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ORIGINAL PAPER

Effects of external ATP on Ca^{2+} signalling in endothelial cells isolated from mouse islets

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Abstract External ATP is believed to initiate and propagate Ca^{2+} signals co-ordinating the insulin release pulses within and among the different islets in the pancreas. The possibility that islet endothelial cells participate in this process was evaluated by comparing the effects on $[Ca^{2+}]_i$ of purinoceptor activation in these cells with those in β-cells. β-Cell-rich pancreatic islets were isolated from *ob/ob* mice and dispersed into single cells/aggregates. After culture with or without endothelial cell growth supplement (ECGS), the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with ratiometric fura-2 technique. Presence of ECGS or prolongation of culture (>5 days) resulted in proliferation of endothelial cells and altered their phenotype from rounded to elongated. Endothelial cells, preliminarily identified by attachment of Dynabeads coated with the *Bandeiraea simplicifolia* 1 lectin (BS-1), responded in a similar way as those stained with CD31 antibodies after measurements of $[Ca^{2+}]_i$. Spontaneous transients and oscillations of $[Ca^{2+}]_i$ were seen in β-cells, but not in endothelial cells exposed to 20 mM glucose. Addition of ATP (10 μM) resulted in pronounced and more extended rise of $[Ca^{2+}]_i$ in endothelial cells than in β-cells. The endothelial cells differed from the β-cells by also responding with a rise of $[Ca^{2+}]_i$ to 10 μM UTP, but not to equimolar ADP and acetylcholine. The results support the idea of mutual interactions between islet endothelium and β-cells based on ATP-induced Ca^{2+} signals. It is suggested that the endothelial cells have a tonic inhibitory action on β-cell P2 purinoceptors resulting in impaired synchronization of the insulin release pulses.

Keywords Acetylcholine · ATP · Ca^{2+} signalling · Islet endothelium · Pancreatic β-cells · Purinoceptors

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Introduction

An increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) stimulates a number of cellular events, including the release of insulin from pancreatic β-cells. Each β-cell is a biological oscillator, responding to glucose stimulation with pulses of insulin release resulting from rhythmic depolarization with subsequent entry of Ca^{2+} [1]. Within the islets external ATP is complementary to gap junctions as a co-ordinator of the oscillatory activity of the β-cells [2–4]. The entrainment of the β-cells into a common rhythm is related to generation of short-lived rises of $[Ca^{2+}]_i$ superimposed on the oscillations [5]. These transients will temporarily interrupt the entry of Ca^{2+} by activating a repolarizing K^+ current [6]. Pancreatic β-cells both receive an ATP message inducing $[Ca^{2+}]_i$ transients and propagate this signal to neighbouring cells [7]. It has therefore been proposed that neural activity with intermittent discharge of ATP adjusts islets in different oscillatory phases into a common rhythm [8].

Accumulating data suggest multiple interactions between islet endothelial cells and β-cells besides those involved in the regulation of blood flow and transport over the capillary wall [9]. However, there is no information whether islet capillary endothelium affects $[Ca^{2+}]_i$ signalling for pulsatile insulin release. Addition of ATP is known to raise $[Ca^{2+}]_i$ in other types of microvascular endothelium [10–12]. Moreover, regenerative ATP release has been

reported to occur in cultures of umbilical vein [13] and cardiovascular endothelial cells [14].

The aim of the study was to explore whether islet endothelial cells respond to ATP and other purinoceptor agonists with an increase of $[Ca^{2+}]_i$ as reported for capillary endothelium from other sources. Testing the effects in co-cultures we have also compared the Ca^{2+} signals generated by the islet endothelial cells with those seen in adjacent insulin-producing β-cells.

Results

Presence of endothelial cells

Islet cell preparations kept in culture up to 5 days in media supplemented with 20% fetal calf serum contained only a few endothelial cells. Presence of ECGS resulted in proliferation of the endothelial cells and altered their phenotype from rounded to elongated (Figs. 1, 2). A similar but less pronounced proliferation was seen after long-term culture (>5 days) in media lacking ECGS. Phase contrast images of endothelial cells after 8 days culture in the presence or absence of 30 μM ECGS are shown in Fig. 2.

Ca^{2+} responses to ATP and other purinoceptor agonists

The effects of ATP and other purinoceptor agonists were tested in the presence of 5 or 20 mM glucose. Results of experiments performed with 5 mM glucose are shown in Figs. 3 and 4. The β-cells responded to 10 μM concentrations of ATP and ADP with an increase of $[Ca^{2+}]_i$, whereas

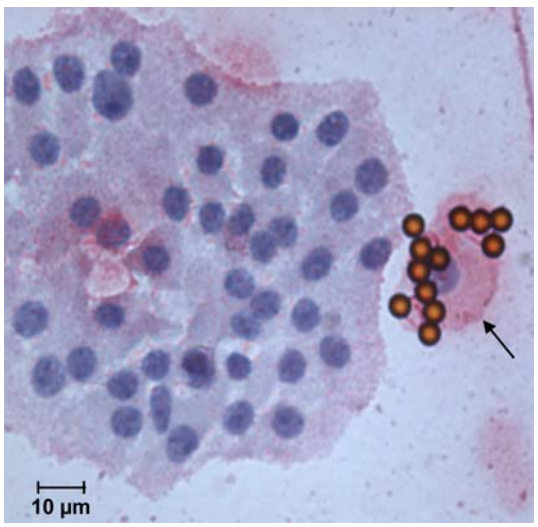

Fig. 1 Aggregate of β-cells (left) and a rounded endothelial cell labelled with Dynabeads coated with BS-1 (right) after 6 days culture in the presence of 30 μM ECGS. The Dynabeads were attached during the final 20 min of Fura-2 loading

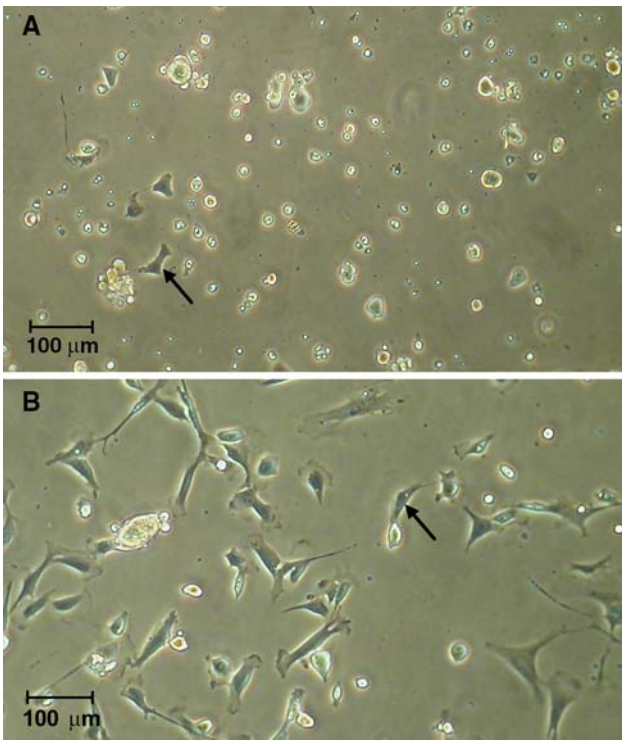

Fig. 2 Phase contrast images showing elongated endothelial cells (arrows) after 8 days culture in the absence (**A**) and presence (**B**) of 30 μM ECGS

UTP, UDP and adenosine lacked effects (Fig. 3). In the endothelial cells, pronounced elevation of $[Ca^{2+}]_i$ was seen during exposure to 10 μM ATP and UTP, but there was no effect of ADP, UDP or adenosine. The ATP-induced rises of $[Ca^{2+}]_i$ were higher and more extended in endothelial cells than in β-cells (amplitudes 302 ± 31 vs. 31 ± 10 nM; $P < 0.001$). The corresponding half-widths for the $[Ca^{2+}]_i$ rises were 4.1 ± 0.4 vs. 0.8 ± 0.2 min ($P < 0.001$).

During exposure to 10 μM UTP, addition of equimolar ATP induced rise of $[Ca^{2+}]_i$ in β-cells but not in endothelial cells (Fig. 4A). In Fig. 4B it is shown that the purinoceptor antagonist suramin (100 μM) prevents the $[Ca^{2+}]_i$ rise obtained with 10 μM UTP and ATP in the endothelial cells and with 10 μM ATP in the β-cells (cf Fig. 3).

Isolated β-cells are known to generate spontaneous $[Ca^{2+}]_i$ oscillations in the presence of 20 mM glucose. The islet endothelial cells lacked this type of $[Ca^{2+}]_i$ rhythmicity (Figs. 5, 6). Studying the Ca^{2+} signalling at 20 mM glucose, it was found that endothelial cells respond with rise of $[Ca^{2+}]_i$ to 10 μM UTP and ATP but not to 10 μM ADP (Figs. 5, 6). The effects of ATP and ADP on β-cells were often manifested as premature oscillations (Figs. 5, 6). Differences between the responses to the nucleotides were seen also when the β-cell oscillations of $[Ca^{2+}]_i$ were suppressed with the Ca^{2+} channel blocker methoxyverapamil (Fig. 7). In this case, pulse addition of 10 μM ADP induced short-lived increase of $[Ca^{2+}]_i$ in

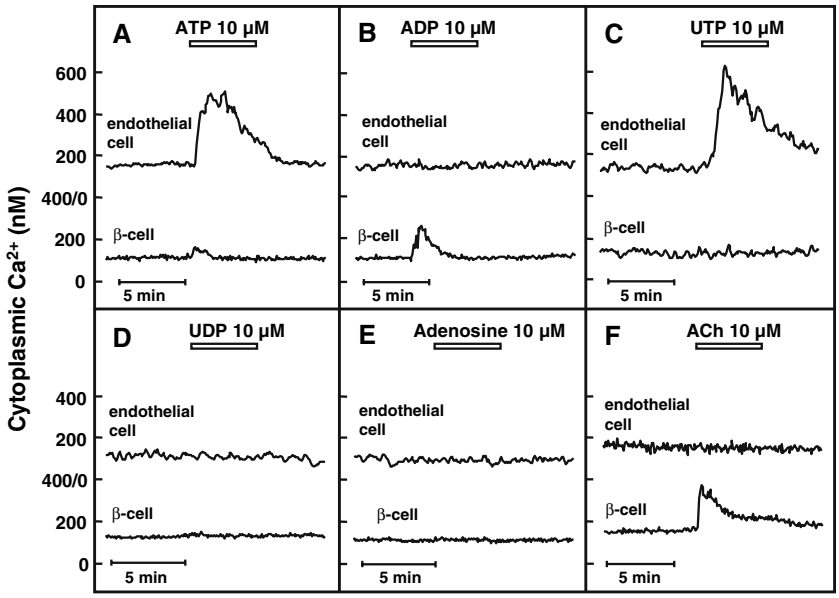

Fig. 3 Effects of ATP, ADP, UTP, UDP, adenosine and acetylcholine (ACh) on $[Ca^{2+}]_i$ in endothelial cells and β-cells superfused with a medium containing 5 mM glucose. The test substances were added at a concentration of 10 μM during a 5 min period. Representative for five experiments

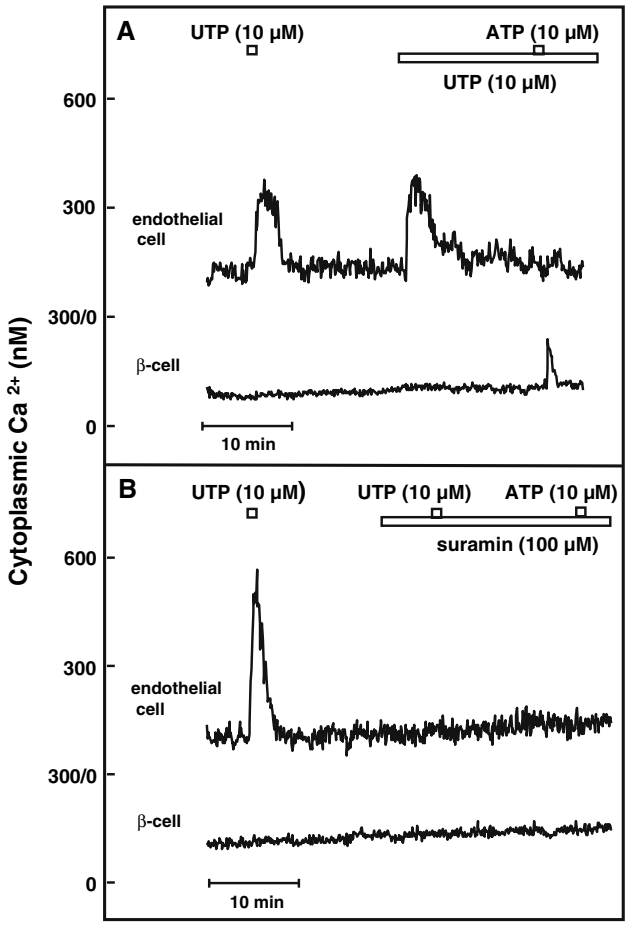

Fig. 4 Effects of 60 s pulses of 10 μM UTP and ATP on $[Ca^{2+}]_i$ in endothelial cells during exposure to 10 μM UTP (A) or 100 μM suramin (B) added to a superfusion medium containing 5 mM glucose. Representative for five experiments

β-cells without affecting the endothelial cells. Using the same experimental model it was found that the presence of UTP prevented the effect of ATP in endothelial cells but not in β-cells (Fig. 7).

The effects of introducing purinoceptor agonists into a medium containing methoxyverapamil were tested also under conditions (20 nM glucagon and 20 mM glucose) known to promote the $[Ca^{2+}]_i$ transients entraining the β-cells into a common rhythm. Endothelial cells differed from β-cells by lacking such transients (Fig. 8). However,

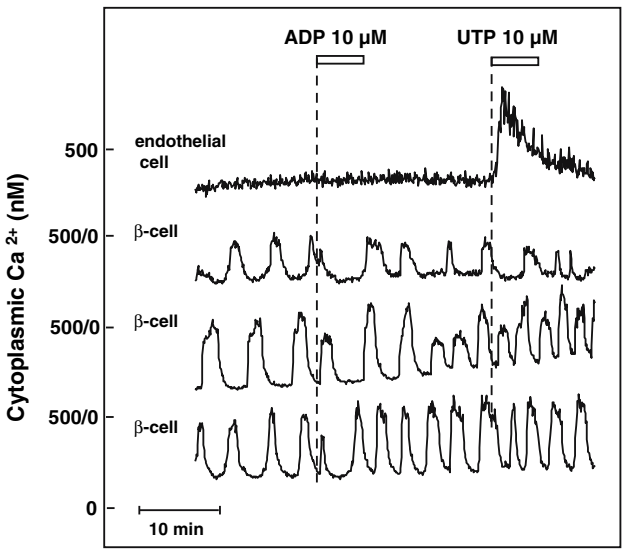

Fig. 5 Effects of 10 μM ADP and UTP on $[Ca^{2+}]_i$ in an endothelial cell and three β-cells superfused with a medium containing 20 mM glucose. The test substances were added during the periods indicated. Endothelial cells differ from β-cells by responding to UTP but not to ADP. Representative for six experiments

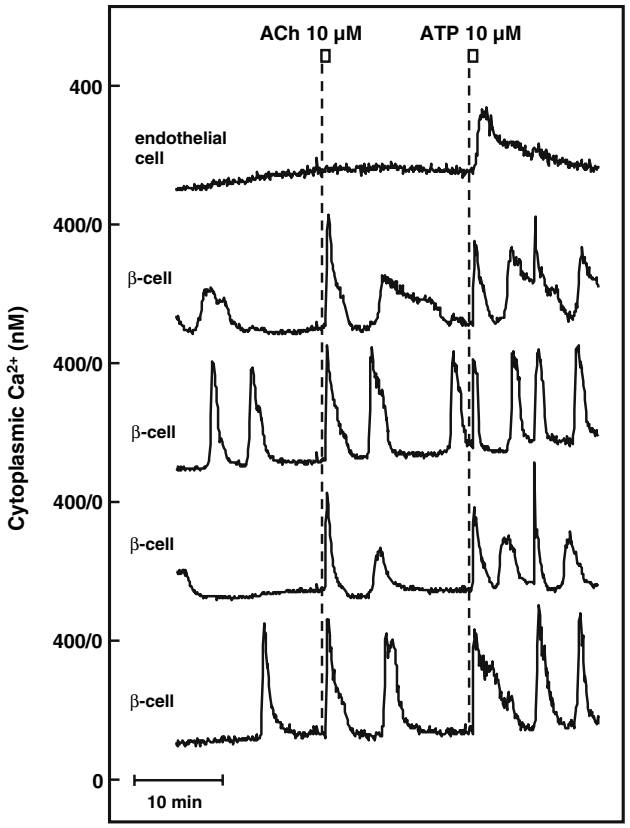

Fig. 6 Effects of 60 s pulses of 10 μM ATP and acetylcholine (ACh) on $[Ca^{2+}]_i$ in an endothelial cell and four β-cells superfused with a medium containing 20 mM glucose. Endothelial cells differ from β-cells in not responding to acetylcholine. Representative for seven experiments

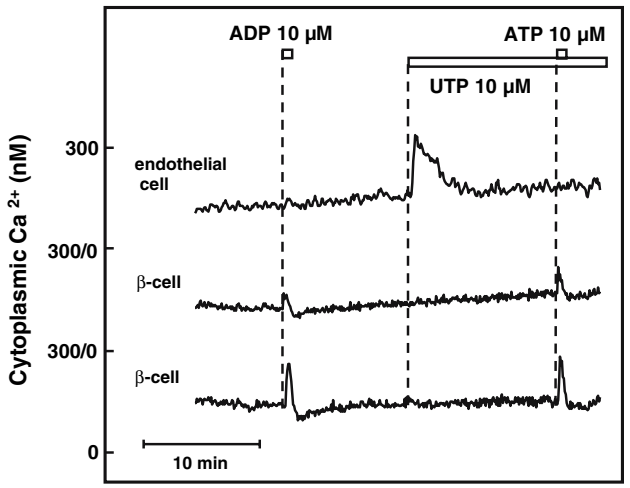

Fig. 7 Effect of a 60 s pulse of 10 μM ATP on $[Ca^{2+}]_i$ in an endothelial cell and two β-cells during exposure to 10 μM UTP added to a superfusion medium containing 50 μM methoxyverapamil and 20 mM glucose. Representative for six experiments. In control experiments, ATP induced $[Ca^{2+}]_i$ rise in endothelial cells exposed to a similar medium devoid of UTP (not shown)

they responded to 10 μM ATP or UTP but not to ADP with extended rise of $[Ca^{2+}]_{i.}$. Addition of 10 μM UTP did not affect the β-cells but the same concentrations of ATP or ADP promptly raised $[Ca^{2+}]_i$ followed by temporary suppression of the spontaneous transients.

Ca^{2+} response to acetylcholine

The effects of acetylcholine on $[Ca^{2+}]_i$ were tested with the same protocol as used for demonstrating effects mediated by ATP and other purinoceptor agonists. Addition of 10 μM acetylcholine did not affect the endothelial cells in the absence (Figs. 3, 6) or presence (Fig. 8) of methoxyverapamil. However, acetylcholine was as efficient as ATP to generate rise of $[Ca^{2+}]_i$ in the β-cells.

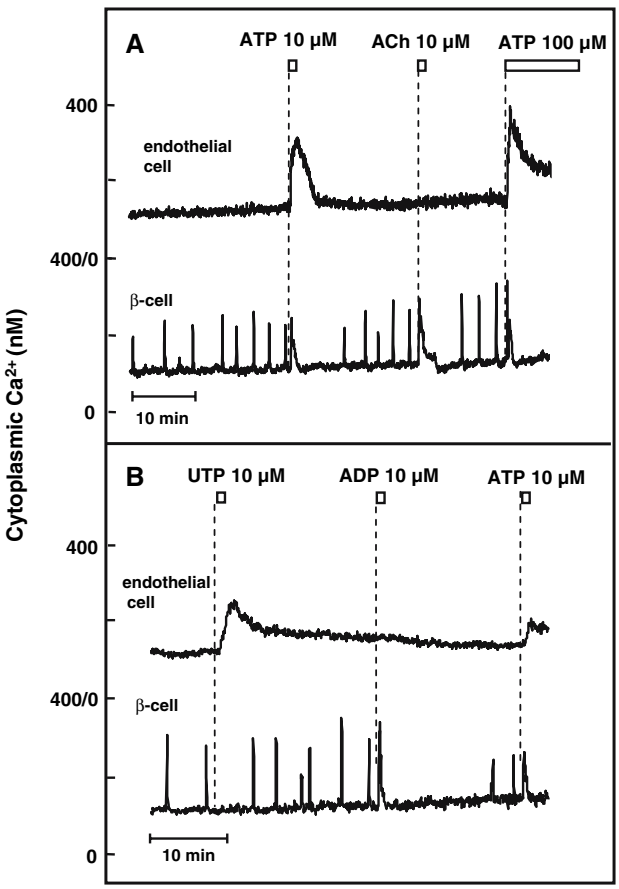

Fig. 8 Effects of ATP, ADP, UTP and acetylcholine (ACh) on $[Ca^{2+}]_i$ in islet endothelial cells and β-cells superfused with a medium containing 20 mM glucose, 20 nM glucagon and 50 μM methoxyverapamil. The test substances were present at concentrations of 10 or 100 μM during the periods indicated. Spontaneous transients are seen in β-cells but not in endothelial cells. The endothelial cells respond with extended rises of $[Ca^{2+}]_i$ to ATP and UTP but not to ADP or acetylcholine. Representative for 5 (**A**) and 4 (**B**) experiments

Discussion

Accumulating data indicate various types of interactions between the islet endothelial cells and adjacent β-cells [9]. Products like NO [15], CO [16] and endothelin 1 [17] are examples of endothelial-derived substances with effects on insulin secretion. It has been proposed that release of VEGF-A from β-cells promotes the development of fenestrations in the islet endothelial cells [18]. We now report that activation of purinoceptors elicits the rise of $[Ca^{2+}]_i$ required for stimulus-response coupling in endothelial cells [19]. Since external ATP triggers $[Ca^{2+}]_i$ increase also in the β-cells, it is likely that this nucleotide has a key role for co-ordinating the activity of the islet endothelium with that of β-cells. In capillary networks endothelial cells co-operate via gap junctions [20]. Accordingly, pulses of depolarization in endothelial cells can be conducted both up and downstream in the capillaries [21] and propagate to the arterioles [22, 23]. It is possible that the electrical coupling enables local responses of endothelial cells to affect the afferent arterioles supposed to regulate the islet blood flow.

Since the original observation of $[Ca^{2+}]_i$ oscillations in pancreatic β-cells [24], the fura-2 indicator has been used to demonstrate spontaneous $[Ca^{2+}]_i$ rhythmicity also in the islet cells producing glucagon and somatostatin [25]. In the present study, restricted uptake of the indicator in the endothelial cells made it necessary to increase the concentration of the fura-2 ester 5–10 times, and include the detergent Pluronic F-127 in the loading medium. Intact islets rapidly lose the capillary endothelial cells when kept in culture [26]. We now observe that preparations of cells, obtained by dispersion of islets and cultured for 1–4 days, are practically devoid of endothelial cells. ECGS has been used for stimulating outgrowth of endothelium from isolated islets [15, 27]. The present study indicates that addition of ECGS stimulates the proliferation of endothelial cells also in cultures of islet cells. Prolongation of culture to 6 days or more made it possible to get endothelial cells suitable for analyses in the absence of ECGS. The latter observation can be expected to reflect the stimulatory action of the high concentration (20%) of fetal calf serum previously used for outgrow of endothelium from intact islets [15, 27].

Vascular endothelial cells respond to stimuli with rise of $[Ca^{2+}]_i$ initiating a number of cellular processes [10–12]. The Ca^{2+} -calmodulin complex is an essential regulator of constitutive endothelial generation of the vasodilator NO [28]. Elevation of $[Ca^{2+}]_i$ is also involved in the production of two other vasodilators of endothelial origin, prostacyclin and endothelium-derived hyperpolarizing factor [29–31]. Released from β-cells in response to depolarization, ATP may not only synchronize the insulin pulses [5, 8], but also induce $[Ca^{2+}]_i$ increase in the islet endothelium. Testing the idea that ATP acts as a messenger between β-cells and islet endothelial cells, we observe that 10 μM of the nucleotide generated a pronounced rise of $[Ca^{2+}]_i$ in both types of cells. Acetylcholine is widely employed for initiating release of endothelial factors regulating the vascular tone [32]. However, 10 μM acetylcholine was found to elicit rise of $[Ca^{2+}]_i$ in β-cells but not in endothelial cells. The observation that islet endothelial cells react with rise of $[Ca^{2+}]_i$ in response to purinergic but not to cholinergic stimuli is not unique for islet capillary endothelium, but is also seen in brain capillary endothelial cells [11].

Several types of purinergic receptors have been identified in a variety of cells [33, 34]. Whereas P1 receptors bind adenosine, the P2 receptors are activated by a number of nucleotides, including ATP and UTP. The P2 receptor family includes ion-channel P2X and G-protein coupled P2Y receptors. The present study indicates that $[Ca^{2+}]_i$ remains unaffected when islet endothelial cells are exposed to 10 μM adenosine or ADP. Therefore, the $[Ca^{2+}]_i$ elevation seen with addition of ATP is not due to adenosine generated by ectonucleotidase activity. Evidence has been provided that activation of subtype A1 of the P1 family contributes to glucose stimulation of the islet blood flow [35]. Nevertheless, there was no increase of $[Ca^{2+}]_i$ when islet endothelial cells were exposed to adenosine. The latter observation suggests that the vasodilator action of adenosine reflects direct interaction with different arterioles rather than with intra-islet capillaries. In many types of endothelial cells, the ATP-induced increase of $[Ca^{2+}]_i$ is mediated by activation of $P2Y_1$ receptors [33]. This receptor subtype may be present in rat islet capillary endothelium, as indicated from immunostaining of the pancreas [36]. However, there was no $[Ca^{2+}]_i$ response to ADP in the islet endothelial cells, indicating that mouse islet endothelium lacks functionally active $P2Y_1$ receptors.

In accordance to what is seen with other types of endothelium [29, 37, 38], UTP was as effective as ATP in raising $[Ca^{2+}]_i$. Added at a concentration of 10 μM, UTP elicited 5–10 min elevation of $[Ca^{2+}]_i$ in the endothelial cells under conditions when the β-cells remained unaffected. Prolongation of the exposure time beyond 60 s had a little effect on the $[Ca^{2+}]_i$ response to ATP and UTP. The observations that ATP lacks effects in the presence of UTP, and that both nucleotides elicit $[Ca^{2+}]_i$ increase with similar kinetics suggest that most of their effects are mediated by the same type of purinoceptor, probably $P2Y_2$. Additional binding to $P2Y_4$ and $P2Y_6$ receptors seems unlikely, since these receptors are relatively resistant to suramin [33]. Moreover, there was no increase of $[Ca^{2+}]_i$ when the islet endothelial cells were exposed to UDP, the principal activator of the $P2Y_6$ receptors [39]. It is well established that the vasoconstriction seen after addition of ATP results from activation of P2X receptors on vascular smooth muscles [38]. Endothelial cells sometimes express P2X receptors, which mediate vasodilation [38, 40]. Since UTP was equally effective as ATP in raising $[Ca^{2+}]_i$, it is likely

that islet endothelial cells lack functionally active P2X receptors.

Increase of $[Ca^{2+}]_i$ is an important trigger of physiological processes, including release of secretory products from insulin-producing β-cells and microvascular endothelial cells. Within the islets, the secretory Ca^{2+} signal propagates from one β-cell to another via gap junctions and diffusible messengers, essentially ATP [4]. We now observe that external ATP is a promotor of $[Ca^{2+}]_i$ increase also in the islet endothelial cells. Endothelial cells release ATP in response to hypoxia and capillary dilation [41, 42], and also when they are exposed to external ATP [13, 14]. Regenerative release of ATP may therefore be a mechanism for propagating Ca^{2+} signals both from one β-cell to another, and between the islet endothelial cells.

A crucial question is whether ATP released from the β-cells has regulatory effects on islet endothelial cells and vice versa. In support for the idea that release of ATP, related to exocytosis of insulin secretory granules, affects the secretory activity in the capillary endothelium it was found that isolated endothelial cells respond to purinoceptor activation with pronounced and extended rise of $[Ca^{2+}]_i$. The presence of such an ATP effect is consistent with reports that β-cells regulate the blood flow within the islets and the endothelial cell production of pro-angiogenic and angiostatic factors [9]. Evaluating the alternatives for how elevation of $[Ca^{2+}]_i$ in the endothelial cells affects insulin secretion, it is important to note that these cells lack spontaneous $[Ca^{2+}]_i$ transients similar to those supposed to entrain the β-cells into a common rhythm. The effect of external ATP on β-cells involves initial stimulation followed by inhibition of the secretory activity with disappearance of the $[Ca^{2+}]_i$ transients [4]. It is possible to counteract the suppressive component by adding ATP in short pulses. Indeed, ATP is intermittently released both from β-cells [43] and nerves [4]. Since the islet endothelial cells respond to purinoceptor activation with extended elevation of $[Ca^{2+}]_i$, it is likely that the accompanying release of ATP is protracted. Accordingly, the endothelial cells may have a tonic inhibitory action on β-cell P2 purinoceptors, resulting in impaired synchronization of the insulin release pulses.

Materials and methods

Chemicals

Reagents of analytical grade and deionized water were used. *Bandeiraea simplicifolia* 1 lectin (BS-1), endothelial cell growth supplement (ECGS), ATP (ultragrade), ADP, UTP, UDP, suramin, adenosine, acetylcholine, glucagon and methoxyverapamil were obtained from Sigma-Aldrich (St Louis, MO). Collagenase, bovine serum albumin (BSA) and HEPES were provided by Roche Diagnostics (Mannheim, Germany). Pluronic F-127 and the acetoxymethyl ester of fura-2 were bought from Molecular Probes (Eugene, OR) and Dynabeads M-450 were provided by Dynal (Oslo, Norway). Fetal calf serum and RPMI 1640 medium were products of Invitrogen Corp. (Carlsbad, Calif) and monoclonal rat anti-CD31 antibody was supplied by HyCult Biotechnology (Uden, The Netherlands). Dacopatts A/S (Glostrup, Denmark) provided kits for immunocytochemical staining with avidin-biotinylated enzyme complex technique (ABC).

Preparation of islet cell culture

The experiments were approved by a local ethical committee and met the guidelines for the care of animals specified by the European Community. Adult *ob/ob* mice were taken from a local colony [44] and killed by decapitation. Islets were isolated from the splenic part of the pancreas with the aid of collagenase. These islets contain >90% β-cells, which have a normal secretory response to glucose [45]. Single cells and small aggregates were prepared by shaking the islets in a Ca^{2+}-deficient medium. After suspension in RPMI 1640 medium containing 11 mM glucose, 20% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, the cells were allowed to attach to the central part of circular coverslips during culture at 37°C in an atmosphere of 5% CO_2 in humidified air.

Measurements of cytoplasmic Ca^{2+}

The experiments were performed with a basal medium containing 0.5 mg/ml BSA, 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$ and 25 mM HEPES with pH adjusted to 7.40 using NaOH. After rinsing, the cells were loaded for 60 min at 37°C with fura-2 in medium supplemented with 6–8 μM of the fura-2 acetoxymethyl ester, 0.01% Pluronic F-127 and 3 mM glucose. The coverslips with the attached cells were then washed and used as exchangeable bottoms of an open chamber connected to a two-channel peristaltic pump. The cells were superfused at a rate of 0.8 ml/min with a medium containing 5 or 20 mM glucose in the presence and absence of 20 nM glucagon (promoter of $[Ca^{2+}]_i$ transients) and 50 μM methoxyverapamil (suppressor of $[Ca^{2+}]_i$ oscillations). The studies were performed with an inverted microscope (Nikon Diaphot) using a climate box maintained at 37 °C. The microscope was equipped for epifluorescence microscopy with a 400 nm dichroic mirror and a 40 × fluor oil immersion objective.

A xenon arc lamp was used for excitation of fura-2 at 340 and 380 nm with a monochromator (Cairn Optoscan, Faversham, Kent, UK). Images were collected through a 30 nm half-bandwith filter at 510 nm with an intensified

CCD camera (Extended Isis-M, Phototonic Science, Robertsbridge, East Sussex, UK). Pairs of 340 and 380 nm images, consisting of 10–16 accumulated video frames, were captured, followed by a delay resulting in measuring cycles of 2–5 s. The specimen were illuminated only during image capture, and excitation light was kept at a minimum. Ratio frames were calculated after background subtraction, and $[Ca^{2+}]_i$ was estimated as previously described [46, 47]. In each experiment, $[Ca^{2+}]_i$ was measured in single endothelial cells and in single β-cells, or small aggregates attached to the same coverslip. Results are presented as mean values ± SEM.

Identification of cells

After measurements of $[Ca^{2+}]_i$, the cells were fixed in situ by addition of ethanol to the superfusion chamber. Photographs taken before and after fixation made it possible to trace the $[Ca^{2+}]_i$ signals to endothelial cells later identified with biotinylated forms of BS-1 [48] or primary antibody against CD31 [49]. The staining was based on ABC technique, using Vectastatin for visualization of BS-1 and Envision + System for demonstration of CD31.

In some experiments, Dynabeads were coated with BS-1 [27] and used for labelling endothelial cells before the measurements of $[Ca^{2+}]_i$. The beads (final concentration about 5×10^4/ml) were allowed to bind to the endothelial cells during 20 min with 150 rpm of orbital shaking (VXR shaker, IKA Werke Gmbh; Staufen, Germany). The binding of beads was maintained during fixation and the following staining (Fig. 1). Control experiments revealed that endothelial cells with attached beads responded with similar $[Ca^{2+}]_i$ increase as seen in cells identified after the measurements.

The insulin-secreting β-cells were recognized by their large size and low nucleo-cytoplasmic volume ratio compared with the cells secreting glucagon and somatostatin [25]. In some experiments BS-1 staining of endothelial cells was combined with immunostaining of β-cells for insulin.

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