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Extracellular ATP-induced nuclear Ca^{2+} transient is mediated by inositol 1,4,5-trisphosphate receptors in mouse pancreatic β-cells

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ABSTRACT

Extracellular ATP (eATP) induces an intracellular Ca^{2+} transient by activating phospholipase C (PLC)-associated P2X4 purinergic receptors, leading to production of inositol 1,4,5-trisphosphate (IP3) and subsequent Ca^{2+} release from intracellular stores in mouse pancreatic β-cells. Using laser scanning confocal microscopy, Ca^{2+} indicator fluo-4 AM, and the cell permeable nuclear indicator Hoechst 33342, we examined the properties of eATP-induced Ca^{2+} release in pancreatic β-cell nuclei. eATP induced a higher nuclear Ca^{2+} transient in pancreatic β-cell nuclei than in the cytosol. After pretreatment with thapsigargin (TG), an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, the amplitude of eATP-induced Ca^{2+} transients in the nucleus was still much higher than those in the cytosol. This effect of eATP was not altered by inhibition of either the plasma membrane Ca^{2+}-ATPase (PMCA) or the plasma membrane Na^+/Ca^{2+} exchanger (NCX) by $LaCl_3$ or by replacement of Na^+ with *N*-Methyl-Glucosamine. eATP-induced nuclear Ca^{2+} transients were abolished by a cell-permeable IP3R inhibitor, 2-aminoethoxydiphenyl borate (2-APB), but were not blocked by the ryanodine receptor (RyR) antagonist ryanodine. Immunofluorescence studies showed that IP3Rs are expressed on the nuclear envelope of pancreatic β-cells. These results indicate that eATP triggers nuclear Ca^{2+} transients by mobilizing a nuclear Ca^{2+} store via nuclear IP3Rs.

Introduction

ATP, an extracellular physiological signal molecule, regulates many cellular processes including secretion and transcription via increasing the concentration of intracellular free Ca^{2+}. Spatio-temporal patterns of Ca^{2+} signals are associated with specific cell functions [1]. In pancreatic β-cells, cytosolic Ca^{2+} is linked to insulin secretion while nuclear Ca^{2+} is associated with gene expression [2]. In pancreatic β-cells, three types of Ca^{2+} channels contribute to increases in $[Ca^{2+}]_i$: the plasma membrane voltage-gated Ca^{2+} channel, sarco-endoplasmic ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP3Rs) [3]. Systems for removal of cytosolic Ca^{2+} include the plasma membrane Na^+/Ca^{2+} exchanger (NCX), plasma membrane Ca^{2+}-ATPase (PMCA), and the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [4]. These on–off Ca^{2+} systems form a cytosolic local Ca^{2+} signal which regulates insulin secretion. Although the presence of RyRs, IP3Rs and SERCA in the nuclear envelope has been implicated [5], and the sarcoplasmic reticulum and nuclear envelope (SR-NE) are an interconnected Ca^{2+} store [6], the regulation of eATP-induced nuclear Ca^{2+} signaling and its relationship to gene expression is poorly understood.

Increases in understanding of nuclear Ca^{2+} regulation have mainly come from studies of isolated nuclei [5,7,8] under non-physiological conditions. Confocal microscopy and appropriate dyes provide the opportunity to compartmentalize nuclear Ca^{2+} in intact cells [2,9,10]. In the present study we have examined alterations in eATP-induced nuclear Ca^{2+} in intact primary pancreatic β-cells using confocal microscopy imaging with the Ca^{2+} indicator fluo-4 AM and the cell permeable DNA dye Hoechst 33342. Agents were applied to selectively block each of the ON and OFF systems. Our results show that nuclear IP3Rs mediate eATP-induced Ca^{2+} transients in pancreatic β-cells.

Materials and methods

Cell preparation. Islets from 129 adult (8–12 week old) male mice killed by cervical dislocation were isolated and then dispersed into single cells according to a published procedure [4]. Briefly, islets were obtained by incubating small pancreatic pieces for 20 min in Hank's buffered solution, containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5 mM D-glucose, 4.2 mM $NaHCO_3$, 1–2 mg/ml

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collagenase P (Boehringer, Germany) and 1 mg/ml BSA. Single cells were dispersed by shaking islets in Ca^{2+}- and Mg^{2+}-free Hank's buffered solution containing 1 mmol EGTA, 10 mmol BSA, and 5 mmol glucose. Isolated cells were plated on coverslips and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 mmol/l glucose in a 37 °C, 5% CO_2 incubator for 1–2 days.

Preparation of nuclei. Nuclei were isolated from about 500 islets by homogenization in a sucrose buffer and centrifugation using a published method [11]. The final pellet of nuclei was resuspended in the following buffer: 140 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 100 μM EGTA, 75 μM $CaCl_2$ and 1 mM ATP, pH 7.2 (adjusted with KOH).

Immunofluorescence. Primary pancreatic β-cells and nuclei plated on coverslips pre-coated with poly-lysine were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, and then incubated with antibodies against IP3Rs (1:100) at room temperature for 2 h. After washing, the cells and nuclei were incubated with FITC-conjugated anti-mouse IgG. Controls in which no primary antibody was used were negative for each experiment.

Confocal microscopy and calcium measurement. For calcium measurements and nuclear compartmentalization, cells were loaded with the Ca^{2+}-sensitive dye fluo-4 AM (2 μM) (Molecular Probes, Eugene, OR, USA) and cell permeable nuclear indicator Hoechst 33342 (10 μg/ml) in a standard solution containing 140 mM NaCl, 5.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 3 mM glucose, pH 7.4 (adjusted with NaOH) at room temperature for 5–10 min. To inhibit IP3Rs, cells were pretreated with 100 μM 2-APB in the standard solution for 20 min at room temperature before each experiment. The coverslips (1 mm) with attached cells were placed at the bottom of a chamber in Ca^{2+}-free standard solution (140 mM NaCl, 5.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 3 mM glucose, 2 mM EGTA, pH 7.4 (adjusted with NaOH)). To inhibit NCX and PMCA, we substituted Na^+ with *N*-Methyl-Glucosamine, and added 200 μM $LaCl_3$ in the Ca^{2+}-free solution [12]. Ca^{2+} was measured by using a laser scanning confocal microscope (FV500, Olympus), which was connected to an Olympus IX-70 inverted microscope, using a Plan Apo ×60 oil objective (1.4 numerical aperture). The excitation wavelengths for detection of Ca^{2+} and Hoechst 33342 were 405 and 488 nm, respectively. A sequential scanning acquisition technique was used in order to avoid cross-talk. Images were collected and analyzed with FLUOVIEW V.4.3 (Olympus). Regions of the nucleus and cytosol were also defined with this software. Under our experimental conditions, fluorescent bleaching was not significant. The Ca^{2+}-dependent fluorescence intensity ratio (F/F_0) was plotted as a function of time.

Chemicals. Hoechst 33342 was from Beyotime, Fluo-4 AM was from Molecular Probes, and 2-APB was from Calbiochem. Mouse anti-IP3Rs monoclonal antibody was from Chemicon. Ryanodine, *N*-methyl-D-glucamine, thapsigargin (TG) and other chemicals were from Sigma.

Statistical analysis. Statistical analysis was performed by using SIGMAPLOT. Values given are means ± SE. Data were tested for significance using the Student's *t*-test. Only results with *P* values <0.05 were considered statistically significant.

Results and discussion

Compartmentalization of eATP-induced nuclear Ca^{2+} transients in pancreatic β-cells

Isolated nuclei are widely used to study nuclear Ca^{2+} regulation, however, such investigations are conduced under non-physiological conditions. Several previous reports [2,9,10] have shown that laser scanning confocal microscopy is a tool with potential for studying nuclear Ca^{2+} in intact cells. To examine nuclear Ca^{2+} in intact pancreatic β-cells in the present study, we used confocal microscopy imaging using the Ca^{2+} indicator fluo-4 AM and the cell permeable, low toxic nuclear indicator Hoechst 33342. When cells were loaded with 2 μM fluo-4 for 5–10 min, the fluorescence of fluo-4 was distributed evenly throughout the pancreatic β-cells (Fig. 1A), and when the incubation time was extended from 5 to 10 min to more than 15 min, the fluorescence was higher in the nucleus (Fig. 1B), suggesting that fluo-4 AM is preferentially accumulated in the nucleus. This result is consistent with previous reports [2,13]. Some reports have shown that differences in the distribution and behavior of Ca^{2+}-sensitive dyes in different subcellular compartments may lead to incorrect conclusions from Ca^{2+} imaging studies [7,14]. To avoid artificial results, cells with evenly distributed fluo-4 fluorescence were used to perform experiments. Hoechst 33342 was used for the first time in this study to compartmentalize nuclear Ca^{2+} changes in pancreatic β-cells. Nuclear and cytosolic Ca^{2+} transients were observed after application of eATP (Fig. 1C).

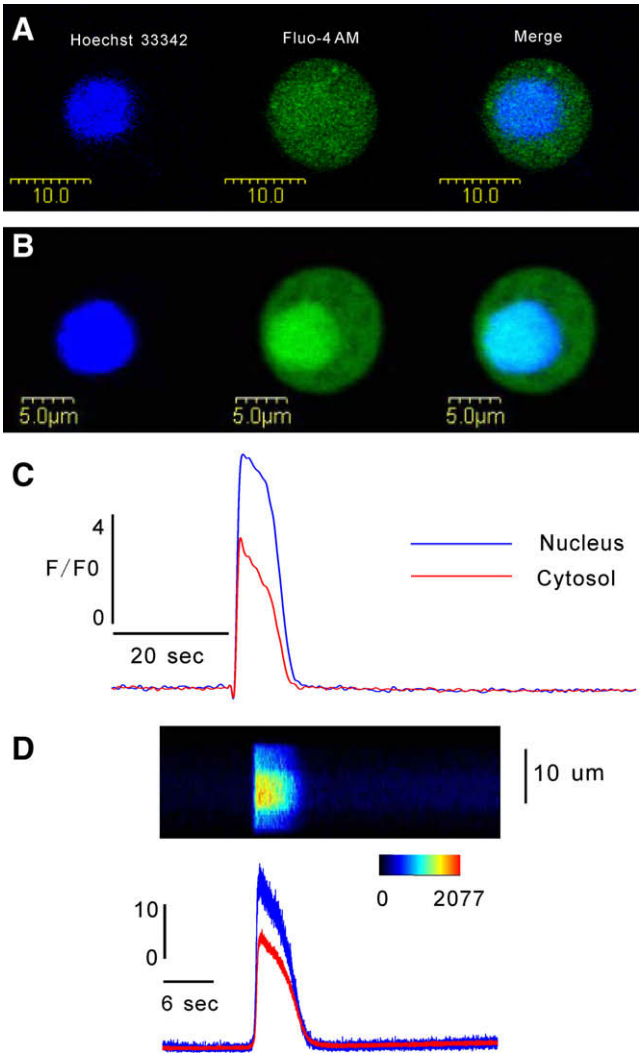

Fig. 1. Compartmentalization of eATP-induced nuclear and cytosolic Ca^{2+} transients. Pancreatic β-cells co-incubated with Hoechst 33342 and fluo-4 AM for 5–10 min (A) and 15–20 min (B), respectively. (C) A representative XYT image of nuclear and cytosolic Ca^{2+} transients; (D) a representative XT image of nuclear and cytosolic Ca^{2+} transients (upper). The profiles (lower) were taken from the above linescan image.

eATP-induced nuclear Ca^{2+} transients in pancreatic β-cells

The single Ca^{2+} transient observed in the nucleus was higher than that of the cytosol in pancreatic β-cells after application of eATP (Figs. 1C and D and 3A). This result partially confirms previous reports that isoform three of IP3R is predominant in pancreatic β-cells [15] and encodes a single Ca^{2+} transient [16]. Some reports have shown that fluo-4 had higher absorbance and fluorescence emission in the nucleoplasm than in the cytoplasm [13,17], and that calibrations of the fluorescence show a lower K_d in the nucleoplasm than in the cytoplasm [14]. However, depolarization of pancreatic β-cells with 25 mM KCl resulted in Ca^{2+} transients with equal amplitudes in the cytosol and nucleus (Supplemental data) under our conditions, consistent with previous calibrated results [14]. These results indicate that the observed eATP-induced higher nuclear Ca^{2+} transient was not artificial.

SERCA, PMCA and NCX were not involved in the lower cytosolic and higher nuclear Ca^{2+} transients induced by eATP in pancreatic β-cells

We examined whether the lower Ca^{2+} transient observed in the cytosol was due to the action of the Ca^{2+} clearance system that exists in the cytosol of pancreatic β-cells. Cells were pretreated with TG, a SERCA inhibitor, and then stimulated with eATP. Under these conditions, a higher nuclear Ca^{2+} transient was still observed (Fig. 2A). Next, we tested the role of PMCA and NCX in the regulation of cytosol and nuclear Ca^{2+} transients induced by eATP using a bath solution in which NaCl was replaced with *N*-Methyl-Glucosamine (140 mM) and LaCl3 (200 μM) was added. Even under these conditions, a higher nuclear Ca^{2+} transient was clearly observed on application of eATP in all cells tested (Fig. 2B), suggesting that SERCA, PMCA, and NCX are not the main causes of eATP-induced lower cytosolic, higher nuclear Ca^{2+} transients. We noticed that TG alone also induced higher nuclear Ca^{2+} transients (Fig. 2A and B), but depolarization of pancreatic β-cells with 25 mM KCl triggered an even amplitude of Ca^{2+} transients in the cytosol and nucleus (Supplemental data). These results indicate that different agents trigger different Ca^{2+} signals in the cytosol and nucleus in pancreatic β-cells.

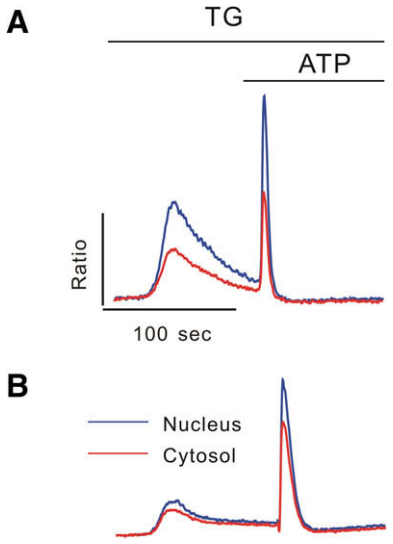

Fig. 2. eATP-induced higher nuclear Ca^{2+} transient. In the presence of SERCA, PMCA and NCX inhibitors eATP could still induce nuclear Ca^{2+} transients. (A) Normal Ca^{2+}-free bath solution (TG was 2 μM); (B) *N*-Methyl-Glucosamine (140 mM) and LaCl3 (200 μM) bath solution. The image presented is representative of 15 experiments.

IP3Rs mediate eATP-induced nuclear Ca^{2+} transients in primary pancreatic β-cells

It has been shown that functional IP3Rs and RyRs exist on nuclear envelopes in pancreatic acinar cells [5] and cardiac myocytes [19]. Here, we examined if these Ca^{2+} release channels are functional in pancreatic β-cell nuclei. As shown in Fig. 3B and D, in 2-APB pretreated pancreatic β-cells both nuclear and cytosolic Ca^{2+} transients were completed abolished. In contrast, the amplitude of nuclear and cytosolic Ca^{2+} transients induced by eATP were not changed in ryanodine-pretreated pancreatic β-cells (Fig. 3C and D) though the expression of RyRs on NEs was also observed (data not shown), indicating that RyRs were not involved in eATP-induced nuclear Ca^{2+} transients. These results suggest that different Ca^{2+} channels, or their specific distribution, contribute to different stimulus-triggered Ca^{2+} transients [14]. The ER-NE is known to be an interconnected Ca^{2+} store [6], therefore the eATP-induced higher nuclear Ca^{2+} transients might be due to specific distribution of IP3Rs.

Expression of P3Rs on the nuclear envelope in primary pancreatic β-cells

Our experiments demonstrate that IP3Rs mediate nuclear Ca^{2+} transients in primary pancreatic β-cells (see above). To confirm these results, we further examined the distribution of IP3Rs in primary pancreatic β-cells. An immuno-fluorescence study was carried out by using a monoclonal antibody that recognizes IP3Rs. As shown in Fig. 4A, staining of IP3Rs was mainly concentrated in the perinuclear area of intact pancreatic β-cells. Similar results were observed in isolated nuclei (Fig. 4B). This result is consistent with previous studies in cardiac myocytes [18] and suggests that IP3Rs are widely expressed on the NE in many cell types.

In conclusion, we have used the nuclear indicator Hoechst 33342 for the first time to compartmentalize eATP-induced nuclear and cytosolic Ca^{2+} transients in primary pancreatic β-cells, and

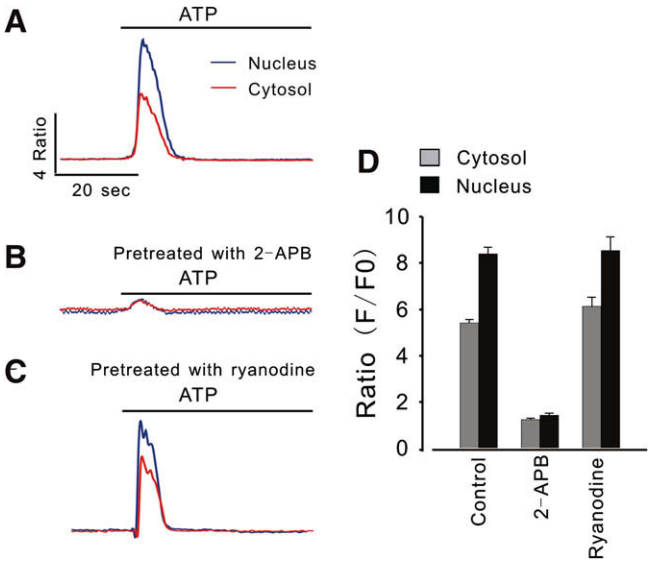

Fig. 3. IP3Rs mediate eATP-induced nuclear Ca^{2+} transients in pancreatic β-cells. (A) Pancreatic β-cell stimulated with 10 μM eATP (representative of 27 cells). (B) Pancreatic β-cell pretreated with 2-APB and stimulated with 10 μM eATP (representative of 15 cells). (C) Pancreatic β-cell incubated with 10 μM ryanodine for 20 min and then stimulated with 10 μM eATP (representative of 21 cells). (D) Summary data of the amplitude of Ca^{2+} transients. In the 2-APB pretreated cells, the cytosolic and nuclear Ca^{2+} amplitudes were reduced to 1.23 ± 0.05 from 5.42 ± 0.15 ($P < 0.01$) and to 1.42 ± 0.09 from 8.39 ± 0.29 ($P < 0.01$), respectively ($n = 15$–27 cells). In ryanodine-pretreated cells, the cytosolic and nuclear Ca^{2+} amplitude was 6.14 ± 0.38 and 8.53 ± 0.58, respectively.

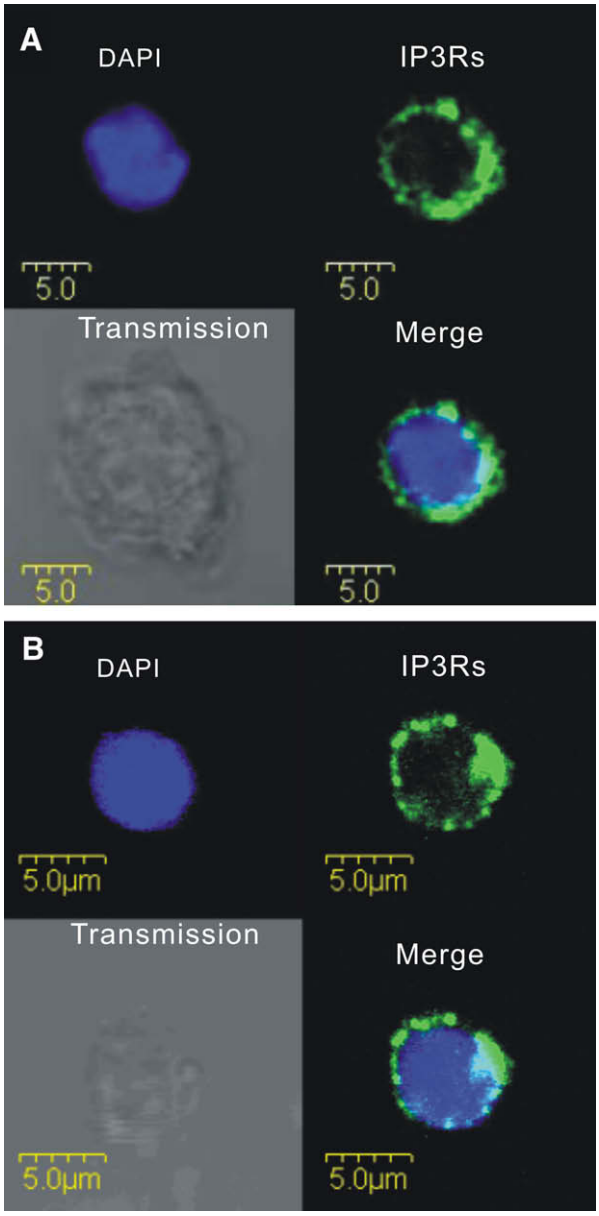

Fig. 4. Expression of IP3Rs on the nuclear envelope of pancreatic β-cells. Intact pancreatic β-cells (A) and isolated nuclei (B) were labeled with DAPI and a monoclonal antibody to IP3Rs.

found that eATP-induced Ca^{2+} transients are significantly higher in the nucleus than in the cytosol. This higher nuclear Ca^{2+} transient is mediated by IP3Rs on the NE. Furthermore, these results indicate that secretory ATP regulates nuclear function by increasing nuclear Ca^{2+} concentration via nuclear IP3Rs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.030.

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