Cell Calcium 57 (2015) 69–75

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

A crosstalk between Na^+ channels, Na^+/K^+ pump and mitochondrial Na^+ transporters controls glucose-dependent cytosolic and mitochondrial Na^+ signals

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ARTICLE INFO

Article history:
Received 22 October 2014
Received in revised form 9 December 2014
Accepted 10 December 2014
Available online 18 December 2014

Keywords:
TTX
Mitochondrial Na^+/Ca^{2+} exchanger
Ouabain
Mitochondrial Na^+/H^+ exchanger

ABSTRACT

Glucose-dependent cytosolic Na^+ influx in pancreatic islet β cells is mediated by TTX-sensitive Na^+ channels and is propagated into the mitochondria through the mitochondrial Na^+/Ca^{2+} exchanger, NCLX. Mitochondrial Na^+ transients are also controlled by the mitochondrial Na^+/H^+ exchanger, NHE, while cytosolic Na^+ changes are governed by Na^+/K^+ ATPase pump. The functional interaction between the Na^+ channels, Na^+/K^+ ATPase pump and mitochondrial Na^+ transporters, NCLX and NHE, in mediating Na^+ signaling is poorly understood. Here, we combine fluorescent Na^+ imaging, pharmacological inhibition by TTX, ouabain and EIPA, with molecular control of NCLX expression, so as to investigate the crosstalk between Na^+ transporters on both the plasma membrane and the mitochondria. According to our results, glucose-dependent cytosolic Na^+ response was enhanced by ouabain and was followed by a rise in mitochondrial Na^+ signal. Silencing of NCLX expression using siNCLX, did not affect the glucose- or ouabain-dependent cytosolic rise in Na^+. In contrast, the ouabain-dependent rise in mitochondrial Na^+ was strongly suppressed by siNCLX. Furthermore, mitochondrial Na^+ influx rates were accelerated in cells treated with the Na^+/H^+ exchanger inhibitor, EIPA or by combination of EIPA and ouabain. Similarly, TTX blocked the cytosolic and mitochondrial Na^+ responses, which were enhanced by ouabain or EIPA, respectively. Our results suggest that Na^+/K^+ ATPase pump controls cytosolic glucose-dependent Na^+ rise, in a manner that is mediated by TTX-sensitive Na^+ channels and subsequent mitochondrial Na^+ uptake via NCLX. Furthermore, these results indicate that mitochondrial Na^+ influx via NCLX is antagonized by Na^+ efflux, which is mediated by the mitochondrial NHE; thus, the duration of mitochondrial Na^+ transients is set by the interplay between these pivotal transporters.

1. Introduction

In pancreatic β cells, glucose uptake and its metabolism are followed by a rise in cytosolic ATP that subsequently binds and inactivates K^+-ATP channels, leading to cell depolarization and the opening of voltage-gated Ca^{2+} channels [1]; this then triggers insulin secretion. In addition to the Ca^{2+} and K^+-ATP channels, β cells also express voltage-gated Na^+ channels.

Glucose-dependent Na^+ currents were shown to occur in pancreatic β cells [2] and to be linked to the control of duration and magnitude of the action potential, leading to insulin secretion [3–5], in similarity to the release of neurotransmitters [6]. Their role in controlling cytosolic and organellar Na^+ responses remain, however, elusive.

While isotopic studies indicate that cytosolic Na^+ transients are promoted by glucose [7], other studies, employing microfluorimetric analysis, suggest that cytosolic Na^+ transients are reduced by the activity of the Na^+/K^+ ATPase pump [8]. Recently, we have shown that the glucose-dependent Na^+ influx through Na^+ channels is followed by a change in the cytosolic and mitochondrial Na^+ transients, in pancreatic β cells [9].

Cytosolic Na^+ transients are propagated into the mitochondria in several cell types [10]. Na^+ influx in the mitochondria is primarily mediated by the mitochondrial Na^+/Ca^{2+} exchanger, NCLX, which is expressed in pancreatic β cells [11], displaying a stoichiometry of at least 3:1 Na^+ to Ca^{2+} ratio [12]. In addition, NCLX can sense the

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http://dx.doi.org/10.1016/j.ceca.2014.12.007
0143-4160/

glucose-dependent cytosolic rise in Na^+, due to an affinity to Na^+ that is close to resting cytosolic Na^+ concentrations (K_M = 12 mM) [9].

Glucose-dependent cytosolic Na^+ rise can be counter-balanced by the Na^+/K^+ ATPase pump, the main route for cytosolic Na^+ efflux, as depicted by an abrupt increase in cytosolic Na^+ levels in the presence of the Na^+/K^+ ATPase inhibitor, ouabain [8]. However, the role of this pump in regulating Na^+ signals in the mitochondria in general, and in pancreatic β cells in particular, is unresolved.

Mitochondrial Na^+ influx through NCLX could be potentially counter-balanced by the activity of a mitochondrial Na^+/H^+ exchanger, NHE, which mediates the mitochondrial Na^+ efflux by utilizing the trans-inner mitochondrial H^+ gradient that is generated by the respiratory chain [13,14]. Although this activity can be blocked by amiloride derivatives, the molecular identity of the mitochondrial NHE remains elusive, and it is unknown whether there is a functional crosstalk between the Na^+/K^+ ATPase pump and either of the two mitochondrial Na^+ transporters, NCLX and NHE.

In the present study, we aim to determine whether glucose-dependent Na^+ influx, mediated by the Na^+ channel, is controlled by the Na^+/K^+ ATPase pump. We further examine whether a crosstalk between the pump and the Na^+ channel, as well as the mitochondrial Na^+ transporters, shapes the profile of mitochondrial Na^+ transients. We observed that Na^+/K^+ ATPase counteracts the Na^+ influx mediated by the TTX-sensitive Na^+ channel. In addition, since the threshold for NCLX activation is determined by cytosolic Na^+, we found that the pump strongly controls mitochondrial Na^+ influx through NCLX, and indirectly affects mitochondrial Na^+ efflux by mitochondrial NHE.

2. Materials and methods

2.1. Mice and islet isolation

Six-eight-week old female DBA/2J mice were purchased from Jackson laboratories, Bar Harbor, ME, USA. Mice were kept in a pathogen-free environment at the Ben-Gurion University of the Negev Research Animal Facility. Animal care and experiments were conducted according to the University's Care and Use of Animals Committee guidelines.

Animals were anesthetized prior to islet isolation by standard ketamine/xylazine injection, as described elsewhere [15]. Briefly, pancreata were inflated with collagenase XI (Sigma–Aldrich, Rehovot, Israel) and then incubated at 37 °C for 13 min. Digested pancreata were washed with cold HBSS containing 0.5% BSA, vortexes and filtered through 500 μm sieves onto 100 μm nylon cell strainers (Falcon; BD Biosciences Discovery Labware). The sieves were turned over a Petri dish and the islets were washed with HBSS and then hand-picked under a stereomicroscope [16].

2.2. Cell culture and transfection

Isolated islets were cultured in RPMI 1640 (Biological Industries, Kibbutz Beit Haemek, Israel) for 2–3 days and MIN6 cells in DMEM (Beit Haemek); both media were supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-Glutamine (all from Beit Haemek) and 5 mM Glucose (Gerbu). Islets were dispersed into single cells using Trypsin-EDTA (Beit Haemek) [17]. Dispersed primary islet cells and MIN6 cells were seeded onto coverslips [18]. According to immunohistochemical analysis for insulin, more than 90% of islet cells in the single-cell suspension were β cells, as previously described [19]. Pancreatic primary β cells and MIN6 cells were then transfected with siRNA NCLX vs. siRNA Control using DharmaFECT siRNATransfection Reagents (Dharmacon, Chicago, IL). siRNA NCLX (*AACGGCCACUCAACUGUCU*) vs. siRNA Control (*AACGCGCAUCCAACUGUCU*) were diluted in DharmaFECT siRNA transfection reagent, incubated for 20 min at room temperature and then added to antibiotic-free medium, as previously described [11]. Transfection efficiency was evaluated by the siRNA marker, siGLO Red (Dharmacon).

2.3. Fluorescent Na^+ imaging

The imaging system consisted of an Axiovert 100 inverted microscope (Zeiss, Oberaue, Germany), Polychrome V monochromator (TILL Photonics, Planegg, Germany) and a SensiCam cooled charge-coupled device (PCO, Kelheim, Germany). Fluorescent images were acquired with Imaging WorkBench 4.0 software (Axon Instruments, Foster City, CA). Fluorescent Na^+ imaging was performed in pancreatic primary β cells and MIN6 cells attached onto coverslips and superfused with Ringer's solution containing (in mM): 126 NaCl (Frutarom), 5.4 KCl (Sigma), 0.8 $MgCl_2$ (Gerbu), 20 HEPES (Amresco), 1.8 $CaCl_2$ (Sigma), 15 Glucose (Gerbu), pH was adjusted to 7.4 with NaOH (Sigma). In glucose dependent experiments, the pancreatic primary β cells or MIN6 cells were pre-washed for 30 min with low glucose (3 mM) followed by high glucose (20 mM) Ringer's solution.

Fluorescent imaging of Na^+ in cytosol and mitochondria was performed using CoroNa Green (Invitrogen, Eugene, OR) and CoroNa Red (Invitrogen, Eugene, OR), respectively. Cytosolic Na^+ response was acquired in cells loaded with CoroNa Green at excitation of 488 nm and imaged at 510 nm long pass filter [20]. Mitochondrial Na^+ signals were monitored in cells loaded with CoroNa Red at excitation of 568 nm and emission at 590 nm, respectively [20].

The traces of all fluorescent imaging experiments were plotted using KaleidaGraph 4.0 (Synergy Software, Reading, PA). The fluorescent Na^+ traces were normalized to the averaged baseline signal (F/F_0), obtained at the beginning of the measurements. The influx and efflux rates were derived from a linear fit of the fluorescence change during 30 s [11,12]. Peak amplitude was established by the difference between the maximal peak heights of the signal to the baseline fluorescence, as described [21]. Averaged rates or amplitudes of the fluorescent Na^+ responses, over *n* (indicated in the figure legends) experiments, are presented in the bar graphs.

3. Results

3.1. Role of Na^+/K^+ ATPase pump vs. mitochondrial Na^+/Ca^{2+} exchanger in the glucose-dependent cytosolic Na^+ response

We initially sought to determine the role of Na^+/K^+ ATPase pump and NCLX in regulating of the glucose-dependent cytosolic Na^+ response. Both Na^+ transporters have the potential to modulate cytosolic Na^+ signals, the former by extruding Na^+ out of the cells while the latter by pumping Na^+ into the mitochondria. Cells were transfected with siNCLX vs. siControl, loaded with CoroNa Green and superfused with low-glucose Ringer's solution, followed by application of high glucose. As shown in Fig. 1A, knockdown of NCLX expression does not affect glucose-dependent initial rate or amplitude of the cytosolic Na^+ rise.

To determine the role of the Na^+/K^+ ATPase pump in pancreatic β cells, glucose-dependent changes in cytosolic Na^+ were compared between non-treated (Control) vs. ouabain-treated cells (Fig. 1B). As shown, application of ouabain (100 μM) was followed by a strong increase in rate and amplitude of the glucose dependent cytosolic Na^+ rise (10 ± 1.3-fold and 7.6 ± 1-fold, respectively) (Fig. 1C and D).

To determine the combined role of NCLX and Na^+/K^+ ATPase pump in glucose-dependent Na^+ changes, cytosolic Na^+ responses were compared between pancreatic primary islet cells transfected