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Ryanodine receptors are involved in nuclear calcium oscillation in primary pancreatic β-cells

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ABSTRACT

Ryanodine receptors (RyRs) are mainly located on the endoplasmic reticulum (ER) and play an important role in regulating glucose-induced cytosolic Ca^{2+} oscillation in pancreatic β-cells. However, subcellular locations and functions of RyRs on other cell organelles such as nuclear envelope are not well understood. In order to investigate the role of RyRs in nuclear Ca^{2+} oscillation we designed and conducted experiments in intact primary pancreatic β-cells.

Immunocytochemistry was used to examine the expression of RYRs on the nuclear envelope. Confocal microscopy was used to evaluate the function of RYRs on the nuclear envelope. We found that RyRs are expressed on the nuclear envelope in single primary pancreatic β-cells and isolated nuclei. Laser scanning confocal microscopy studies indicated that application of glucose to the cells co-incubated with Ca^{2+} indicator Fluo-4 AM and cell-permeable nuclear indicator Hoechst 33342 resulted in nuclear Ca^{2+} oscillation. The pattern of glucose-induced Ca^{2+} oscillation in the nucleus and cytosol was similar. The reduction of Ca^{2+} oscillation amplitude by ryanodine was much greater in the nucleus though both the cytosol and the nucleus Ca^{2+} amplitude decreased by ryanodine.

Our results suggest that functional ryanodine receptors not only exist in endoplasmic reticulum but are also expressed in nuclear envelope of pancreatic β-cells.

1. Introduction

In pancreatic beta cells, physiological regulation of insulin synthesis and secretion by glucose depends on nuclear and cytosolic Ca^{2+} oscillation. Two major sources of Ca^{2+} are involved in this process. The first is extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels and the second is release from intracellular Ca^{2+} stores, including the endoplasmic reticulum and other organelles such as the nuclear envelope (NE), and release through ryanodine receptors (RyRs) or inositol (1,4,5)-triphosphate receptors (IP3Rs). RyRs are mainly located on the ER (SR) in most types of cells and their function is clear. However, the subcellular location and function of RyRs in other cell organelles such as the NE are not well understood. Functional studies indicate that RyRs are present on the NE in cardiac myocytes, pancreatic acinar cells [2] and neurons [3]. Here we report for the first time that RyRs also exist on the NE and have functions in pancreatic β-cells.

Increases in our understanding of nuclear Ca^{2+} regulation have mainly come from the study of isolated nuclei [2,4,5], under non-physiological conditions. Confocal microscopy and appropriate dyes provide the possibility of studying the compartmentalization of nuclear Ca^{2+} in intact cells [6,7]. Here we use confocal microscopy imaging to monitor the specific distribution of fluo-4 AM [8–10] and the cell-permeable DNA dye Hoechst 33324 to examine the role of RyRs in nuclear Ca^{2+} oscillation in intact primary pancreatic β-cells. The initiation of glucose-induced Ca^{2+} oscillation in the nucleus and cytosol was found to be simultaneous, and their patterns were similar. However, the amplitude of nuclear Ca^{2+} elevation was higher than that of the cytosol. Blockade of RyRs with ryanodine did not influence nuclear or cytosolic Ca^{2+} oscillation frequency, but reduced nuclear Ca^{2+} elevation to a much greater extent than that of cytosol. Our results demonstrate that ryanodine-sensitive Ca^{2+} stores exist and have function in the pancreatic β-cell nucleus.

2. Material and methods

2.1. Cell preparation

Islets from 129 adult (8–12 weeks old) male mice killed by cervical dislocation were isolated and then dispersed into single cells

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according to a published procedure [11]. Briefly, islets were obtained by incubating small pancreatic pieces in Hank's buffered solution, containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2{\cdot}2H_2O$, 0.8 mM $MgSO_4{\cdot}7H_2O$, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4{\cdot}12H_2O$, 5 mM D-Glucose, 4.2 mM $NaHCO_3$, 1–2 mg/ml collagenase P (Boehringer, Germany) and 1 mg/ml BSA for 25 min. Single cells were dispersed by shaking islets in Ca^{2+}- and Mg^{2+}-free Hank's buffered solution containing 1 mM EGTA, 10 mM BSA, and 5 mM 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 mM glucose for 1–2 days at 37 °C, 5% CO_2.

2.2. Preparation of nuclei

Nuclei were isolated from about 500 islets by homogenization in a sucrose buffer and centrifugation using a published method [12]. 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.

2.3. Double staining study of insulin and ryanodine receptors

Single cells isolated from pancreatic islets were fixed in 4% paraformaldehyde in 0.1 M PBS for 20 min. Fixed samples were washed three times with PBS (pH 7.4). The cells were incubated with 0.1% triton X-100 for 10 min, followed by incubation with insulin antibody (1:100, Cell Signaling) in 1% BSA PBS for 2 h and wash three times at room temperature. Thereafter, cell preparations were then indirectly immunolabelled with a dilution 1:400 insulin anti-rabbit antibody (labeled with Rhodamine Red-X) for one and half hour at room temperature. Primary pancreatic β-cells and nuclei plated on coverslips pre-coated with poly-lysine were loaded with 1 μM BODIPY FL-X Ryanodine (Molecular Probes, Eugene, OR, USA) in Hank's Buffered solution for 30 min at room temperature. The solution was changed to dye-free Hank's Buffered solution and perfused for 20 min before excitation with the 488 nm line of an argon-ion laser of a confocal microscope (FV 500 or FV1000, Olympus).

2.4. Calcium measurement

For calcium measurements and compartmentalization of the nucleus, 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) for 10–15 min at room temperature in a standard solution containing 140 mM NaCL, 5.5 mM KCl, 2 mM $CaCl_2{\cdot}2H_2O$, 1 mM $MgCl_2$, 10 mM HEPES, 3 mM Glucose, pH 7.4 (adjusted with NaOH). To inhibit RyRs, cells were pretreated with 10 or 100 μM ryanodine for 30–40 min at room temperature in standard solution before the beginning of the experiment. The coverslips (1 mm) with attached cells were placed at the bottom of a perfusion chamber with a perfusion rate of 1.5 ml/min. Ca^{2+} was measured by laser scanning confocal microscopy performed on an Olympus IX-70 inverted microscope, using a Plan Apo ×60 oil objective (1.4 numerical aperture). 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 (F1/F0) was plotted as a function of time.

2.5. Chemicals

Hoechst 33342 was from Beyotime. Insulin antibody was from Cell Signaling. BODIPY FL-X Ryanodine and Fluo-4 AM were from Molecular Probes. Poly-lysine and other chemicals were from Sigma.

2.6. Statistical analysis

Statistical analysis was performed using SIGMAPLOT. Values are means ± SE. Data were tested for significance using the Student's *t* test. Results with P values of <0.05 or lesser were considered statistically significant.

3. Results and discussion

3.1. RyRs exist on the nuclear envelope of primary pancreatic β-cells

Accumulating experimental evidences obtained using molecular techniques [13–15], immunocytochemistry [16], and pharmacological tools [17–19] suggest that RYRs are present in pancreatic β-cells. To observe the distribution of RyRs in primary pancreatic β-cells, BODIPY FL ryanodine which possesses a high binding affinity for RyRs was used in the present study. A perinuclear ring-like pattern of fluorescence was observed in both insulin staining positive pancreatic β-cell nuclei (Fig. 1a and b) and isolated nuclei (Fig. 1c), suggesting that RyRs are not only located on the ER but are also present on the NE. This result is consistent with previous reports from pancreatic acinar cells [2] and other tissues [4]. Our RT-PCR results confirmed also the existence of RYRs in pancreatic β-cells (Fig. 1B). However, the density and the functional role of RyRs on the NE may be different in different cell types [1]. In pancreatic β-cells K_{ATP} channels located in the NE trigger nuclear Ca^{2+} elevation through a calcium release channel, which could be RyRs. However, the role of RyRs in nuclear Ca^{2+} regulation has not been examined in intact cells.

3.2. Compartmentalization of glucose-induced nuclear Ca^{2+} oscillation

Isolated nuclei are widely used to study nuclear Ca^{2+} regulation even though this is a non-physiological treatment condition. Several previous reports [6,7,20] have indicated that laser scanning confocal microscopy can be used to study nuclear Ca^{2+} in intact cells. However, spot confocal microscopy can not be used to detect real Ca^{2+} changes within the whole nucleus. Similarly, care should be taken when using ethidium bromide as an indicator to compartmentalize nuclear Ca^{2+}. To examine nuclear Ca^{2+} oscillation in intact pancreatic β-cells a combination of confocal microscopy imaging, the Ca^{2+} indicator fluo-4 AM [8–10,21–24] and the cell-permeable, low toxicity nuclear indicator Hoechst 33342 were used in the present study. Interestingly, a nuclear-preference fluorescence pattern for fluo-4 AM was observed in pancreatic β-cells (Fig. 2Ab). The high intensity fluorescence of fluo-4 AM co-localized with that of Hoechst 33342 (Fig. 2Ac). This result showed that fluo-4 AM preferentially accumulated in the nucleus of pancreatic β-cells, which is consistent with previous reports for other cell types [25]. We also used either Hoechst 33342 or the specific distribution of Fluo-4 to compartmentalize nuclear Ca^{2+} changes. When the glucose concentration was increased from 3 to 20 mM, oscillations in nuclear and cytosolic Ca^{2+} were clearly observed (Fig. 2B), further confirming the presence and possible function of RYRs in the nucleus of pancreatic β-cells.

3.3. Inhibition of RYRs reduces glucose-induced nuclear Ca^{2+} increase in primary pancreatic β-cells

Our results indicating the presence of RYRs in the nucleus encouraged us to further investigate the role of RyRs in nuclear Ca^{2+} oscillation. First we examined the effect of high concentrations

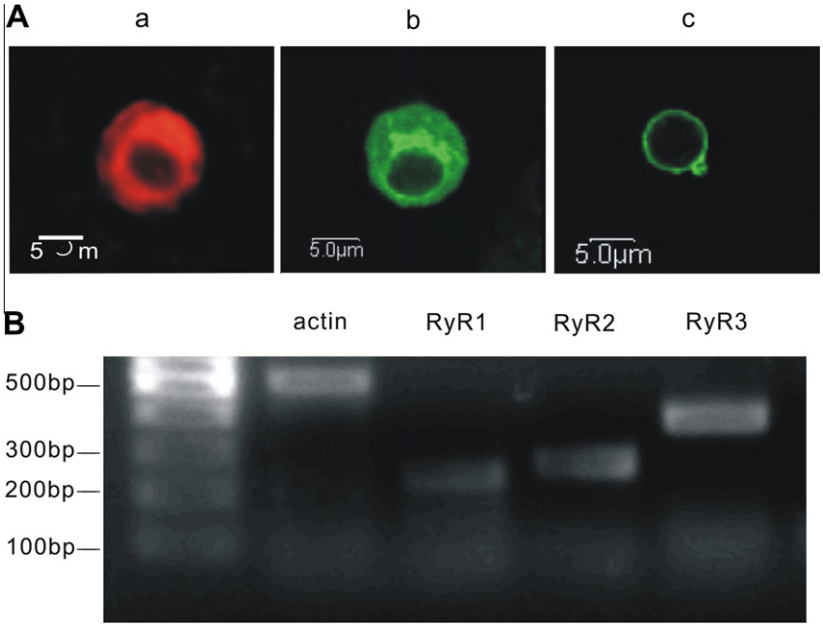

Fig. 1. Idenfication of RyRs in pancreatic β-cells. (A) double staining of insulin (a) and RyRs (b & c). (B) RT-PCR results of RyRs in pancreatic β-cells.

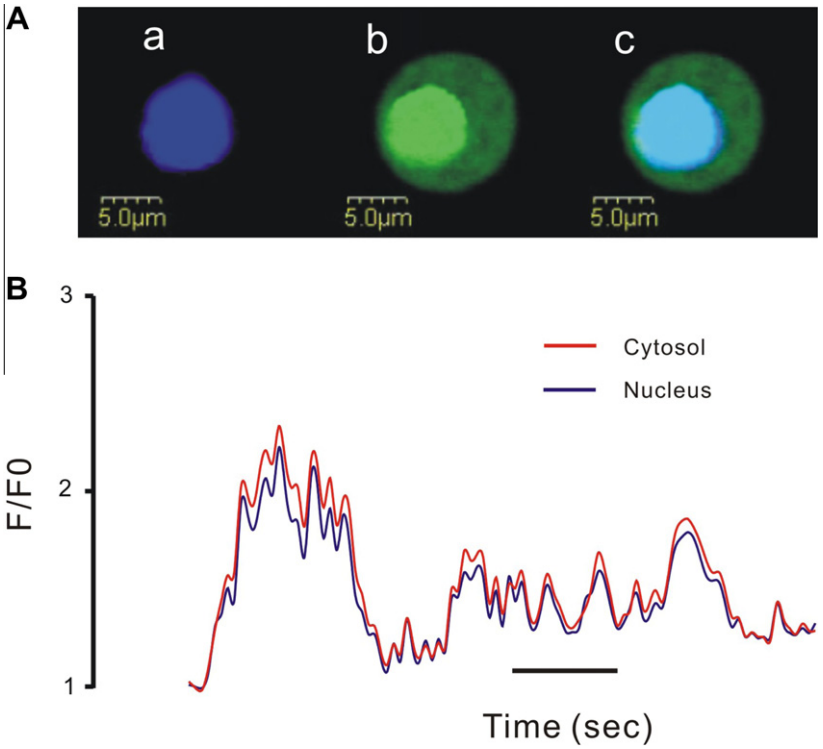

Fig. 2. Glucose-induced nuclear and cytosolic Ca^{2+} oscillation. (A) Images collected from the nucleus and cytosol. Aa: Hoechst 33324; Ab: fluo-4 AM; Ac: merge; (B) a sample recording of cytosolic and nuclear Ca^{2+} oscillation time courses.

of glucose on Ca^{2+} oscillation in the nucleus. When glucose concentration was increased from 3 to 20 mM nuclear Ca^{2+} oscillation was clearly observed in most of the cells tested. The pattern of Ca^{2+} oscillation occurring in the nucleus was similar to that which occurred in the cytosol. However, the amplitude of nuclear Ca^{2+} oscillation was higher than that of the cytosol in 79% of the cells (22/28 cells, Fig. 3A) though there was no significant difference in Ca^{2+} elevation between the nucleus and the cytosol (Fig. 3C), suggesting that there was a variation in the response of cells to glucose. This result explains why there are different opinions on the relationship between nuclear and cytosolic Ca^{2+} oscillation [6], though different cell preparations and methods of Ca^{2+} measurement undoubtedly also contribute to the problem. The conclusion that the amplitude of nuclear Ca^{2+} elevation was higher than that of the cytosol at the single-cell level (Fig. 3D) implies that the NE, acting as an independent Ca^{2+} store, contributes to the higher amplitude of nuclear Ca^{2+} oscillation.

In the next series of experiments we tested the inhibitory effect of ryanodine ($10 \sim 100\ \mu M$) on nuclear Ca^{2+} oscillation. First we sought to confirm the effect of ryanodine on RyRs in adult mouse

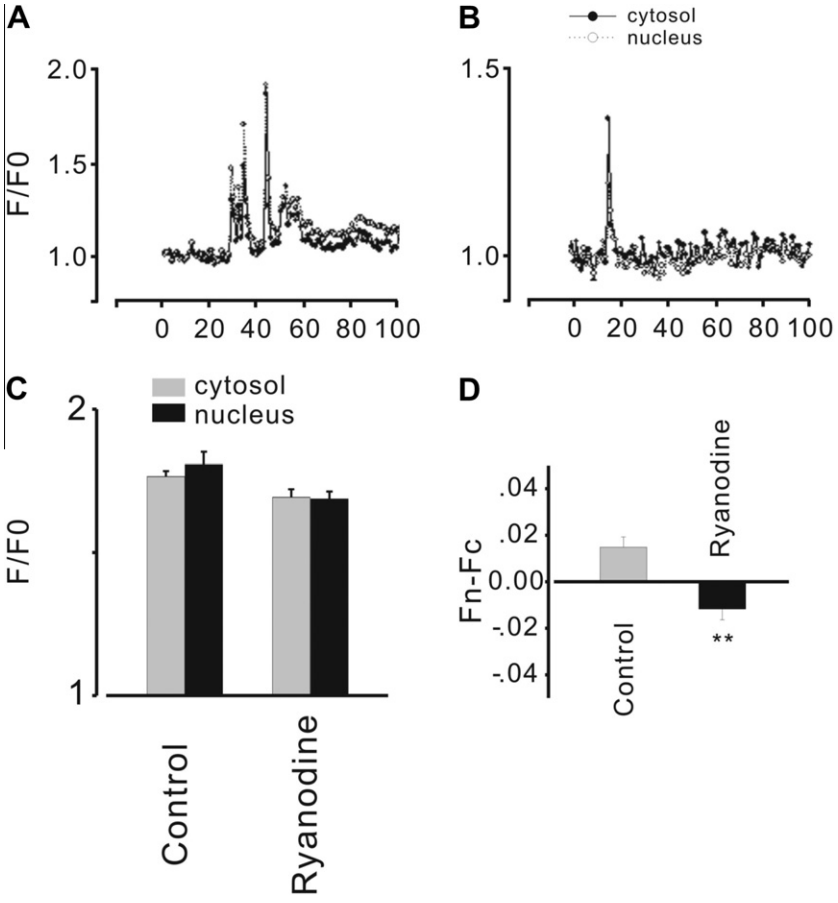

Fig. 3. Inhibition of glucose-induced nuclear Ca^{2+} elevation by ryanodine. (A) A pancreatic β-cell stimulated with 20 mM glucose (image is representative of 22 out of 28 cells). (B) A pancreatic β-cell pretreated with 100 μM ryanodine for 30–40 min was stimulated with 20 mM glucose (image is representative of 21 out of 29 cells). (C) Comparison of the average amplitude of Ca^{2+} oscillation in the presence and absence of ryanodine. In the presence of ryanodine the cytosolic and nuclear Ca^{2+} amplitudes were reduced to 1.06 ± 0.006 from 1.17 ± 0.02 ($P < 0.01$), and to 1.05 ± 0.008 from 1.23 ± 0.04 ($P < 0.01$), respectively ($n = 28–29$ cells). (D) Comparison of nuclear and cytosolic Ca^{2+} elevation with or without ryanodine pretreatment. Rn−Rc is the average amplitude of nuclear Ca^{2+} minus the average amplitude of cytosol Ca^{2+} ($P < 0.01$, $n = 27–29$).

cardiac myocytes. Increased application of ryanodine for 15 min resulted in the disappearance of spontaneous Ca^{2+} waves and sparks (data not shown). After per-incubation of pancreatic β-cells with 10 or 100 μM ryanodine for 30–40 min, nuclear and cytosolic Ca^{2+} oscillation could still be observed. However, the average increase in Ca^{2+} in the nucleus was significantly reduced by ryanodine treatment compared to that in the cytosol (14.88% versus 9.57%, $P < 0.01$; Fig. 3C). Furthermore, the amplitude of nuclear Ca^{2+} oscillation was lower than that in the cytosol in 72% of the cells (21/29 cells tested, Fig. 3B). Thus, these results indicate that the glucose-induced nuclear Ca^{2+} elevation mainly came from the cytosol due to Ca^{2+} influx in the presence of the ryanodine receptor blocker.

From the above results, we conclude that functional RyRs are present on the NE.

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