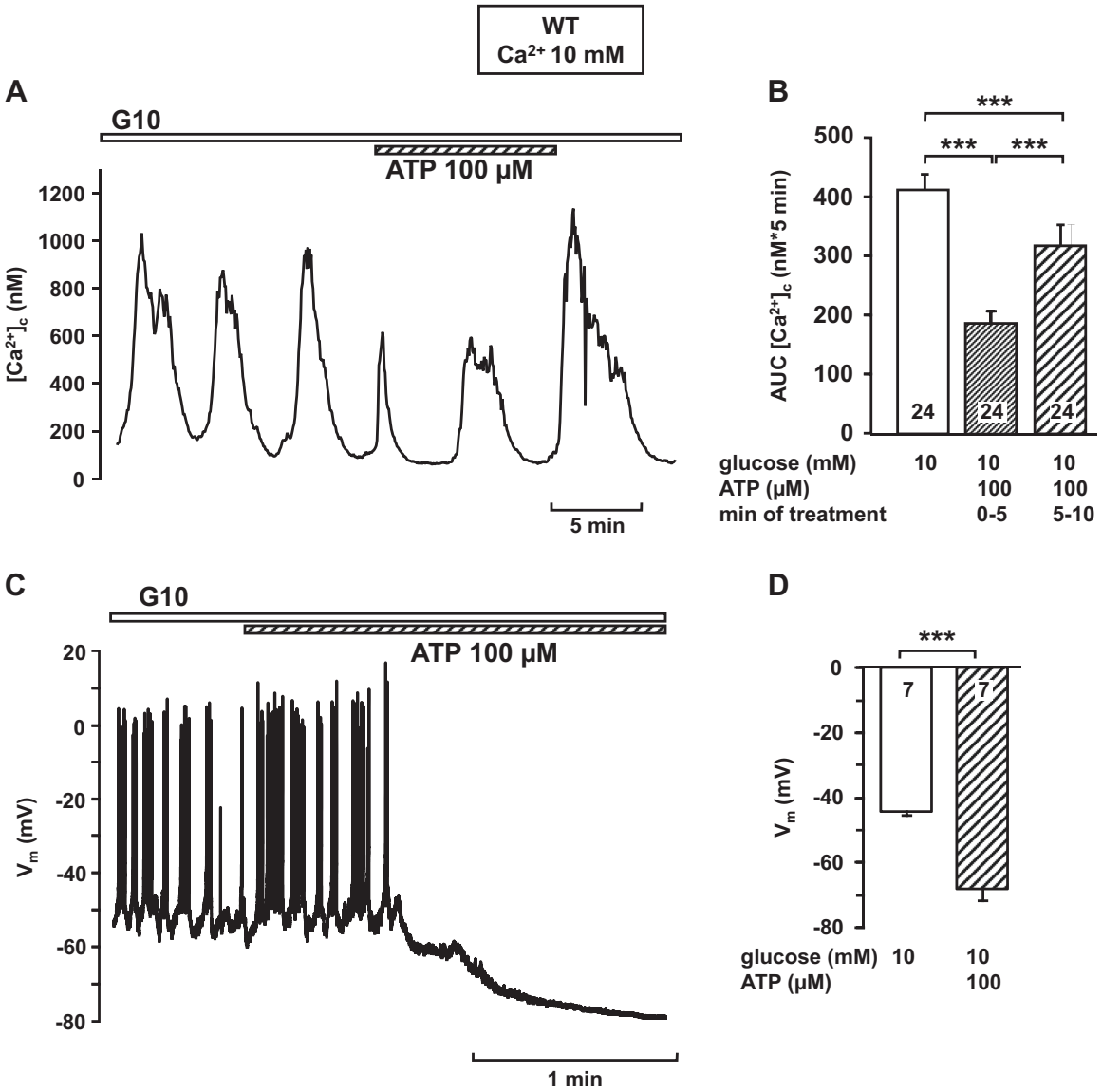

Fig. 2 Enhancing the negative feedback of Ca^{2+} on SSC augments the effectiveness of extracellular ATP on V_m and $[Ca^{2+}]_c$. ATP clearly affects oscillations of $[Ca^{2+}]_c$ and V_m in the presence of 10 mM glucose and 10 mM Ca^{2+}. **a** Representative recording showing regular oscillations of $[Ca^{2+}]_c$. ATP addition leads to a reduction in the AUC of $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after addition of ATP. **c** ATP hyperpolarizes the cell membrane potential (V_m). Representative recording measured in the perforated-patch configuration showing slow waves in response to 10 mM glucose and 10 mM Ca^{2+}. **d** Summary of the quantitative analysis of V_m measurements. Values under control condition were taken at the plateau potential, values after application of ATP at the maximal hyperpolarization. The numbers in the columns indicate the number of experiments with different cell clusters from at least three different mice. $***P \le 0.001$

glucose metabolism and ATP production [27] because the electrochemical proton gradient across the inner mitochondrial membrane determines the activity of the F1/F0-ATPase. Increasing glucose concentration caused a hyperpolarization of $\Delta\Psi$, which is indicated by a decrease in rhodamine 123 fluorescence signal and reflects ATP production. On average, the fluorescence signal was lowered from 502 ± 47 a.u. in the presence of 0.5 mM glucose to 412 ± 32 a.u. upon an increase of the glucose concentration to 10 mM ($P \le 0.001$, $n = 28$). ATP (100 µM) had no effect on $\Delta\Psi$ (409±32 a.u.) ($n = 28$, n. s., data not shown). The experiment was repeated with 500 µM ATP and additionally in 5 mM glucose to create conditions where $\Delta\Psi$ is not that hyperpolarized but ATP did not show any effect (G10/Ca10: 530±35 a.u., G10/Ca10 + 500 µM ATP: 528±34 a.u., $n = 27$, n.s.; G5/Ca10: 550±45 a.u., G5/Ca10 + 500 µM ATP: 552±45 a.u., $n = 16$, n.s., data not shown). Thus, extracellular ATP seems not to affect mitochondrial ATP production.

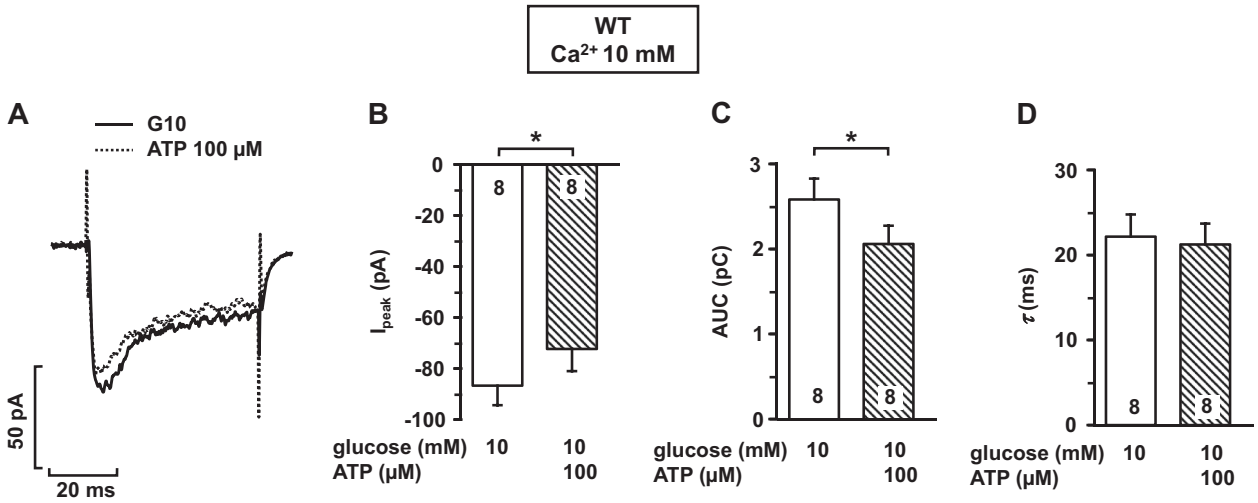

Fig. 3 Extracellular ATP influences currents through L-type Ca^{2+} channels. **a** Representative recordings of I_{Ca} under control conditions (black curve) and after addition of ATP (dotted curve). Currents were elicited by 50 ms voltage steps from –70 to 0 mV. **b–d** Summary of the quantitative analysis of the peak current (I_{peak}) (**b**) and the charge movement (determined by the area under the curve (AUC)) (**c**) as well as the inactivation of the currents determined by the time constant τ (**d**). The numbers in the columns indicate the number of experiments with different cell clusters from at least three different mice. *$P \leq 0.05$

Current through voltage-dependent Ca^{2+} channels

Next, a possible influence of ATP on Ca^{2+} currents was studied. In Fig. 3a, a typical Ca^{2+} current is shown which was elicited by a 50 ms voltage step from −70 to 0 mV. Under control conditions (black curve) opening of L-type Ca^{2+} channels led to a marked and rapid Ca^{2+} influx followed by a slow current decay due to Ca^{2+}-dependent current inactivation. Addition of ATP (100 µM, dotted curve) reduced the peak Ca^{2+} current (I_{peak}) from −87 ± 8 to −72 ± 9 pA (Fig. 3b) and the charge movement, measured as area under the curve (AUC), from 2.6 ± 0.3 to 2.1 ± 0.2 pC (Fig. 3c). The Ca^{2+}-dependent inactivation of the channels [28] which was determined by calculating the time constant τ was not influenced by ATP (22 ± 3 ms under control conditions vs. 21 ± 2 ms with ATP) (Fig. 3d).

Extracellular ATP reduces insulin secretion

The effect of ATP on insulin secretion was studied in steady state as well as in perifusion experiments. As seen in Fig. 4a, after 1 h steady-state incubation in G15/Ca10, ATP (500 µM and 1 mM) reduced glucose-induced insulin secretion by 26% and 33%, respectively. ATP in a concentration of 100 µM was without effect in these experiments. In contrast to the experiments described before, insulin secretion measurements are performed with whole islets which are encircled by a capsule of connective tissue. Evidently, this capsule impedes diffusion of ATP to the islets cells, an observation that confirms earlier findings [29]. To test for this, the Ca^{2+} experiments illustrated in Fig. 2a, b were repeated with whole islets instead of dispersed cells. Indeed, 100 µM ATP did not influence $[Ca^{2+}]_c$ measured with whole islets (as fluorescence ratio F340/F380) (10 mM glucose: 0.48 ± 0.09; after ATP application: 0.48 ± 0.09, $n = 6$ different islets, not shown). This suggestion is supported by the finding that pretreatment of islets for 1 h with 100 and 200 µM ATP, respectively, reduced the response to a subsequent glucose stimulus (Fig. 4b).

The inhibitory effect of ATP on insulin secretion was not mimicked by AMP, ADP, or adenosine (Suppl. Figure 1). In contrast to ATP, concentrations of adenosine higher than 100 µM increased insulin secretion. Perifusion experiments disclosed that ATP diminished the first and second phase of insulin secretion. Rising the glucose concentration from 3 to 15 mM glucose in the absence (solid curve) and presence of ATP (dotted curve) revealed that the first phase of insulin secretion was markedly reduced by ATP (Fig. 5a). The quantitative analyses of the AUC demonstrated that ATP decreased the first phase of insulin secretion from 61 ± 14 ng insulin/(50 islets × 30 min) to 41 ± 10 ng insulin/(50 islets × 30 min) (Fig. 5b). In Fig. 5c the glucose concentration was first raised from 3 to 15 mM glucose showing the typical biphasic pattern of insulin secretion. The figure shows the typical steep, transient rise during the first phase followed by a lower but still elevated plateau in the second phase. Addition of ATP at the steady state during the second phase reduced insulin secretion. For further quantification the AUC was calculated for the last 10 min in the absence and presence of ATP, respectively. It decreased from 15 ± 2 ng insulin/(50 islets × 10 min) to 12 ± 2 ng insulin/(50 islets × 10 min) (Fig. 5d).

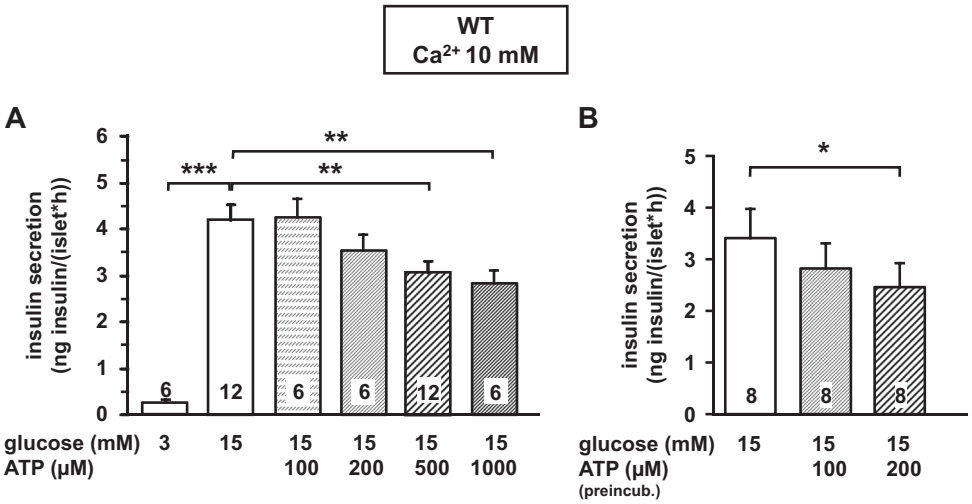

Fig. 4 Extracellular ATP reduces steady-state insulin secretion. **a** ATP dose-dependently decreases insulin secretion in the presence of 15 mM glucose after 1 h incubation under steady-state conditions. **b** Pre-treatment with ATP for 1 h reduces the response to a subsequent glucose stimulus. The numbers in the columns indicate the number of mice. $*P \le 0.05$, $**P \le 0.01$, $***P \le 0.001$

Extracellular ATP mediates its effects by activation of P2X receptors

The $P2X_{1,3}$ agonist αβ-MeATP was used to test whether ATP-induced effects on SSC are mediated via P2X receptors. As shown in Fig. 6a αβ-MeATP mimicked the effect of ATP on $[Ca^{2+}]_c$ oscillations. αβ-MeATP led to a decrease in the AUC of $[Ca^{2+}]_c$ (Fig. 6b) from 345 ± 22 nM × 5 min under control conditions to 264 ± 33 and 287 ± 30 nM × 5 min, respectively, during the first and second 5 min period in the presence of αβ-MeATP. As observed with ATP, duration of the interburst phase directly after addition of αβ-MeATP was prolonged compared to the mean interburst phase before application of the $P2X_{1,3}$ agonist (240±54 vs. 123±17 s, $n = 16$, $P \le 0.05$). Furthermore, αβ-MeATP reduced glucose-induced insulin secretion by 36% (from 3.1 ± 0.4 ng insulin/(islet × h) under control conditions to 2.0 ± 0.1 ng insulin/(islet × h) in the presence of αβ-MeATP) after 1 h steady-state incubation in 15 mM glucose and 10 mM Ca^{2+} (Fig. 6c). Insulin secretion experiments with the $P2X_1$ antagonist NF-279 revealed that ATP is no longer able to reduce insulin secretion when $P2X_1$ channels are blocked (Fig. 6d). Insulin secretion in the presence of NF-279 was 2.2 ± 0.2 ng insulin/(islet × h) before and 2.2 ± 0.1 ng insulin/(islet × h) after addition of ATP. In contrast, the $P2X_3$ antagonist RO-3 was not able to suppress the inhibitory effect of extracellular ATP (Fig. 6e). Insulin secretion in the presence of RO-3 was 3.9 ± 0.7 ng insulin/(islet × h) before and 2.4 ± 0.3 ng insulin/(islet × h) after addition of ATP.

The most prominent receptor of the P2Y family in β-cells is the $P2Y_1$ receptor [12, 13, 30]. This receptor family is Gq protein-coupled and thus affects intracellular Ca^{2+} stores. We observed a short Ca^{2+} transient after ATP administration (Fig. 2a). This was also present when L-type Ca^{2+} channels were blocked (Suppl. Figure 2A, B) and is due to ER store depletion (Suppl. Figure 2C, D). However, pre-treatment of β-cells with the SERCA inhibitor thapsigargin did not affect the inhibitory effect of ATP on insulin secretion (Suppl. Figure 2E). Moreover, the inhibitory effect of ATP on insulin secretion was not influenced by the specific $P2Y_1$ receptor antagonist MRS-2179 (Suppl. Figure 2F). Obviously, the P2Y receptor family does not essentially contribute to the inhibitory effect of ATP.

Possible involvement of Ca^{2+}-dependent potassium channels

As P2X receptors are unspecific cation channels leading to a depolarizing cation influx, activation of these receptors seems hard to reconcile with the above-mentioned negative influences of ATP and αβ-MeATP on parameters of SSC. In β-cells several types of Ca^{2+}-dependent K^+ channels are expressed mediating K^+ outflux upon activation including BK channels with large conductance and SK4 channels with intermediate conductance. Both channel types are involved in the regulation of β-cell function [31, 32]. To test whether extracellular ATP activates these channels to mediate a negative feedback on SSC, we used β-cells from BK-KO mice and TRAM-34 as a pharmacologic inhibitor of SK4 channels.

Figure 7a shows a measurement of V_m in the perforated-patch configuration with BK-KO β-cells. After SK4 channel inhibition the BK channel-deficient β-cells are rather depolarized and show continuous spike activity. Administration of ATP (100 µM) only led to a transient hyperpolarization of −10.7 ± 2.6 mV in six out of nine experiments (from −39 ± 3 mV in the presence of TRAM-34 in 10 mM glucose and 10 mM Ca^{2+} to −50 ± 3 mV after application of ATP (Fig. 7b)). A slight depolarization (from −34 ± 3 mV in the presence of TRAM-34 to −29 ± 3 mV) was

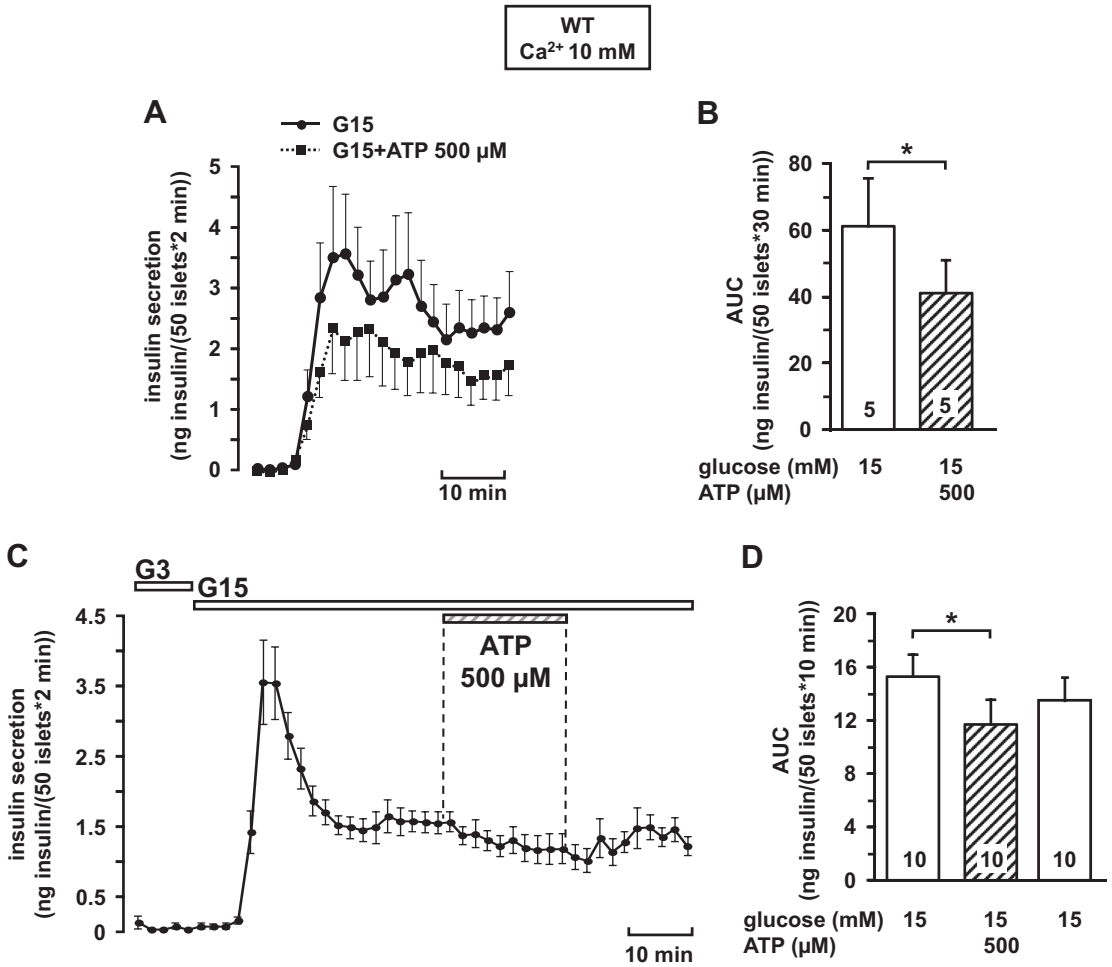

Fig. 5 Extracellular ATP diminishes first and second phase insulin secretion. **a** ATP reduces the first phase of insulin secretion. Curves represent the mean values of first phase insulin secretion. Samples were taken every 2 min. Isolated islets were either perifused with 15 mM glucose without (black curve) or with ATP (dotted curve). In both cases, islets were silenced with 3 mM glucose (±ATP 500 µM) before solution change. **b** Summary of the quantitative analysis determined by the AUC of the first 30 min after changing the glucose concentration from 3 to 15 mM (±ATP 500 µM). **c** ATP reduces the second phase of insulin secretion. The curve represents the mean values of the first and second phase of insulin secretion under control conditions. Administration of ATP in the second phase leads to reduction of insulin secretion. Samples were taken every 2 min. **d** Summary of the quantitative analysis determined by the AUC of the last 10 min under control conditions and ATP administration, respectively. The numbers in the columns indicate the number of mice. *$P \leq 0.05$

observed in all nine measurements during the last 3 min of ATP addition (Fig. 7c).

In addition, the effect of ATP on Ca^{2+} oscillations is reduced after inhibition of SK4 channels in BK-KO cells. Figure 7d shows Ca^{2+} oscillations in β-cells from BK-KO mice in the presence of TRAM-34. Under these conditions ATP reduced the AUC of $[Ca^{2+}]_c$ only transiently despite of the high extracellular Ca^{2+} concentration (Fig. 7e) which fits to the transient effect of ATP on electrical activity. The AUC decreased from 472 ± 29 nM × 5 min under control conditions with TRAM-34 to 339 ± 20 nM × 5 min in the presence of ATP during the first 5 min application period. In the second 5 min period of ATP addition the AUC of $[Ca^{2+}]_c$ amounted to 439 ± 21 nM × 5 min.

In agreement with the influence of BK and SK4 channels on ATP-induced alterations in $[Ca^{2+}]_c$, the effect of ATP on insulin secretion was reduced after inhibition of these channels. Five hundred micromolar ATP led to a significant reduction of glucose-stimulated insulin secretion which amounted to 34% under control conditions but only to 23% after pharmacological blockage of BK channels with 100 nM iberiotoxin and SK4 channels with 10 µM TRAM-34. In this series of experiments, insulin secretion (15 mM glucose) was reduced by ATP from 4.5 ± 0.5 to 2.9 ± 0.1 ng insulin/(islet × h) ($n = 6$, $P \leq 0.05$). In the presence of TRAM-34 and iberiotoxin the effect of ATP was lower (reduction from 4.2 ± 0.3 to 3.1 ± 0.2 ng insulin/(islet × h), $n = 6$, $P \leq 0.05$, data not shown).

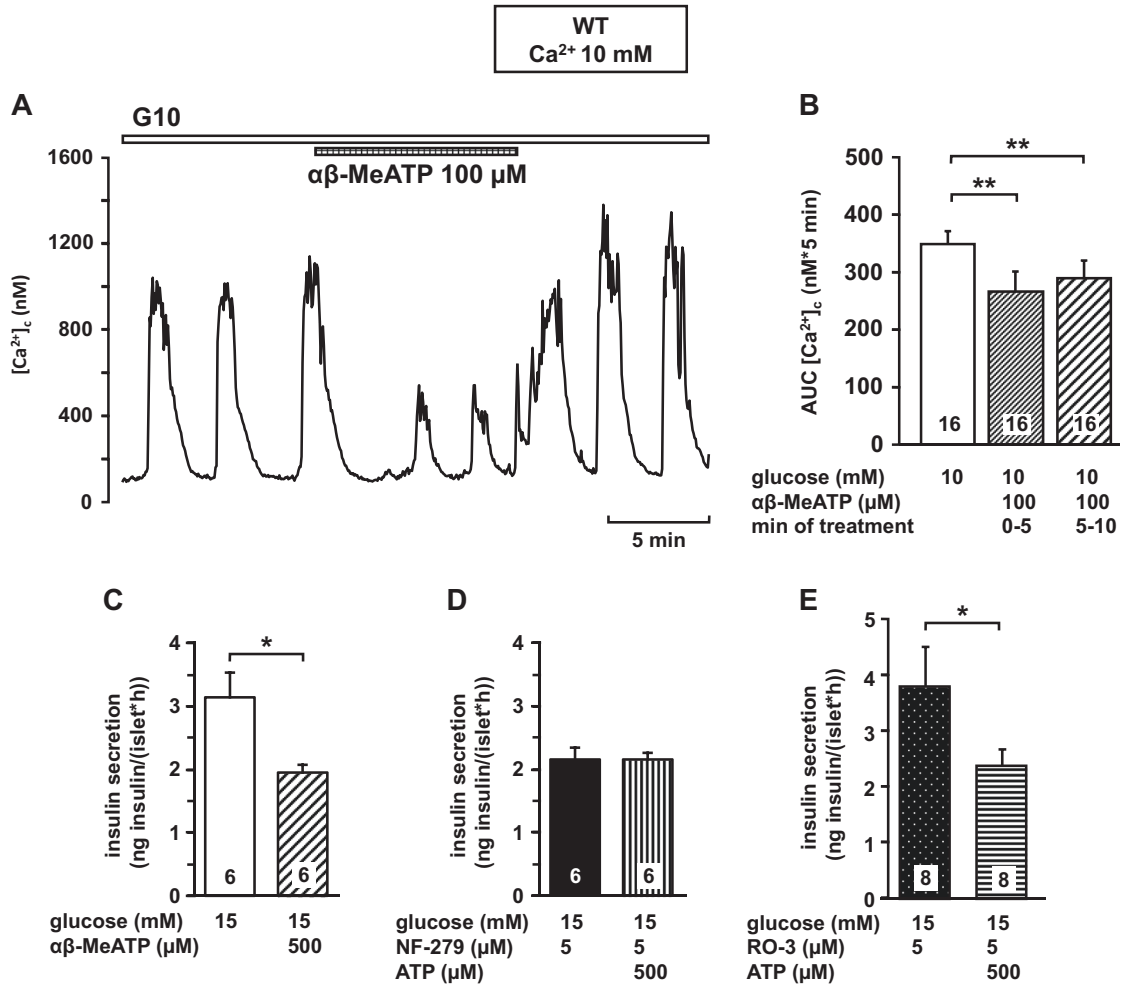

Fig. 6 Extracellular ATP mediates its effects by activating P2X receptors. The $P2X_{1,3}$ agonist αβ-MeATP (100 µM) mimics the effect of ATP on oscillations of $[Ca^{2+}]_c$. **a** Representative recording showing regular oscillations of $[Ca^{2+}]_c$. αβ-MeATP (100 µM) reduced the AUC of $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after application of αβ-MeATP. **c** αβ-MeATP (500 µM) reduces insulin secretion in the presence of 15 mM glucose after 1 h incubation under steady-state conditions. **d** After inhibition of $P2X_1$ channels with the specific antagonist NF-279 (5 µM) ATP has no effect on insulin secretion in 15 mM glucose. **e** The $P2X_3$ receptor blocker RO-3 does not alter the effect of ATP on insulin secretion. The numbers in the columns indicate the number of experiments with different cell clusters from three different mice (**a**, **b**) or the number of mice used for islet preparations (**c**–**e**). $^*P \le 0.05$, $^{**}P \le 0.01$

Blockage of $P2X_1$ channels increased electrical activity and $[Ca^{2+}]_c$

According to our hypothesis inhibition of the $P2X_1$ channels should increase electrical activity, the AUC of $[Ca^{2+}]_c$, and insulin secretion. Figure 8a, b reveals that NF-279 indeed augmented the AUC of $[Ca^{2+}]_c$ from 592 ± 35 nM × 10 min under control conditions to 632 ± 45 nM × 10 min in the presence of 5 µM NF-279. Accordingly, the FOPP increased from 46 ± 9 to 56 ± 11% after addition of NF-279 (Fig. 8c, d). Paradoxically, insulin secretion was reduced by NF-279 from 2.81 ± 0.30 to 2.16 ± 0.18 ng insulin/(islet × h) ($n = 6$, $P \le 0.05$). Since this is unexpected and does not fit to the results obtained with NF-279 on $[Ca^{2+}]_c$ and V_m, it is suggested that this effect is an unspecific Ca^{2+}-independent interaction with the exocytotic machinery. To further strengthen this conclusion, we tested suramin on $[Ca^{2+}]_c$, another frequently used blocker of $P2X_1$ channels, although less specific. Suramin enhanced the AUC of $[Ca^{2+}]_c$ during a 5 min application period in 9 out of 13 cells from 323 ± 29 nM × 5 min to 404 ± 42 nM × 5 min. Subsequent addition of ATP for 5 min in the presence of suramin did not significantly alter the AUC of $[Ca^{2+}]_c$ (Fig. 8e, f).

Discussion

The inhibitory signaling pathway of extracellular ATP

It is well known that signaling through purinergic receptors affects insulin secretion and that ATP released from

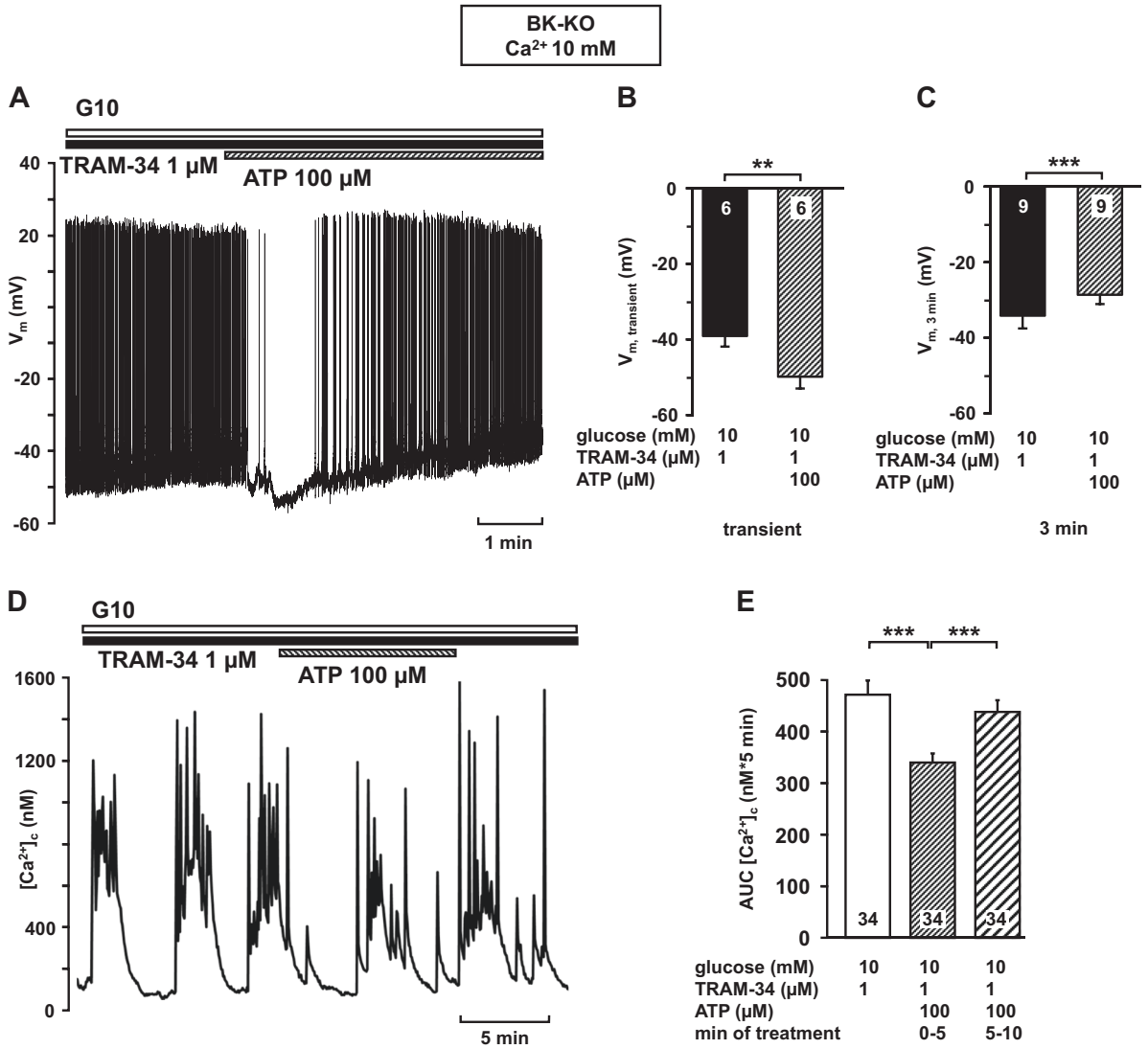

Fig. 7 Possible involvement of Ca^{2+}-dependent potassium channels. **a** The effect of ATP on V_m is reduced after elimination of BK and SK4 channels. Representative recording showing continuous spike activity in β-cells of BK-KO mice after addition of the SK4 channel blocker TRAM-34 measured in the perforated-patch configuration. ATP hyperpolarizes V_m only transiently and leads to a slight depolarization during the last period of administration. **b**, **c** Summary of the quantitative analysis of V_m measurements. Values under control condition were taken at the plateau potential; values under ATP were determined at the minimum of hyperpolarization (**b**) and over the last 3 min of ATP administration (**c**). **d** The effect of ATP on Ca^{2+} oscillations is reduced after inhibition of SK4 channels in BK-KO β-cells. Representative recording showing regularly oscillations of $[Ca^{2+}]_c$ in β-cells of BK-KO mice treated with the SK4 channel blocker TRAM-34. ATP does not cause a significant delay of the start of the next oscillation after ATP administration and reduces its amplitude less pronounced compared to untreated WT β-cells. **e** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after addition of ATP. The numbers in the columns indicate the number of experiments with different cell clusters from three different mice. $^{**}P \le 0.01$, $^{***}P \le 0.001$

secretory granules or nerve endings can activate these receptors. Effects on β-cells are mediated by P2X and P2Y receptors but not by P1 receptors (for review see [33]). The physiological significance of activation of purinergic receptors is still debated; stimulation and inhibition of insulin secretion have been reported. The situation is complex because each receptor has many subtypes and subtype expression varies between species and between primary β-cells and insulin-secreting tumor cells.

We have chosen a protocol for our experiments where we stimulated β-cells and islets by glucose and raised Ca^{2+} influx by increasing the electrochemical gradient for Ca^{2+}. This protocol enhances the Ca^{2+}-mediated negative feedback on β-cell function [26] which is most prominent during burst phases with Ca^{2+} action potentials. Under these conditions, extracellular ATP inhibited SSC of β-cells as evidenced by hyperpolarization of V_m, reduced Ca^{2+} influx and AUC of $[Ca^{2+}]_c$ and decreased insulin secretion. This effect was

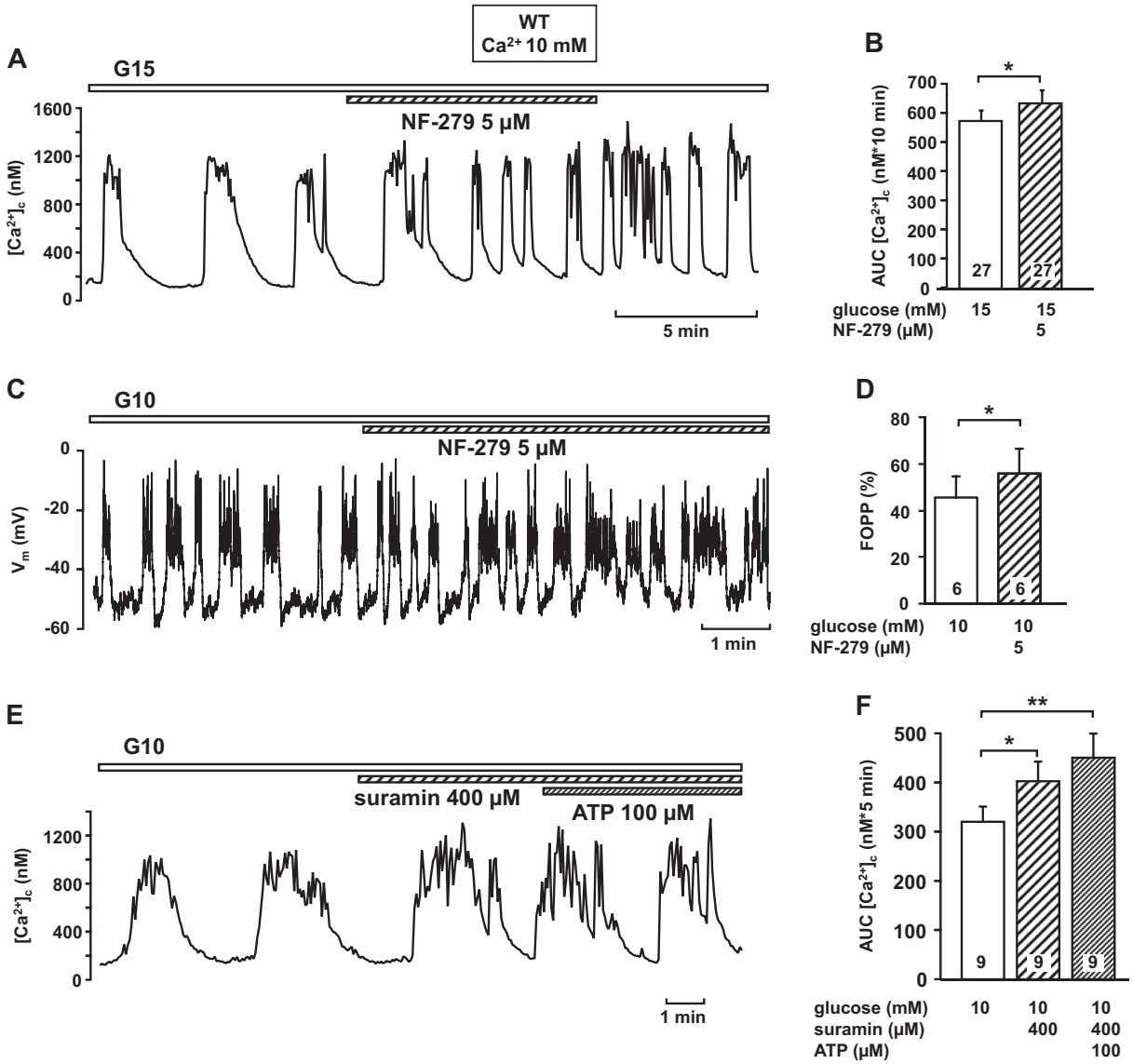

Fig. 8 Blockage of $P2X_1$ channels augments the fraction of plateau phase and the AUC of $[Ca^{2+}]_c$. **a** Representative recording showing regular oscillations of $[Ca^{2+}]_c$. NF-279 (5 µM) increases $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after application of NF-279. **c** Representative recording showing oscillations of V_m with burst and interburst phases. Application of NF-279 (5 µM) augments the fraction of plateau phase (FOPP). **d** Summary of the quantitative analysis of the FOPP before and after application of NF-279. **e** Representative recording demonstrating the effect of suramin (400 µM) on the AUC of $[Ca^{2+}]_c$. **f** Quantitative analysis of the data of this series. The numbers in the columns indicate the number of experiments with different cell clusters from three different mice. $^*P \le 0.05$, $^{**}P \le 0.01$

specific for ATP and not mimicked by AMP, ADP, or adenosine suggesting interference of ATP with specific receptors. Our results point to the involvement of $P2X_1$ receptors under these conditions: (1) The $P2X_{1,3}$ agonist αβ-MeATP mimicked the effect of extracellular ATP on insulin secretion. (2) The $P2X_1$ receptor antagonist NF-279 suppressed the effect of ATP on insulin release but not the $P2X_3$ receptor antagonist RO-3. (3) The $P2Y_1$ antagonist MRS-2179 did not influence the effect of ATP on insulin secretion.

The $P2X_1$ receptor is a non-specific cation channel with a relatively high permeability to Ca^{2+} [34]. $P2X_1$ receptor activation by extracellular ATP leads to Ca^{2+} influx and the local enhancement of Ca^{2+} in the sub-membrane space obviously influences the activity of other ion channels: (1) Extracellular ATP reduced the charge movement through L-type Ca^{2+} channels. This may be explained by a reduction of the driving force for Ca^{2+} due to the local increase of the intracellular Ca^{2+} concentration after $P2X_1$ receptor activation. Since Ca^{2+} influx through L-type Ca^{2+} channels constitutes the major trigger signal for insulin secretion [35], reduced influx would diminish secretion. (2) The effectiveness of ATP to inhibit SSC was reduced after

genetic and pharmacological deletion of two Ca^{2+}-activated K^+ channels present in β-cells, the BK and SK4 channels. An increase in the current through these channels after $P2X_1$ receptor activation would hyperpolarize the membrane and in turn lower insulin secretion contributing to the negative feedback induced by ATP.

Since the early paper of Gylfe and Hellman [36], who demonstrated that extracellular ATP induces Ca^{2+} release from ER Ca^{2+} stores, a variety of studies has shown similar results. These observations are confirmed by our experiments. Notably, store depletion does not affect the effectiveness of ATP to inhibit insulin secretion. This indicates that the sub-membrane increase in Ca^{2+} is decisive for the effect of ATP.

Physiological significance of extracellular ATP for β-cell oscillations

Oscillatory activity of β-cells is a prerequisite for normal action of insulin and glycemic control. Disturbances in the fluctuations of the parameters of SSC profoundly impair insulin signaling in peripheral tissues and are early events in diabetes [37]. We have extensively studied the mechanisms underlying these oscillations earlier [27, 31]. In the present paper we suggest a negative feedback for extracellular ATP in synergism with Ca^{2+}, i.e., a role for ATP in the termination of burst phases with action potentials. Partial inhibition of this feedback by blockade of $P2X_1$ channels indeed prolonged burst phases and thus increased electrical activity and the AUC of $[Ca^{2+}]_c$.

An extracellular ATP concentration of 100 µM, as used in the present work, seems not to be extraordinarily high considering that the ATP concentration within exocytotic granules of β-cells is around 3.5 mM [2]. In 1998 Hazama and co-workers [38] demonstrated that the ATP concentration at the cell surface is above 25 µM after stimulation of rat β-cells with glucose. They concluded that ATP can reach concentrations high enough to stimulate purinergic receptors during insulin secretion. Therefore, we used 100 µM ATP to realize an effect of ATP additional to that evoked by endogenous ATP from the granules. In experiments with whole islets, even higher concentrations of ATP (500 µM) were needed, which is most likely due to the capsule of connective tissue surrounding the islets which can form a barrier for molecules like ATP (see also Extracellular ATP reduces insulin secretion). Interestingly, Weitz et al. [6] recently demonstrated that tissue-resident macrophages of islets sense the interstitial ATP concentration. It is concluded that macrophages use ATP as a signal to estimate the activity state of the β-cells via purinergic receptors in order to balance their secretion products which influence β-cell proliferation. To achieve maximum Ca^{2+} signals in macrophages concentrations of ATP up to 1 mM were used.

In most experiments we have used a high extracellular Ca^{2+} concentration of 10 mM to augment the negative feedback mediated by Ca^{2+} which is most important during the burst phases. This feedback is thought to contribute to the initiation of burst termination. Exocytotic vesicles contain 120 mM Ca^{2+} which is mostly bound. Anyhow, the free granular Ca^{2+} concentration reaches 10 mM [2] suggesting that the local Ca^{2+} concentration on the surface of the cells is higher than the bulk Ca^{2+} concentration of the extracellular space. Enhanced local Ca^{2+} concentration steepens the gradient driving Ca^{2+} entry.

Ion flux through P2X receptors is dependent on the extracellular Ca^{2+} concentration, i.e., Ca^{2+} influx through P2X increases concomitantly with rising extracellular Ca^{2+} concentration [39] initiating an inhibitory signaling pathway (see above).

It has been demonstrated that a rise in the mitochondrial Ca^{2+} concentration during a burst phase increases K_{ATP} current and hyperpolarizes V_m to initiate the interburst [40]. Based on our current results, it can be concluded that extracellular ATP and fluctuations of the intracellular Ca^{2+} concentration act synergistically to exert a negative feedback that terminates the burst phases and perpetuate oscillatory activity.

Proposed model

The negative effects of extracellular ATP on SSC described in the present paper are in accordance with numerous reports in the literature, e.g. [8, 9, 19, 41]. This contrasts with findings about stimulating effects of ATP on β-cells in mouse and other species [11, 15, 16, 42–44]. To reconcile these observations it is hypothesized that short ATP pulses stimulate β-cell SSC [42], while longer exposure and higher concentrations induce a negative feedback [19–21, 30]. The authors suggest that this dual effect of extracellular ATP supports the coordination of Ca^{2+} oscillations. Furthermore, it is assumed that short pulses of ATP evoked by kiss-and run exocytosis [45] (where only small molecules like ATP but no insulin is released) or by exocytosis at the beginning of a burst phase activate P2Y receptors. Activation of these receptors increases $[Ca^{2+}]_c$ via Ca^{2+} release from the ER, an effect also seen in our experiments. However, this is not sufficient to potentiate the first phase of insulin release.

Our observation that the effect of extracellular ATP depends on the concentration of extracellular Ca^{2+} underlines the complexity of the action of ATP. At a low extracellular Ca^{2+} concentration ATP hardly affects β-cell SSC. Obviously, an increased extracellular Ca^{2+} concentration is necessary to unveil the ATP-induced negative feedback, i.e. extracellular ATP amplifies the negative feedback on β-cell function induced by high Ca^{2+}, thus contributing to the termination of bursts and the maintenance of β-cell oscillations.

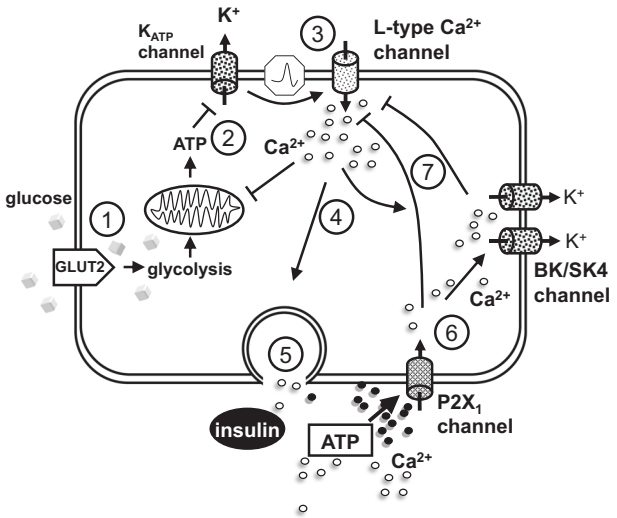

Fig. 9 Proposed model. For details see text

According to our model (Fig. 9) the effect of ATP essentially depends on V_m and activity of Ca^{2+}-dependent ion channels during burst phases. During burst phases quickly enhanced extracellular ATP and Ca^{2+} concentrations and thus marked ATP-mediated Ca^{2+} influx through $P2X_1$ receptors prevail, exerting a negative feedback limiting the burst phase. This involves opening of SK4 and BK channels. The novel proposed feedback mechanism of ATP may offer new approaches to influence the regulation of β-cell activity.

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Author's contribution C.B., J.K., and J.S. researched data; P.K.-D. evaluated data and edited the manuscript; M.D. contributed to discussion and study design and edited the manuscript; G.D. designed the study, wrote and edited the manuscript, and contributed to discussion. G.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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