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Imaging of extracellular and intracellular ATP in pancreatic beta cells reveals correlation between glucose metabolism and purinergic signalling

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ABSTRACT

Adenosine triphosphate (ATP) is a universal energy molecule and yet cells release it and extracellular ATP is an important signalling molecule between cells. Monitoring of ATP levels outside of cells is important for our understanding of physiological and pathophysiological processes in cells/tissues. Here, we focus on pancreatic beta cells (INS-1E) and test the hypothesis that there is an association between intra- and extracellular ATP levels which depends on glucose provision. We imaged real-time changes in extracellular ATP in pancreatic beta cells using two sensors tethered to extracellular aspects of the plasma membrane (eATeam3.10, iATPSnFR1.0). Increase in glucose induced fast micromolar ATP release to the cell surface, depending on glucose concentrations. Chronic pre-treatment with glucose increased the basal ATP signal. In addition, we co-expressed intracellular ATP sensors (ATeam1.30, PercevalHR) in the same cultures and showed that glucose induced fast increases in extracellular and intracellular ATP. Glucose and extracellular ATP stimulated glucose transport monitored by the glucose sensor (FLII12Pglu-700uDelta6). In conclusion, we propose that in beta cells there is a dynamic relation between intra- and extracellular ATP that depends on glucose transport and metabolism and these processes may be tuned by purinergic signalling. Future development of ATP sensors for imaging may aid development of novel approaches to target extracellular ATP in, for example, type 2 diabetes mellitus therapy.

1. Introduction

ATP is the molecule of life – inside of cells it is an energy supply, building block, regulator of K⁺ and Cl⁻ channels (e.g., KATP and CFTR) and precursor for intracellular signalling. Outside of cells it is a ubiquitous signalling molecule, as it activates purinergic receptors, and following hydrolysis to adenosine, adenosine activates adenosine receptors. Balance between ATP and adenosine is important in tissue microenvironment as, for example, ATP is pro-inflammatory, while adenosine is promoting immune evasion [1–3]. ATP is released from cells both under physiological and pathophysiological conditions. Several release mechanisms have been proposed, including plasma membrane channels/transporters, such as pannexin-1, and vesicular transporters, like VNUT [4–6]. However, one of the challenging problems is to quantify ATP changes at the outer surface of plasma membranes, before it is degraded by nucleotidases. Monitoring and quantification of extracellular ATP is important for a number of reasons: (i) to predict which P2 receptors may be stimulated; (ii) to estimate whether the ATP release is physiological or indicating cells in stress or dying; and (iii) to estimate whether there is a correlation between cellular ATP production and ATP release. The latter question is especially relevant for cells with high metabolism such as pancreatic beta cells, adipocytes, neurons, cancer cells, and muscle cells.

The most broadly used method to detect ATP is the bioluminescence reaction using luciferin and soluble firefly luciferase and, as recently developed, luciferase targeted to the plasma membrane of specific cells [3,7,8]. The bioluminescence is very sensitive as it can detect pM-nM ATP but saturates at μM-mM ATP concentrations. Due to the fact that

Abbreviations: ATP, adenosine triphosphate; eATP, extracellular ATP; iATP, intracellular ATP; AUC, area under the curve; CFP, cyan fluorescence protein; CFTR, cystic fibrosis transmembrane regulator; CLSM, confocal laser scanning microscopy; 2-DOG, 2-deoxy-glucose; FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; PRET, Förster Resonance Energy Transfer; dF, change in fluorescence; F0, fluorescence at time zero; GFP, green fluorescence protein; VNUT, vesicular nucleotide transporter; YFP, yellow fluorescence protein.

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the luminescence reaction emits light within 1 cm radius, the spatial resolution is limited and one cannot resolve which cells release ATP, unless a special setup with single photon counting is employed [9], or one utilizes fluorescence of the substrate luciferin [10]. To provide better spatiotemporal resolution, there has been a focus on the development of fluorescent ATP sensors that would be suitable for in vitro and in vivo experiments. Several studies reported development of genetically encoded fluorescent ATP sensors that are targeted to the outer aspect of the plasma membrane and report extracellular ATP changes. These sensors are based on bacterial F$_{0}$-ATP synthase ε subunit linked to fluorescent proteins such as CFP/YFP FRET pair or modified GFP and they require transfection and expression in host cells [11,12]. Very recently, another approach was to hybridize F$_{0}$-ATP synthase ε subunit with small fluorophore Cy3 (ATPOS), and by using Alexa488 biotin-streptavidin link and anchoring of the ratiometric ATPOS complex to the surface of neurons, it was possible to visualize ATP waves in mouse brains following electrical stimulation [13,14].

Our research focus is on pancreatic beta cells which release ATP in response to glucose via vesicular ATP release and panexin-1 [19–21]. ATP/purinergic signalling can then via P2X and P2Y receptors regulate a number of processes, including insulin release, cell survival, cytokine release, macrophage activation that can have physiological role or be involved in pathophysiological processes relevant to diabetes [22,23].

Some of the important receptors sensitive to low ATP concentrations are the P2Y1 and P2X3 receptors and those to high ATP concentrations are the P2X7 receptors [21,24–28]. Since the 1990s, it has been known that P2 receptors can upregulate insulin secretion [23] and a recent study revives interest in the P2Y1 receptor as a promising diabetes target to reverse beta cell failure to secrete insulin [28]. On the other hand, P2X7 receptors could be involved in islet autoimmune response and inflammation [29–32]. Obesity and tissue inflammation are predisposing factors for development of type 2 diabetes [33], and we proposed that high nutrient uptake/or high cell metabolism with increased ATP production could lead to increased levels of extracellular ATP [29,34]. Therefore, it is important to estimate ATP concentrations at the plasma membrane of beta cells, determine whether there is any correlation with glucose levels and intracellular ATP concentrations, and in future determine whether these factors could be indicative of pathophysiological conditions of beta cells or whole pancreatic islets.

Our aim was to estimate and quantify ATP on the outer aspect of plasma membrane (i.e., peri-cellular) of pancreatic beta cells in response to acute and chronic glucose loads and, if possible, to find whether ATP release is related to intracellular ATP levels. For this purpose, we cloned the “extracellular” ATP sensor which was based on intracellular ATeam3.10 [35] and similar to that reported [11], and we also used iATPSnFR1.0 sensor [12]. In addition, we deployed the intracellular ATP sensors ATeam [35] and PercevalHR [36]. The sensors monitoring ATP on the plasma membrane we denote “extracellular” ATP sensors or eATP sensors henceforth in this paper. For these studies we used INS-1E cell line, which is a clonal cell rat insulinoma cell line derived from INS-1 cells, which retains glucose-induced cellular responses and insulin release over many passages [37]. INS-1 cells are well-established model for mechanistic studies and share many characteristics with pancreatic beta cells, including release of ATP [21,38,39].

Our results show that beta cells release ATP in response to increases in glucose and we estimate that the concentrations are in the range 20–100 μM at the plasma membrane. However, eATP sensors become less responsive in cells exposed to high glucose conditions – indicating high “basal” ATP release. Furthermore, we show that glucose-induced changes in intra- and extracellular ATP concentrations follow each other in the time domain, which indicates coupling or association between ATP production and ATP release.
3. Results

3.1. Extracellular ATP sensors detect exogenous ATP on INS-1E cells

We generated in our lab FRET-based extracellular ATP sensor based on the ATeam intracellular sensor ATeam3.10 (Imamura H., et al., 2009) and named it eATeam3.10 (see Methods). EATeam3.10 sensor was expressed in INS-1E cells and microscopic examination in both widefield fluorescence (and CLSM) microscopes showed sensor localization to the plasma membranes (Fig. 1a). First, using widefield fluorescence imaging we performed experiments testing whether the eATeam3.10 sensor was capable of detecting exogenous ATP concentrations in cells exposed to control buffer solution containing 5.5 mM glucose. Prior to this, INS-1E cells were pre-conditioned in in media containing 5.5 mM glucose for one day. Exogenous ATP was added sequentially in steps to the standing bath until the final ATP concentration of 4.6 mM ATP. The sensor responded to increased ATP concentrations with increasing FRET signals (Fig. 1b–c, f). For time-course experiments in Fig. 1, we show non-normalized signals. Fig. 1b, c shows that at low ATP concentrations, the FRET response was transient, indicating that some ATP could have been degraded by ecto-nucleotidases, or that the sensor bound ATP transiently. Indeed, addition of apyrase, an ATP hydrolase, decreased the FRET response to ATP (Fig. 1d). In addition, we performed experiments with a double-mutant eATeam1.03R122K/R126K that does not bind ATP, and as predicted, INS-1E cells expressing this mutant did not respond to ATP nor glucose (Fig. 1e). INS-1E cells expressing eATeam3.10 sensor responded to exogenous ATP and the dose-response curve showing the peak FRET responses/ratios for various ATP concentrations are depicted in Fig. 1f. Relatively high EC50 values indicate relatively low sensitivity of the sensor, issue addressed in section 3.3 below.

In order to validate extracellular ATP sensing in INS-1E cells, we also employed another eATP sensor, i.e., the GFP-based sensor iATPSnFR1.0 described by Lobas and co-workers [12]. The iATPSnFR1.0 sensor was localized on the cell membrane as detected by widefield (and CLSM see below) fluorescence (Fig. 2a). We recorded time responses of INS-1E cells to a stepwise increase in ATP concentrations from 1 μM upwards until the final ATP concentration of about 40 mM (Fig. 2b, c). Since iATPSnFR1.0 is a single wavelength sensor and there was slight bleaching, the fluorescence was normalized to F0 (120 s) values and depicted as dF/F0. The GFP-based iATPSnFR1.0 sensor displayed a dose-dependent increase in GFP fluorescence (Fig. 2d). The iATPSnFR1.0 sensor has a high EC50 compared to the FRET sensor, but it had a strong fluorescence, and it could be easily excited in the confocal microscope (cf. Figs. 1 and 2), and thus was useful in further experiments reported below.

3.2. High glucose stimulates ATP release in INS-1E cells

We have previously reported that INS-1E cells exposed to 16.7 or 25 mM glucose released ATP, as detected by on-line luciferin/luciferase luminescence assay [21]. Since the concentration of ATP depends on the number of cells and volume of the supernatant (and ecto-nucleotidase activity), actual ATP concentrations at the cell surface are unknown. Therefore, our aim here was to quantify the ATP release by live-cell imaging with FRET-based eATeam3.10 or GFP-based iATPSnFR1.0 sensors. INS-1E cells transfected with either sensor were imaged by epifluorescence or confocal fluorescence microscopy. Cells were equilibrated in a nominally glucose-free imaging buffer for 20–30 min and sequentially exposed to the control buffer (negative control) that did not affect fluorophore intensity, followed by addition of glucose so that the final concentration in the bath was 5.5 mM and 25 mM, and lastly 100 μM ATP was added as positive control. Fig. 3a shows averaged traces for the FRET ratio of the eATeam3.10 expressing cells. Recording in 40 cells from 14 different experiments shows clear response to 25 mM glucose and ATP. Fig. 3b depicts average FRET ratios from these and additional control.

2.3. Imaging

The first live imaging experiments were carried out in Nikon Eclipse Ti microscope with 40× or Oil NA1.3 objective. Cells expressing the extracellular sensors eATeam3.10 and eATeam1.03R122K/R126K were illuminated at λex = 430 and 500 nm and emission (5 s/frame) was collected at λem = 470 and 530 nm (DV Photometrics) for the CFP and CFP-YFP FRET channels, and YFP respectively, by an EMCCD camera (Andor iXon3 897) and digitized by IFI image processing system (ThermoFisher Scientific). LA Live Acquisition software was used to both control the monochromator and the CCD camera. After confirming that the sensor was expressed in plasma cell membranes) image analysis was performed on RIOs on whole cells. For analysis we used FRET/CFP ratio. Cells where ratios were higher than 8 or where YFP signal alone changed were discarded from analysis. Cells expressing the iATPSnFR1.0 sensor were illuminated at λex = 470 nm and emission was collected at λem = 530 nm at 0.5 s per frame. The green fluorescence intensity change was depicted as dF/F0, where F0 was the resting fluorescence signal over 120 s.

Confocal microscopy was performed on an inverted Leica SP5-X MP (Leica Microsystems) with HCX PL APO lambda blue 63.0× 1.20 water objective or 40× oil objective. The ATeam sensors (eATeam3.10 and eATeam1.03) were excited with a 405 diode laser, and emission was collected for CFP (450–490 nm in PMT1) and FRET (530–580 nm on PMT3 or HyD detector). Recordings were taken every 5–15 s/frame and 4 lines were averaged. Green sensors iATPSnFR1.0 were excited with 490 nm WLL, and emission collected with PMT3 at 500–530 nm. PercevalHR was excited with 405 and 490 nm lasers and image was collected at 500–560 nm and emission ratio (490/405) was calculated. Glucose CFP/ YFP sensor was imaged with similar settings as the other FRET sensors. When two sensors were imaged, this was done sequentially line by line with an average of 4 lines. Images were analyzed using the LAS AF software. Fluorescent images were corrected for background, if relevant, ratio was calculated, and fluorescent signal was normalized for each cell.

2.4. ATP assay by luminescence

ATP concentrations in the media of cells grown with 5.5 and 25 mM glucose for 72 h was measured using luciferin/luciferase luminescence kit (Sigma ATP Bioluminescence Assay Kit HS II). ATP concentrations were normalized to 10⁴ cells/ml, after the cell number was determined by hemocytometry. Indeed, addition of apyrase, an ATP hydrolase, decreased the FRET response to ATP (Fig. 1d). In addition, we performed experiments with a double-mutant eATeam1.03R122K/R126K that does not bind ATP, and as predicted, INS-1E cells expressing this mutant did not respond to ATP nor glucose (Fig. 1e). INS-1E cells expressing eATeam3.10 sensor responded to exogenous ATP and the dose-response curve showing the peak FRET responses/ratios for various ATP concentrations are depicted in Fig. 1f. Relatively high EC50 values indicate relatively low sensitivity of the sensor, issue addressed in section 3.3 below.

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experiments, which had different time protocols but included all steps tested. Addition of 5.5 mM glucose had a very modest effect, perhaps cells were not sufficiently adapted to low glucose before the glucose step (see below), but there was a clear response to 25 mM glucose. Fig. 3 c depicts ΔFRET/CFP ratio, which shows that on the paired basis 5.5 mM glucose, 25 mM glucose and 100 μM ATP caused rise in ΔFRET/CFP ratio by 0.09, 0.20 and 0.55, respectively.

In parallel experiments, we tested the same sensor in CLSM, where 405 nm laser was used to excite the eATeam3.10 sensor. Timeline of experiments and summarized responses to glucose steps and added ATP are shown in Suppl. Fig. 1 a-c. Although CFP excitation with 405 nm laser was not optimal, responses to glucose and ATP were qualitatively similar to above, and on paired basis ΔFRET/CFP ratio increased by 0.02, 0.05 and 0.18 with 5.5 mM and 25 mM glucose and 100 μM ATP, respectively.

We performed similar experiments with the iATPSnFR1.0 sensor and detected GFP epifluorescence. Fig. 3 d shows the timeline and response in cells corresponding dF/F0 and ΔdF/F0 analysis in Fig. 3 e-f. Fluorescence of the sensor (ΔdF/F0) analysis in Fig. 3 e-f.

Fig. 1. (a) Representative images of INS-1E cells expressing the eATeam3.10 sensor. Images were taken in a CLSM (upper images) and widefield fluorescence (lower images) microscopes and show CFP/YFP (FRET) and CFP images in cells with and without ATP (pseudocolor scale). The scale bar is 20 μm. (b-c) Original recordings of FRET/CFP ratios and means ± SEM in cells expressing eATeam3.10 sensor in response to sequential addition of exogenous ATP (indicated by +) until the final accumulated concentration of 4.6 mM ATP (n = 34, N = 4). CTL denotes addition of a control buffer. (d) Effect of apyrase on FRET/CFP ratio given and mean ± SEM (n = 15, N = 2). (e) FRET/CFP ratio responses in cells expressing the ATP-insensitive sensor eATeam1.03R122K/R126K (n = 44, N = 3) (f) Dose-response curve fitted with log(agonist) vs. response curve with variable slope (four parameters)(n = 32, N = 4).
Taken together, an increase of glucose from 5.5 to 25 mM resulted in an increase in the FRET/CFP ratio (eATeam3.10) and the GFP signal (iATPSnFR1.0 sensor) that were about one third of the response elicited by 100 μM ATP. Therefore, a very simple estimate based on within experiment calibrations indicate that acute glucose exposure (5.5 to 25 mM) stimulated INS-1E cells to release approximately 20–60 μM ATP to the outer plasma membrane (see also below).

3.3. Dose-response of ATP sensors depends on pre-conditioning of cells to different glucose levels

Next, we considered whether the “basal” signal of the eATP sensors could reflect glucose/metabolic status of cells, and therefore affect (calibration of) the sensor with added/exogenous ATP. Hence, we examined whether pre-conditioning of INS-1E cells to acute exposures to glucose affects dose-response to ATP. INS-1E cells transfected with either eATeam3.10 or iATPSnFR1.0 and tested for ATP responses in imaging buffer containing either 5.5, 11 (or 10) or 25 mM glucose (Fig. 4). As Fig. 4a shows, the FRET/CFP ratio increased in response to increasing ATP doses in cells imaged in buffers containing 5.5, 11 and 25 mM glucose. Interestingly, there was a difference in the ratios detected in the basal/low ATP conditions, depending on the glucose concentrations. Equilibration of INS-1E cells in 11 mM glucose level raised the “basal” FRET/CFP ratio and responses to increasing ATP concentrations. This effect was even more pronounced in 11 mM glucose-long experiments, where cells were first tested for acute glucose responses (as in Fig. 3), then rinsed and then tested for ATP responses. This protocol might have conditioned or stressed cells and the released ATP already occupied the sensor. However, perhaps unexpectedly, cells equilibrated in 25 mM glucose had lower basal FRET/CFP ratio and somewhat lower responses to ATP standards. We determined that the half-maximal ATP responses (EC50) of the sensor from these raw not normalized values, and these were 297, 54 and 300 μM ATP for cells that were imaged in 5.5, 11 and 25 mM glucose buffers, respectively. EC50 values and the corresponding Hill coefficients are given in Suppl. Table 1. Notably, a change in the ratio of about 0.2 (as seen with a 5 to 25 mM glucose step) would correspond to increase of ATP to about 100 μM. When the FRET/CFP ratios were normalized, as is often done in other studies [11,35], the basal state of the ATP sensing (in our case due to different glucose conditions) are not obvious. However, some differences in ATP response curves became more obvious, e.g., low maximal response of the sensor in 11 mM glucose-long and high EC50 in 25 mM glucose experiments (Fig. 4b).

Furthermore, we explored whether pre-conditioning of INS-1E cells with chronic exposure to 25 mM glucose affected kinetics of the sensors. INS-1E cells expressing either eATeam3.10 or iATPSnFR1 were pre-conditioned in 25 mM glucose for three days and then imaged in 25 mM glucose solution. Fig. 4a shows that the basal FRET ratio was very high, indicating ATP binding to the sensor, and in the normalized FRET ratio, the response to exogenous ATP appeared flat and EC50 estimate was very high and Hills coefficient decreased, indicating a decreased affinity of added ATP binding (Suppl. Table 1). Similarly, in cells expressing the iATPSnFR1.0 sensor, chronic 25 mM glucose adaptation affected the form of the ATP dose-response, which was difficult to fit, but it is clear that estimated EC50 was right-shifted. Taken together, the above experiments show that high glucose conditions lead to release of ATP that

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**Fig. 2.** (a) Widefield fluorescence images of iATPSnFR1.0 sensor expressing cells. The scale bar is 20 μm. (b-c) Original recordings and means ± SEM of df/F0 fluorescence in cells expressing iATPSnFR1.0 sensor in response to sequential addition of exogenous ATP (indicated by +) to solutions containing 10 mM glucose, until the final accumulated concentration was 39.6 mM (n = 118, N = 8). (d) Dose-response curve fitted with asymmetric sigmoidal 5P curve.
binds to the two types of sensors and desensitizes the sensors and/or cells to addition of exogenous ATP (i.e., flattens and right shifts the dose-responses) and thus makes the calibration problematic. In order to test whether INS-1E cells chronically adapted to high glucose do release more ATP to the extracellular space, we measured ATP concentrations in the collected culture media using luciferin/luciferase assay. Indeed, glucose-adapted cells release more ATP as shown in Fig. 4d. This correlates well with the high basal ATP levels detected by the FRET sensor signal in high-glucose adapted cells (Fig. 4a).

3.4. Correlation between intracellular and extracellular ATP

One important question in our study was whether there is a correlation between glucose metabolism and ATP release, reflected by intracellular and extracellular ATP concentrations, and whether this could be resolved in a time domain. Since beta cells respond to glucose loads very fast (e.g., electrical activity and calcium changes), they should have fast glucose utilization and intracellular ATP production. Therefore, our aim in the following experiments was to reveal whether there is a correlation between ATP production and ATP release.
Since it is not easy to resolve both iATP and eATP in the same cells, as the spectra of our sensors are overlapping, we adopted the following approach. After splitting, half of the INS-1E cells were transfected with intracellular ATeam1.30 sensor and the other half with extracellular iATPSnFR1.0 sensor and then they were co-cultured and imaged in CLSM. Fig. 5a shows images of cells reporting iATP and eATP. Increase in glucose from 5 to 25 mM caused both increase in iATP and eATP in INS-1E cells (Fig. 5b). The insert with expanded time-axis (Fig. 5c) shows that both intracellular ATP and extracellular ATP increased with overall similar kinetics in neighbouring cells, though eATP responses seem faster compared to iATP responses (estimated \( \tau \) of 20 and 32 s, respectively). Subsequent addition of exogenous ATP increased eATP but not iATP. Metabolic inhibitors (i.e., FCCP and sodium azide) reduced iATP, as expected, but eATP increased, most likely because released ATP accumulated extracellularly (i.e., outer aspects of the plasma membranes) and “poisoned” cells were not able to hydrolyse this ATP.

To verify the above data, we performed similar experiments, but this time we adapted the cells to nominal 0 mM glucose (30–60 min) and used another iATP sensor – PercevalHR and images and data are shown in Fig. 6. Again, there was an increase in iATP and eATP with glucose steps (Fig. 6b), eATP but not iATP sensor responded to high extracellular ATP (not shown in this figure), and metabolic inhibitors decreased iATP while eATP increased. Zooming on the time response to the glucose step (Fig. 6c), it appears that the 5 mM glucose response of the eATP sensor was slightly faster than the response of Perceval (estimated \( \tau \) of 7 and 193 s), while responses to 25 mM glucose responses were similar.

In the last series of experiments, we tried to resolve whether glucose uptake is stimulated by eATP. Here we used the intracellular glucose sensor denoted iGlucose, FLII12Pglu-700uDelta6 [40] and data are shown in Fig. 7. Glucose uptake was induced with 5 mM glucose step and further uptake with extracellular ATP (Fig. 7a left panel). Cells that were shortly pre-treated with 2-deoxy-glucose (2-DOG) had reduced glucose uptake and ATP effects (Fig. 7b right panel). Summary of differences between control and inhibitor responses to glucose and ATP.
given as AUC, are depicted in Fig. 7b. 2-DOG is transported by the glucose transporter and it inhibits glycolysis and decreases iATP [18, 41]. Our data indicates that production of iATP is required for glucose transport and that addition of exogenous ATP, most likely acting via P2 receptors, increased glucose influx.

4. Discussion

In this study we have used two different eATP sensors to monitor live-cell release of ATP from pancreatic beta cells in response to glucose. We combined these eATP sensors with sensors monitoring intracellular ATP and glucose and based on our results we propose that there is fast and coordinated coupling between cell glucose metabolism, ATP production and ATP release.

One of the advantages of fluorescent ATP sensors is that one can visualize individual cells and their status, i.e., healthy vs dying cells. The sensor that we have generated, eTEAM3.10 is similar to the one published earlier and named ecAT3.10 and expressed in Neuro2A cells [11]. In INS-1E cells imaged in our epifluorescence experimental set up, this sensor had relatively higher EC_{50} of about 300 μM (Figs. 1 and 4 and Suppl. Table 1), compared to 12 μM reported in the Conley study [11], but our sensor responded fast to exogenous glucose in experiments conducted at 37 °C. The second eATP sensor was a single wavelength GFP based sensor iATPSnFR1.0 developed by Lobas and colleagues [12]. In their studies EC_{50} was between 350 and 630 μM depending on cells expressing it, e.g., HEK293, astrocytes or neurons [12]. In INS1-E cells, EC_{50} was relatively high, about 2000 μM as estimated from epifluorescence recordings (Figs. 1 and 4, Suppl. Table 1). However, the sensor
was useful for laser excitation of our confocal microscope (Figs. 5 and 6). In general, eATP sensors respond fast to a step-pulse of exogenous ATP, but under physiological conditions the response depends on host cells and the stimulus eliciting ATP release [11–13]. Bioluminescence sensors (see Introduction) or intracellular sensors linked to plasma membrane receptors, like P2XR-GCaMP sensors [42], report more sensitive dose-response curves and presumed lower eATP values. Therefore, more research with a variety of eATP sensors and cell types will be needed to resolve the question of how much eATP is on cell surface in physiological and pathophysiological conditions.

The main point of our study was to test whether a physiological stimulus, i.e., glucose, induced ATP release in beta cells. Clear increases in both eATP and iATP were observed with a glucose step from 5 mM to 25 mM (Fig. 3, Suppl. Fig. 1). Using ATP calibrations from the same experiments, we could approximate that increase in glucose causes release of about 20–60 μM ATP at the plasma membrane. Somewhat higher values, about 100 μM ATP, could be derived from dose-response curves (see below for discussion of limitations). Similar high concentrations of ATP (>25 μM) were detected on beta cells stimulated with glucose and detected with electrophysiological response of sensor cells [43]. Also pancreatic acinar cells release up to 20 μM ATP on cell surface when stimulated with acetylcholine and detected by luciferin fluorescence [10]. Pancreatic beta cells, like pancreatic acinar cells, contain ATP in their granules accumulated by VNUT and ATP release by exocytosis would be one mechanism [44–46]. However, pancreatic beta cells also express pannexin-1 [21] and this channel could mediate fast ATP release (see below). Relatively high eATP concentrations around pancreatic cells would imply that a wide spectrum of P2 receptors could be activated, even the low sensitivity P2X7 receptors, which at least in beta cells/INS-1E cells can potentiate insulin release, cytokine balance (IL-1β and IL-1Ra) and cell survival [21,27]. Whether it is the P2X7 receptor that contributes to ATP release or the receptor-pannexin-1 activation that causes ATP release is unclear [21]. Nevertheless, beta cells express both P2X7 receptors and pannexin-1,
inhibitors thereof reduced ATP release as monitored on a population of cells with luciferin/luciferase luminescence reaction [21].

Several studies report fluorescence signals of ATP sensors normalized to the “basal level”, presumed to be non-stimulated state [11–13]. One important observation in our study is that the signal of the eATP sensors depends on the glucose concentration in the buffer or medium (Fig. 4), best illustrated by raw and non-normalized data. Therefore, calibration of sensors to absolute/exogenous ATP concentrations may be problematic (also for other cell types that are basally stimulated with whatever stimulus), and this may contribute to the fact that in our cells EC50 values are higher than those reported by Lobas and co-workers and Conley and co-workers for other cell types (see above) [11, 12]. The important physiological/pathophysiological message is that in beta cells glucose load, especially the chronic one, induces high ATP release as detected by our eATP sensors at the plasma membrane and also by luciferin/luciferase assay in the medium (Fig. 4). Nevertheless, released ATP (μM) concentrations are only a fraction of intracellular ATP (mM) and beta cells retain stable mitochondrial ATP levels [47].

The second observation that stems from these high-glucose experiments implicates that eATP can be influenced or related to iATP. Therefore, we tested this hypothesis by making dynamic measurements of iATP and eATP signals in parallel and using iATP sensor detecting capable of detecting mM ATP concentrations and eATP sensor detecting μM concentrations. First of all, these ATP sensors do keep to their side of the plasma membrane as they respond differentially to addition of extracellular ATP and inhibitors (Figs. 5 and 6). We cannot exclude that our acquisition rate and sensitivity is too slow, or the sensors alone have different kinetic responses, but some interesting observations may be worthwhile considering. With the intracellular ATP sensor ATeam1.03, we were able to detect small but significant increase in iATP with glucose steps, and similar with the ATP/ADP sensor, PercevalHR, which appeared to react a little slower (Figs. 5, 6). These data agree with the studies of Tanaka and colleagues [48] using similar ATeam sensors in MIN6 cells/islets and Merglen and colleagues [37] using luciferase in INS-1E cells and Perceval [49–51] on pancreatic beta cells.

Most importantly, we found that increase in eATP (reported by both eATTeam3.10 and iATPSnFR1.0) in response to glucose was fast, at least as fast or perhaps even preceding that of an increase in iATP (reported by Ateam1.03 and PercevalHR) (Figs. 5 and 6). This was rather surprising as we expected that metabolically produced ATP, i.e., iATP, would precede ATP release, i.e., eATP. Instead, it appears as if glucose-induced ATP release is very fast and an early step in the chain of events. Also, glucose influx reported by iGluc sensor (FLII12Pglu-700uDelta6) was fast and inhibited by a glycolysis blocker 2-DOG (Fig. 7), which reduced ATP release [21]. This data indicates that the release of ATP and/or production of iATP are important for stimulation of glucose transport, presumably through GLUT2. We think that this would be mediated by P2 receptors, as also addition of exogenous ATP increased glucose influx (Fig. 7). Interestingly, one study on white adipocytes reports that pannexin-1 was important for glucose uptake in these cells, though there it was mediated by insulin-sensitive GLUT4 transporter [52]. In contrast, our study on white adipocytes shows that insulin

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Fig. 7. Glucose uptake and effect of inhibitors. (a) Intracellular glucose (iGlucose) was monitored with the glucose sensor (FLII12Pglu-700uDelta6) and timeline of responses to increasing glucose concentration from 0 to 5 mM (0G, 5G) and ATP (100 μM) is shown. Cells were untreated (Control) or pre-treated with 2-deoxy glucose (2-DOG, 5 mM) 20–30 min or 3–5 min before imaging. Time course of responses is given as means ± SEM (n = 16–31, N = 6–8). (b) Analysis of glucose or ATP responses (means ± SD) given as area under the curve AUC (300–600 s, or 600–900 s), corrected for baseline of 1) and significance (unpaired t-test) is indicated.

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signalling down-regulates ATP release via pannexin-1 [34].

In studies on various preparations of beta cells, it has been documented that increase in iATP precedes voltage and calcium oscillations [37,49,53]. Our observation that ATP release is fast, most likely via a channel Pannexin-1 rather than VNUT, makes us suggest that ATP/purinergic signalling, via whatever receptors these might be, contributes to feed-forward signalling in beta cells, perhaps mediating the first steps, e.g., the calcium peak and regulating ion channel activity, metabolism and insulin release. Longer term exposures to ATP does not seem to be detrimental to INS-1E cells, these remain viable and proliferate with exogenous ATP concentrations up to 1 mM [21]. However, effect of ATP on cell viability depends on the origin of beta cells (species, cell lines) and complement of purinergic receptors expressed [21,22].

Apart from an effect of eATP on beta cells, high eATP concentrations may however affect more sensitive neighbouring cells, such as T-cells and macrophages, and thus lead to autoimmune response and low level of inflammation in pancreatic islets and beta cell dysfunction [29,32,54]. In study of white adipocytes, we showed that glucose load increases but insulin decreases pannexin-1 mediated ATP release, and that modulates macrophage migration and lipolysis [54]. Other ATP sensitive cells in pancreas are pancreatic stellate cells that secrete collagen I and IL-6 in response to ATP but high ATP concentrations affect cell survival [55,56]. In a broader perspective, it would be interesting to see whether correlation between increased nutrient load, iATP and eATP exists in other tissues that are involved in glucose body homeostasis and thus could be important factors in connecting inflammation, obesity, and type 2 diabetes mellitus.

In summary, our study shows that in the time domain glucose transport, iATP and eATP are occurring almost simultaneously, as detected in our imaging system in these INS-1E cells. These cells secrete insulin and are good models for imaging experiments with multiple sensors. Nevertheless, our results will have to be validated on native beta cells from mice or human islets. The physiological importance of this study is that there is a very close connection between iATP and eATP and glucose transport. We have some indications that release of ATP occurs in an early step and we suggest that glucose transport and ATP transport may be closely connected, and released ATP then via P2R stimulate iATP production and downstream events leading to insulin release. The study also shows that increased glucose load leads to higher iATP and eATP and thus may have implication for whole body metabolism as excess energy intake and increased metabolic load promotes ATP release that not only affects beta cells but may promote low-level inflammation, macrophage recruitment or activity and impaired beta cell function as hypothesized [29].

5. Conclusions

In this study, we used intra- and extracellular fluorescent sensors for ATP on a model of pancreatic beta cells. We show that in beta cells there is a fast and dynamic relation between intra- and extracellular ATP that depends on glucose transport and metabolism and these processes may be tuned by purinergic receptors and signalling. We propose that these fundamental processes may apply to other tissues and play a role in whole body metabolism. Future development of sensors for imaging extracellular ATP may aid development of novel approaches to target ATP release and relevant P2 receptors in, for example, type 2 diabetes mellitus therapy.

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CRediT authorship contribution statement

Seyed M. Ghiasi: Writing – original draft, Visualization, Formal analysis, Data curation. Yynne M. Christensen: Writing – review & editing, Methodology, Investigation, Data curation. Per A. Pedersen: Resources, Methodology. Emil Z. Skovhej: Investigation, Data curation.

Ivana Novak: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data supporting the finding of this study are available within the paper and its Supplementary Material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cels.2024.111109.


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