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Pancreatic β cell Na^+ channels control global Ca^{2+} signaling and oxidative metabolism by inducing Na^+ and Ca^{2+} responses that are propagated into mitochondria

Iulia I. Nita[1], **Michal Hershfinkel**[1], **Chase Kantor**[2], **Guy A. Rutter**[2], **Eli C. Lewis**[3], and **Israel Sekler**[1]

[1]Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

[2]Section of Cell Biology, Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine, Imperial College London, London, UK

[3]Department of Clinical Biochemistry, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Summary

Na^+ channels are abundantly expressed in pancreatic β cells and prone to prolonged activation. However, their role in regulating cellular Na^+ fluxes or mitochondrial Ca^{2+} transients, and thereby oxidative metabolism, has not been explored. Here, we combined fluorescent Na^+, Ca^{2+} and ATP imaging, electrophysiological analysis and molecular manipulation of channel expression to study the communication between Na^+ channels and mitochondria in these cells. We show that tetrodotoxin (TTX) inhibits glucose-dependent depolarization, blocks cytosolic Na^+ and Ca^{2+} responses and their propagation into mitochondria. TTX-sensitive mitochondrial Ca^{2+} influx was largely blocked by knockdown of the mitochondrial Ca^{2+} uniporter, MCU expression. Knockdown of the mitochondrial Na^+/Ca^{2+} exchanger (NCLX), and Na^+ dose response analysis, demonstrated that NCLX mediates the mitochondrial Na^+ influx and is tuned to sense the TTX-senstive cytosolic Na^+ responses. Finally, the TTX-dependent mitochondrial Ca^{2+} rise upregulated mitochondrial metabolism and enhanced ATP production. Taken together, our results show that Na^+ channels initiate cytosolic Na^+ and Ca^{2+} signals that are propagated by MCU and NCLX into mitochondria, thereby shaping both global Ca^{2+} transients and metabolism in β cells.

Keywords

TTX; β cells; metabolism; NCLX; MCU; mitochondrial Ca^{2+} shuttling

Introduction

A functional interaction of cell membrane and mitochondria is required for Ca^{2+} signaling linked to insulin secretion in β cells. Uptake of glucose is followed by mitochondrial ATP production leading to closure of the K^+-ATP channel thereby to β cell depolarization that triggers Ca^{2+} rise by the voltage gated Ca^{2+} channels (Ashcroft et al., 1973).

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A poorly undersood aspect of mitochondria in pancreatic β cells is their role as a direct cellular Ca^{2+} signaling hub. Powered by the steep mitochondrial membrane potential, Ca^{2+} permeates into the mitochondria via a Ca^{2+} channel traditionally called the mitochondrial Ca^{2+} uniporter, MCU (Baughman et al., 2011)(De Stefani et al., 2011) and it is then extruded by the mitochondrial Na^{+}/Ca^{2+} exchanger, NCLX (Palty et al., 2010). This mitochondrial Ca^{2+} shuttling is linked to several aspects of metabolic and global Ca^{2+} regulation. At least 3 enzymes of Kreb's cycle are activated by an intramitochondrial Ca^{2+} rise (Rutter, 1990) thus linking Ca^{2+} signaling to ATP production (Denton, McCormack, 1985, PMID: 4010776). Mitochondrial Ca^{2+} shuttling also controls the magnitude and duration of cytosolic Ca^{2+} transients and the refilling of the ER Ca^{2+} stores (Poburko et al., 2009). In addition, because Ca^{2+} channels are strongly controlled by cytosolic Ca^{2+}, mitochondria modulating local Ca^{2+} concentration at the plasma membrane micro-domains can control rates of Ca^{2+} influx (Rizzuto et al., 2012). Importantly, the recent molecular identification of MCU (Baughman et al., 2011)(De Stefani et al., 2011) and NCLX (Baughman et al., 2011)(De Stefani et al., 2011) has been instrumental in allowing the roles of each to be dissected using RNA interference (RNAi) in the beta cell {Tarasov,2012} {Tarasov,2013, Pflug Arch}{Alam..Grier,2012, JBC}{Palty,2010}.

Ca^{2+} extrusion mediated by NCLX is coupled and powered by a reciprocal exchange of 3 Na^{+} per Ca^{2+}. However, the occurrence or importance of Na^{+} signaling is still poorly understood. Thus, although Na^{+} is distributed at steep gradients across cell membranes, it has been thought for many years that cytosolic Na^{+} transients are subtle and that a rise in cytosolic Na^{+} is primarily linked to pathophysiological syndromes such as brain or cardiac ischemia (Murphy and Eisner, 2009). At least some of the uncertainties regarding the magnitude of cytosolic changes in Na^{+} are related to the less than ideal properties of available Na^{+}-sensitive fluorescent dyes (Meier et al., 2006). Nevertheless, more recent studies have indicated that cytosolic Na^{+} transients are encountered during many physiological processes and in diverse cell types. For example, in the synaptic cleft Na^{+} influx is required to enhance mitochondrial Ca^{2+} extrusion, thereby controlling Ca^{2+} transients (Yang et al., 2003), whilst neuronal firing is linked to Na^{+} transients in axonal initiating segments (Fleidervish et al., 2010). Similarly, in astroglia, robust cytosolic Na^{+} transients activate the mitochondrial Na^{+}/Ca^{2+} exchanger leading to an enhanced Ca^{2+} response that augments neurotransmitter release (Verkhratsky et al., 2012).

Despite the high firing frequency of Na^{+} channels in pancreatic β cells (Dunne et al., 1990) and the occurrence of glucose-dependent prolonged depolarization episodes, that can potentially trigger their intense activation, their role in shaping glucose dependent Ca^{2+} signaling is still controversial and poorly understood. Early studies failed to find a role for the voltage-gated Na^{+} channels in mouse β cells (Plant, 1988). Later studies, however, suggested that TTX-sensitive Na^{+} channels are required in rat beta cells to maintain robust electrical activity and a high rate of insulin secretion (Hiriart and Matteson, 1988). Later analysis further suggested that, by modulating the electrical activity, permeation of Na^{+} is necessary for the glucose-dependent cytosolic Ca^{2+} response in clonal rat beta cells (Dunne et al., 1990).

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It is further unclear if, in addition to modulating electrical activity and Ca^{2+} fluxes, pancreatic beta cell Na^+ channels can mediate cytosolic Na^+ responses. Thus, while some studies support such glucose-dependent Na^+ transients (Kawazu et al., 1978), others suggested that Na^+ transients are masked by the activity of the Na^+/K^+ pump (Grapengiesser, 1998). Here, we asked whether glucose triggers cytosolic and mitochondrial Na^+ as well as Ca^{2+} signals, and studied their effect on pancreatic β cell metabolism. We find that TTX-sensitive Na^+ channels trigger a cytosolic Na^+ response and upregulate the Ca^{2+} signal. The former ion is propagated by NCLX and the latter by MCU into the mitochondrial matrix. We further show that these mitochondrial Ca^{2+} and Na^+ transients control metabolism and global Ca^{2+} signaling in β cells.

Materials and Methods

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. A limited number of experiments were also performed using islets from C57BL/6 mice (10-14 weeks of age), sacrificed by cervical dislocation as approved by the United Kingdom Home Office (HO) Animal Scientific Procedures Act, 1986.

Animals were anesthetized prior to islet harvest by standard ketamine/ xylazine injection (Lewis et al., 2005). Isolation of islets was performed as previously described (Salvalaggio et al., 2002). Briefly, islets were isolated by collagenase digestion and transferred to 50mL tubes, incubated at 37°C for 13min. The digested pancreata were washed with cold HBSS containing 0.5% BSA, vortexed and filtered through 500 μm sieves. The sieves were turned over a Petri dish and the islets were washed with HBSS and then hand-picked under a stereomicroscope.

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% Pencillin/Streptomycin, 1% L-Glutamine, 5mM Glucose. Islets were then hand-picked under a stereomicroscope and dispersed into single cells using Trypsin-EDTA {PMID: 20204627}. Dispersed primary islet cells and MIN6 cells were seeded onto coverslips for imaging experiments (Jonkers et al., 1999). Immunohistochemical analysis of insulin was performed and revealed that more than 90% of pancreatic islets cells in the culture were β cells as previously described (Lindskog et al., 2012). Pancreatic primary β cells and MIN6 cells cultured on glass coverslips were transfected with siRNA NCLX or siRNA MCU vs. siRNA Control using DharmaFECT siRNATransfection Reagents (Dharmacon, Chicago, IL). siRNA NCLX (*AACGGCCACUCAACUGUCU*) or siRNA MCU (GCCAGAGACAGACAAUA) vs. siRNA Control (*AACGCGCAUCCAACUGUCU*) were diluted in DharmaFECT siRNA transfection reagent, incubated for approximately 20 min at room temperature and then

added to the antibiotics-free medium as previously described (Nita et al., 2012). The fluorescence of siRNA marker, siGLO Red (Dharmacon) was used to assess transfection efficiency.

Fluorescent Ca^{2+} and 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). Ca^{2+} 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, 5.4 KCl, 0.8 $MgCl_2$, 20 HEPES, 1.8 $CaCl_2$, 15 Glucose, pH was adjusted to 7.4 with NaOH or NMDG in Na^+ free Ringer's solutions. 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.

For cytosolic Ca^{2+} measurements, pancreatic primary β cells or MIN6 cells were loaded with Fura 2AM (Teflabs), excited with 340/380nm wavelength light and imaged using a 510nm long pass filter as previously described (Jonkers and Henquin, 2001). Mitochondrial Ca^{2+} measurments were performed in MIN6 cells expressing ratiometric mitochondrial pericam, which is targeted solely to the inner membrane of mitochondria. The mitochondrial pericam fluorescence in MIN6 cells was acquired at 430nm excitation and 550nm emission as previously described (Nagai et al., 2001).

Cytosolic Na^+ levels were recorded in CoroNa Green (1μM) (Invitrogen, Eugene, OR) loaded pancreatic primary β cells and MIN6 cells, excited with 488nm and imaged at 510nm longpass filter (Poburko et al., 2007). Mitochondrial Na^+ signals were monitored in cells loaded with CoroNa Red (Invitrogen, Eugene, OR) at excitation of 568nm and emission at 590nm, respectively (Poburko et al., 2007).

Fluorescent measurements of Na^+ dependent Ca^{2+} efflux

Mitochondrial Ca^{2+} efflux was monitored in digitonin-permeabilized (0.007% Digitonin) MIN6 cells transfected with mito-pericam (see Fluorescent Na^+ and Ca^{2+} imaging) as previously described (Palty et al., 2010) in a sucrose buffer containing (in mM): 220 Sucrose, 0.01 Ca^{2+} free, 10 HEPES, 5 Succinate, 2.5 KH_2PO_4, 0.4 EGTA and 0.001 cyclosporine A, pH adjusted to 7.4 with KOH. Then different concentrations between 0 and 80 mM NaCl were applied to permeabilzed MIN6 cells.

Electrophysiology

Electrophysiological recordings were done in whole-cell perforated-patch configuration, using an EPC9 patchclamp amplifier controlled by Pulse acquisition software (HEKA Elektronik) essentially as described {Tarasov,PLoSOne,2012}. The pipette tip was dipped into pipette solution, and then back-filled with the same solution containing 0.17 mg/ml amphotericin B. Series resistance and cell capacitance were compensated automatically by the acquisition software. Data were filtered at 1 kHz, and digitized at 10 kHz. Analysis of all

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patch-clamp data was performed using Clampfit (pCLAMP version 9.2; Axon Instruments Inc.). The pipette solution contained (in mM): 76 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, 5 HEPES (pH 7.35 with KOH). The extracellular bath solution contained (in mM): 120 NaCl, 4.8 KCl, 0.5 Na_2HPO_4, 24 $NaHCO_3$ (saturated with CO_2), 5 HEPES (pH 7.4 with NaOH), 2.5 $CaCl_2$, 1.2 $MgCl_2$. All experiments were conducted at 32–33 °C, and the bath solution was perifused continuously.

Fluorescent imaging of metabolic rate, mitochondrial membrane potential and ATP production

Metabolic rate was monitored by following the changes in NAD(P)H intrinsic fluorescence in pancreatic primary islets cells (360 nm excitation and 440 nm emission) (Rocheleau et al., 2004). Mitochondrial membrane potential was measured in pancreatic primary islets cells loaded and superfused with TMRM (545 nm excitation and 570 nm emission) (Brennan et al., 2006). For glucose-dependent ATP measurements, MIN6 cells were transfected with the Förster resonance energy transfer- (FRET) based probe, AT 1.03 (435 nm excitation; 475nm and 527 nm emission) (Imamura et al., 2009).

Fluorescence imaging was performed on an inverted microscope (see Fluorescent Ca^{2+} and Na^+ imaging). The metabolic rate, mitochondrial membrane potential and ATP production were calibrated at the end of experiments by superfusing the cells with, the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (5uM FCCP, 5 uM), as previously described (Akhmedov et al., 2010).

Statistical analysis

The traces of all fluorescence imaging experiments were plotted using KaleidaGraph 4.0 (Synergy Software, Reading, PA). Apparent influx of Na^+ or Ca^{2+} into the cytosol or mitochondria consistently began after superfusion of pancreatic β cells with high glucose. Similarly, Ca^{2+} efflux of the mitochondria followed the influx phase (Palty et al., 2010). The fluorescent Na^+ or Ca^{2+} signals were normalized to the averaged signal, obtained at the beginning of the measurements. The influx and efflux rates were derived from a linear fit of the fluorescence change slope during 30 seconds following the stimulation with high glucose. Peak amplitude was defined according to established protocols (Akhmedov et al., 2010), comparing the maximal peak height of the signal to the background fluorescence. Changes in rate and amplitude of the fluorescence of Na^+ or Ca^{2+} responses (Ca^{2+} or Na^+ cytosolic rates or peak amplitude) were calculated by averaging either rates or the amplitude of the fluorescent Na^+ or Ca^{2+} response, respectively, corresponding to number of experiments, n, indicated in the figure legends. The results of the experiments are presented as the mean ± S.E.M. (standard error of the mean) of at least three independent experiments (n), using 20-30 cells in each. Statistical significance for all experiments was determined using a one-way ANOVA test followed by Bonferroni post-hoc analysis. * Significantly different ($p<0.05$) compared to control.

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Results

Glucose-dependent cytosolic Na^+ entry is mediated by TTX-sensitive Na^+ channels

To determine if high glucose can trigger Na^+ influx via the TTX sensitive Na^+ channels, changes in cytosolic Na^+ were monitored in MIN6 and pancreatic primary mouse islets cells, respectively (Fig. 1A, B) preloaded with the cytosolic Na^+ sensitive dye, CoroNa Green. Cells were superfused first at low (3mM) glucose followed by high (20mM) glucose in Ringer's solution (see Methods). In islet/MIN6 cells (?) the application of high glucose was followed by an increase in the rate and amplitude of the cytosolic Na^+ response by 22 ± 2 fold and 1.6 ± 0.1 fold, respectively compared to the low glucose Na^+ phase (Fig. 1A, B). In contrast, in the presence of the Na^+ channel blocker TTX (1μM), the high glucose-dependent change in rate and amplitude of cytosolic Na^+ rise were largely blocked by 20 ± 3 and 1.6 ± 0.2 fold, respectively (Fig. 1D, E), compared to fluxes in the absesnce of TTX.

These results indicate that high glucose triggers Na^+ entry primarily via voltage-dependent TTX-sensitive Na^+ channels in both primary mouse and MIN6 beta cells.

Activation of TTX-sensitive Na^+ channels is triggered by glucose-dependent cell depolarization (Hiriart and Aguilar-Bryan, 2008). We therefore reasoned that the depolarization mediated by K^+-ATP channels contributes to the Na^+ influx. To determine the role of K^+-ATP channel independently of glucose in Na^+ signaling, we compared the cytosolic Na^+ transients in the presence or absence of the K^+-ATP channel blocker, glibenclamide (Fig. 1C). Application of glibenclamide (2 μM) was followed by a rise in rate (25 ± 2.5 fold) and amplitude (2.16 ± 0.5 fold) of the cytosolic Na^+ response compared to the low glucose phase. The cytosolic Na^+ rise triggered by glibenclamide was blocked in the presence of TTX by 5 ± 1.7 fold and 2 ± 0.1 fold, respectively (Fig. 1D, E), compared to the absence of TTX.

Altogether these results indicate that Na^+ influx via TTX-sensitive Na^+ channels is preceeded by inactivation of the ATP dependent K^+ channel, which triggers depolarization and opening of the voltage-gated Na^+ channels.

Cytosolic Na^+ rises are followed by mitochondrial Na^+ influx

We then asked if the cytosolic Na^+ rise is followed by mitochondrial Na^+ uptake. Mitochondrial Na^+ fluxes were monitored in MIN6 cells loaded with the mitochondrial Na^+ sensitive dye, Corona Red. Following the same paradigm described in Fig. 1, the switch from low to high glucose was followed by a strong increase in mitochondrial Na^+ influx rate and amplitude of 13 ± 2.5 fold and 1.1 ± 0.5 fold, respectively in islet/MIN6 (?) cells (Fig. 2A). The rates and amplitudes of mitochondrial Na^+ responses were, however, strongly inhibited, by 5 ± 1.7 fold and 1.05 ± 0.1 fold, respectively, in TTX-treated cells vs. non-treated cells, indicating that Na^+ influx via TTX-sensitive Na^+ channels into the cytosol is followed by Na^+ influx into the mitochondria (Fig. 2C,D). The mitochdondrial exchanger is the major Na^+ influx pathway in cardiac mitochondria (Murphy and Eisner, 2009). To determine if in β cells mitochondrial Na^+ uptake is mediated by NCLX we then compared the rate and amplitude of mitochondrial Na^+ influx in cells transfected with siNCLX vs. siControl (Fig. 2B). The silencing of NCLX expression was followed by a 6 ± 0.8 fold

inhibition in the rate and amplitude of the mitochondrial Na^+ influx, compared to siControl, indicating that NCLX is the major pathway for mitochondrial Na^+ influx (Fig. 2C,D).

If NCLX mediates the cross talk between plasma membrane and mitochondrial Na^+ signaling, then it may sense and respond to glucose dependent cytosolic Na^+ transients. To determine the apparent affinity of the exchanger to cytosolic Na^+, an analysis of the dose dependence on Na^+ was conducted in mito-pericam expressing cells permeabilized with digitonin, thus allowing an unbiased control of cytosolic Na^+ concentration. Initially, MIN6 cells were superfused with Na^+ -free sucrose buffer, containing 10 μM buffered free Ca^{2+} and then the indicated Na^+ concentrations were added in a Ca^{2+}-free sucrose buffer. In the absence of Na^+, the rate of mitochondrial Ca^{2+} efflux was negligible, consistent with the dominant role of Na^+ dependent exchange in mitochondrial Ca^{2+} efflux (Prentki et al., 1983). An incremental rise in cytosolic Na^+ concentration was followed by a faster rate of mitochondrial Ca^{2+} efflux that eventually reached saturation (Fig. 2E). The calculated apparent K_M for mitochondrial Ca^{2+} efflux was ~12 mM, a value slightly higher than the 8mM reported for the purified and reconstituted exchanger (Paucek and Jaburek, 2004) (Fig. 2F).

Cumulatively, these results indicate that cytosolic Na^+ rises are followed by mitochondrial Na^+ uptake, largely mediated by the mitochondrial Na^+/Ca^{2+} exchanger, NCLX. Our results further suggest that the affinity of the mitochondrial Na^+/Ca^{2+} exchanger to Na^+ is tuned to sense the glucose-dependent cytosolic Na^+ changes.

Glucose-dependent cytosolic Na^+ entry via TTX-sensitive Na^+ channels shapes the cytosolic and mitochondrial Ca^{2+} transients

We next asked if the activation of Na^+ channels participates in cytosolic and mitochondrial Ca^{2+} signaling. Cytosolic Ca^{2+} was assessed in MIN6 cells loaded with Fura-2 AM. Consistent with previous studies (Kennedy et al., 1996), the application of high glucose was followed by a 14 ± 5 fold rise in rate and 1.15 ± 0.5 fold rise in amplitude, compared to low glucose Ca^{2+} response (Fig. 3A). In contrast, in cells treated with TTX, and consistent with previous studies (Dunne et al., 1990), the cytosolic Ca^{2+} signal was reduced in rate and amplitude by 2.0 ± 0.7 and 1.09 ± 0.3 fold, respectively, compared to untreated cells (Fig. 3C,D).

We next sought to determine the role of the TTX sensitive Na^+ pathways in controlling mitochondrial Ca^{2+} influx and efflux, using cells expressing the ratiometric mitochondrial targeted Ca^{2+} sensor, mito-pericam (pubmed/12874292). Consistent with previous studies (Nita et al., 2012), the application of glucose was followed by a robust mitochondrial Ca^{2+} influx and efflux (Fig. 3B). In the presence of TTX, however, mitochondrial Ca^{2+} influx was reduced by 3.4 ± 0.6 fold compared to untreated cells (Fig. 3E). Thus, the increase in the glucose-dependent cytosolic Ca^{2+} rise triggered by the TTX-sensitive pathways is followed by enhanced mitochondrial Ca^{2+} uptake. Remarkably, a much greater effect of TTX was observed on mitochondrial Ca^{2+} efflux that was reduced by 17 ± 0.2 fold compared to TTX-untreated cells (Fig. 3F) and thus virtually eliminated.

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We next reasoned that the glucose-dependent cytosolic Ca^{2+} rise might be facilitated by the activation of the voltage-gated Na^{+} channels by promoting plasma membrane depolarization. This question was examined by electrophysiological analysis conducted on primary mouse β cells by monitoring plasma membrane potential (Vm) using perforated patch clamp in current clamp mode (Tarasov et al., 2006). Application of high glucose generated a train of action potentials (APs) with an average frequency of 143 APs/min, originating at -51 ± 6 mV and reaching up to -11 ± 3 mV (n=6). Addition of TTX (500 nM) was initially followed by periods of electrical quiescence or substantially slower action potentials occurring at irregular intervals (54 ± 11 APs/min) (Fig. 3G,H). Of six cells analysed in detail, prolonged application of TTX (>8 min) resulted in a strong (87%) reduction of action potential rate (12 ± 4 APs/min; $p < 0.05$, n=4) and even a complete block of AP firing in two cases.

Altogether, the results of the above analysis suggest that the cytosolic Ca^{2+} rise is facilitating by enhanced depolarization mediated by TTX-sensitive Na^{+} channels. These findings further show that the cytosolic Ca^{2+} and Na^{+} rise induced by the TTX-sensitive Na^{+} pathway has a dual effect of first increasing mitochondrial Ca^{2+} influx followed by a Na^{+}-dependent acceleration of mitochondrial Ca^{2+} efflux.

TTX-sensitive Na^{+} channel activity is required for both mitochondrial Ca^{2+} influx by MCU and Na^{+} dependent Ca^{2+} efflux through NCLX

The above results indicate that Ca^{2+} and Na^{+} responses upregulated by TTX-sensitive Na^{+} channels are required to trigger and control mitochondrial Ca^{2+} transients respectively. We therefore asked next which mitochondrial transporters were linked to the influx and efflux of Ca^{2+} regulated by the TTX-sensitive Na^{+} pathway. We first focussed on MCU {destefani}{Baughmann} given that this emerges as the molecular moiety likely to correspond to the Ca^{2+} uniporter in β {Tarasov,2012}{Tarasov,2013}{Alam,Graier,2012} and other cells by comparing the rate of mitochondrial Ca^{2+} influx in siRNA Control vs. siRNA MCU transfected cells and co-transfected with mito-pericam. Knockdown of MCU expression was followed by a strong inhibition of mitochondrial Ca^{2+} influx rate by 5 ± 0.3 fold compared to control indicating that enhanced TTX-dependent mitochondrial Ca^{2+} influx is mediated by MCU (Fig. 4A,B,C). We then examined the rate of Ca^{2+} efflux. Notably, despite the decreased rate in Ca^{2+} influx, the rate of Ca^{2+} efflux remained unaffected in MCU siRNA-transfected cells. The application of TTX, however, abolished mitochondrial Ca^{2+} efflux indicating that, consistent with the data shown in Fig. 3, the cytosolic Na^{+} rise triggered by the TTX pathway is independently required for activation of NCLX, thereby for mitochondrial Ca^{2+} efflux. We then asked if TTX dependent mitochondrial Ca^{2+} influx via MCU is linked to glucose dependent ATP production by comparing ATP levels in siRNA Control vs siRNA MCU transfected MIN6 cells and co-transfected with the AT 1.03, an intracellular ATP indicator. The knock down of MCU expression was followed by a strong inhibition of glucose dependent ATP production (Fig. 4D). A similar decrease in ATP was shown in siRNA MCU transfected cells and treated with TTX, indicating that TTX dependent cytosolic Ca^{2+} rise was transmitted to the mitochondria and increased glucose-dependent ATP production.

Taken together, the results of the analysis above suggest that, by enhancing cytosolic Ca^{2+} responses, TTX-sensitive Na^+ channels increase mitochondrial Ca^{2+} influx via MCU and, by augmenting cytosolic Na^+ influx, control mitochondrial Ca^{2+} efflux by activating NCLX. In addition, the TTX-sensitive pathway regulates ATP production by increasing mitochondrial Ca^{2+} influx through MCU.

TTX sensitive Na^+ channels upregulate mitochondrial metabolism, membrane potential and ATP production

Considering the major effect of TTX in shaping mitochondrial Ca^{2+} influx (see above), and the role of intra-mitochondrial Ca^{2+} in the activation of key mitochondrial dehydrogenases (Rutter, 1990), we next sought to determine the general effect of the TTX-sensitive pathway on mitochondrial membrane potential, metabolism and ATP synthesis. Mitochondrial metabolism was determined by monitoring changes in NAD(P)H-dependent autofluorescence in pancreatic primary islets cells. Consistent with previous studies (Nita et al., 2012), application of high glucose triggered a robust rise in intrinsic NAD(P)H fluorescence. Conversely, in the presence of TTX, the formation of NAD(P)H was strongly decreased (Fig. 5A). We further monitored the changes in mitochondrial membrane potential in MIN6 cells loaded with the mitochondrial membrane potential sensitive dye tetramethylrhodamine methylester (TMRM, 0.05 μM). Consistent with previous studies (Akhmedov et al., 2010), application of high glucose trigered mitochondrial hyperpolarization that was reduced in cells treated by TTX (Fig. 5B).

Glucose-dependent ATP production was next measured in MIN6 cells transfected with AT 1.03. Application of high glucose triggered a corresponding rise in fluorescence, consistent with a rise in ATP production. Notably, the rise in ATP production triggered by high glucose was largely inhibited in cells that were superfused with high glucose in the presence of TTX (Fig. 5C).

This set of results suggests that TTX-sensitive Na^+ channels participate in glucose-dependent mitochondrial hyperpolarization, accelerated metabolism and enhanced ATP production in β cells.

Li^+ regulates the cytosolic and mitochondrial Ca^{2+}transients

A unique feature of the mitochondrial exchanger NCLX is that it is non selective for Na^+ and Li^+ while the plasma membrane NCX is highly selective for Na^+ (ref) Thus, the use of Li^+ provides an effective control to investigate the Na^+ channel-mitochondrial axis and distinguish between a role of NCX and NCLX. We initially compared the glucose dependent Ca^{2+} response in MIN6 cells loaded with Fura 2 AM that were superfused with either Na^+, Li^+ or N-methyl D-glucamine (NMDG)-containing Ringer's solution. A similar increase in rate (10 ± 5 fold) and amplitude (1.14 ± 0.5 fold) of glucose-dependent Ca^{2+} increase was monitored in cells exposed to either Na^+ or Li^+. In contrast, in the presence of NMDG, the rate and amplitude of cytosolic Ca^{2+} change were reduced by 3.3 ± 0.3 and 1.1 ± 0.2 fold, respectively compared to cells exposed to Na^+ (Fig. 6A,C,D). In addition, the inclusion of either Na^+ or Li^+ led to a similar recovery of the mitochondrial Ca^{2+} transients. In constrast,

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when both ions were replaced by NMDG, mitochondrial Ca^{2+} transients were inhibited by 4 ± 0.3 and 18 ± 0.4 fold, compared to the presence of Na^{+} (Fig. 6B, E, F).

Thus, our results indicate that Li^{+}, but not NMDG, can fully rescue the Na^{+} -dependent cytosolic and mitochondrial Ca^{2+} response, identifying NCLX as the exchanger responsible.

To determine the distinct role of NCLX in mediating the cytosolic and mitochondrial effects of Li^{+}, we applied the same experimental paradigm described in Fig. 6A, controling NCLX expression by using siRNA NCLX vs. siRNA Control. Knock down of NCLX expression resulted in a 3.0 ± 0.2 fold decrease in the rate of glucose-dependent cytosolic Ca^{2+} increase, compared to the siRNA Control rate (Fig 6G,I,J). Note, however, that the amplitude of the cytosolic Ca^{2+} response was unaffected.

To determine if NCLX mediates Li^{+}/Ca^{2+} exchange in pancreatic β cells we next compared the Li^{+}-dependent mitochondrial Ca^{2+} responses in cells co-transfected with either siRNA NCLX or siRNA Control and mito-pericam. Intense mitochondrial Ca^{2+} influx was monitotred in siRNA NCLX-expressing cells superfused with high glucose (20mM) Ringer's solution. In contrast, the mitochondrial Ca^{2+} efflux was strongly inhibited by 4 ± 0.5 folds compared to siRNA Control, when the expression of NCLX was knockdown by siRNA NCLX. Thus, Li^{+} failed to enhance mitochondrial Ca^{2+} efflux when the expression of NCLX was reduced. This finding is consisted with the essential role of NCLX in catalysing mitochondrial Ca^{2+} efflux and its capacity also to carry out Li^{+}/Ca^{2+} exchange (Fig. 6H,K,L).

Discussion

We sought here to explore the degree to which metabolic cross talk between the plasma membrane and mitochondria, mediated through cytosolic Ca^{2+} signaling, is required for glucose-dependent insulin secretion.

We show that a pathway involving TTX-sensitive Na^{+} channels leads to enhanced glucose-dependent cytosolic Na^{+} and Ca^{2+} responses followed by enhanced mitochondrial metabolic and Ca^{2+} signaling activity in β cells. In particular, we demonstrate that high glucose-dependent Na^{+} influx through Na^{+} channels contributes to cell depolarization and subsequently the rise in both cytosolic Na^{+} and Ca^{2+}. These Na^{+} and Ca^{2+} signals are then propagated into mitochondria. Calcium enters these organelles via the mitochondrial Ca^{2+} uniporter, MCU, while Na^{+} enters via the mitochondrial exchanger, NCLX, in exchange for Ca^{2+}. Importantly, it appears that the acceleration of mitochondrial Ca^{2+} uptake due to Na^{+} channel-dependent increases in cytosolic Ca^{2+}, and hence MCU activity, exceeds that of NCLX-mediated Ca^{2+} efflux such that the net effect is an increase in mitochondrial Ca^{2+} concentration. This, in turn, upregulates glucose-dependent mitochondrial metabolic activity and thereby ATP production (Scheme 1).

Previous comparisons of Na^{+} channel activity based on electrophysiological studies in β cells suggest that their density at the cell membrane reaches ~50% than that of mouse neocortical neurons (Dunne et al., 1990). However, while activation of neuronal Na^{+} channel is brief, lasting only a few milliseconds (Fleidervish et al., 1996), the much longer

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repolarization/depolarization cycles induced by glucose in β cells predicts a longer open dwell time and thus a higher influx of Na^+ into the cytoplasm through this channel.

Our results, showing a substantial increase in cytosolic Na^+, higher than those previously observed for example in axonal regions (Fleidervish et al., 2010), are consistent with this scenario in β cells. Thus, our findings indicate that TTX-sensitive Na^+ channels not only participate in the control of electrical activity in pancreatic mouse β cells, but can also trigger a glucose-dependent cytosolic Na^+ change.

Importantly, we also demonstrate that the glucose-dependent rise in cytosolic Na^+ is then followed by mitochondrial Na^+ uptake. Several mitochondrial pathways may account for this Na^+ uptake among them most notably the Na^+/H^+ exchanger and NCLX (Murphy and Eisner, 2009). We find the knockdown of NCLX expression leads to a strong inhibition of the glucose-dependent mitochondrial Na^+ response indicating that, in β cells, the mitochondrial Na^+/Ca^{2+} exchanger is the major mitochondrial Na^+ influx pathway under these conditions. In this respect, pancreatic β cells appear to be similar to cardiomyocytes in which the mitochondrial exchanger represents the major Na^+ influx pathway (Bernardinelli et al., 2006).

Our results further show that the cytosolic Na^+ rise triggered by Na^+ influx via TTX-sensitive Na^+ channels precedes, and is required for, the removal of mitochondrial Ca^{2+} by activating NCLX, thereby accelerating mitochondrial Ca^{2+} efflux and shuttling. Thus, for the mitochondria, the cytosolic Na^+ rise provides a self-inhibiting feedback loop aimed, on the one hand, to prevent a potentially toxic surge in mitochondrial Ca^{2+} (Rizzuto R. et al, 2012, Nature), (Duchen, 2000) but on the other a longer duration of mitochondrial Ca^{2+} increase. Recent studies have also identified an important link between mitochondrial Na^+ and H^+ homeostasis (Murphy and Eisner, 2009). Although beyond the scope of the present paper, our results in this context predict that the robust mitochondrial Na^+ influx mediated by NCLX may also participate in the pH homeostasis of the mitochondrial matrix by promoting H^+ influx by the mitochondrial Na^+/ H^+ exchanger.

Our results further suggest that the strong stimulatory effect of the cytosolic Na^+ rise on NCLX activity is related to the low affinity (K_M ~ 12mM) of NCLX towards cytosolic Na^+ and is consistent with previous studies on the cardiac mitochondrial exchanger (Paucek and Jaburek, 2004). Thus, NCLX is largely inactive at resting Na^+ concentrations, but it is strongly activated by a glucose-dependent cytosolic Na^+ rise. Therefore, the results indicate that the glucose-dependent cytosolic Na^+ rise is a signaling event that triggers the activation of NCLX and that this exchanger is tuned to sense and respond to cytosolic Na^+ transients triggered by glucose. Interestingly, previous studies (pubmed/12663468) in clonal INS1 β cells have suggested that inhibition of mitochondrial Na^+-Ca^{2+} exchange using the pharmacological agent CGP37157 leads to a potentiation of glucose-stimulated insulin secretion attributed to enhanced mitochondrial calcium accumulation. On the other hand, subsequent studies (pubmed/17719029) using primary mouse and human β cells revealed that this agent inhibited insulin secretion, findings consistent with those reported here, and with the view that mitochondrial Na^+-Ca^{2+} exchange is required to ensure normal fluxes of

Ca^{2+} across the mitochondrial membrane, and hence metabolism-dependent hormone release.

Our findings here do not exclude the possibility that transmembrane Na^+ gradients may also affect the activity of the plasma membrane NCX members. In particular, an inward shift in Na^+ gradient may promote the reversal of these exchangers and a rise of Ca^{2+} (Herchuelz et al., 2013). We find that Li^+, a substrate cation of NCLX but not of NCX or NCKX (Palty et al., 2004), can fully replace Na^+ in triggering the cytosolic and mitochondrial Ca^{2+} responses. Thus, and consistent with previous studies (Gall and Susa, 1999)(Van Eylen et al., 2002), our results do not show a strong NCX-dependent change in rodent pancreatic β cells. Our results utilizing Li^+, while controlling the expression of NCLX, further indicate that the mitochondrial Na^+/Ca^{2+} exchanger maintains mitochondrial Ca^{2+} shuttling and are thereby consistent with previous studies increase the rate of the glucose dependent cytosolic Ca^{2+} signal (Nita et al, 2012) [the meaning of this sentence is a little unclear to me.].

Consistent with previous studies in a rat insulinoma-derived β cell line (Dunne et al., 1990), our electrophysiological analysis indicated that, by maintaining the frequency of action potential firing, TTX-sensitive Na+ channels increase the magnitude of cytosolic Ca^{2+} influx into primary mouse β cells. An important conclusion of this study is that the substantial rise in cytosolic free Ca^{2+} triggered by TTX-sensitive Na^+ channels is followed by a rise in mitochondrial Ca^{2+}. These results, consistent with our previous studies (Tarasov et al., 2012), show that mitochondrial Ca^{2+} uptake is mediated by MCU, and accelerates mitochondrial oxidative metabolism thereby promoting ATP production. Thus, voltage-sensitive Na^+ channels may promote cytosolic Ca^{2+} rise not only by direct depolarization but also by stimulating mitochondrial Ca^{2+} dependent ATP production, that further and independently contributes to the cytosolic Ca^{2+} rise by raising ATP and blocking K^+-ATP channels

Finally, we note that diabetes is associated with cardiovascular morbidity and is often treated with Na^+ channel blockers (). Our results suggesting a role of the Na^+ channel in glucose-dependent cytosolic and mitochondrial Ca^{2+} signaling in β cells, albeit at a cellular level nevertheless warrants that therapeutically used Na^+ channels blockers may have an effect on β cell secretory processes that warrants further testing in vivo models or through epidemiological studies in man.

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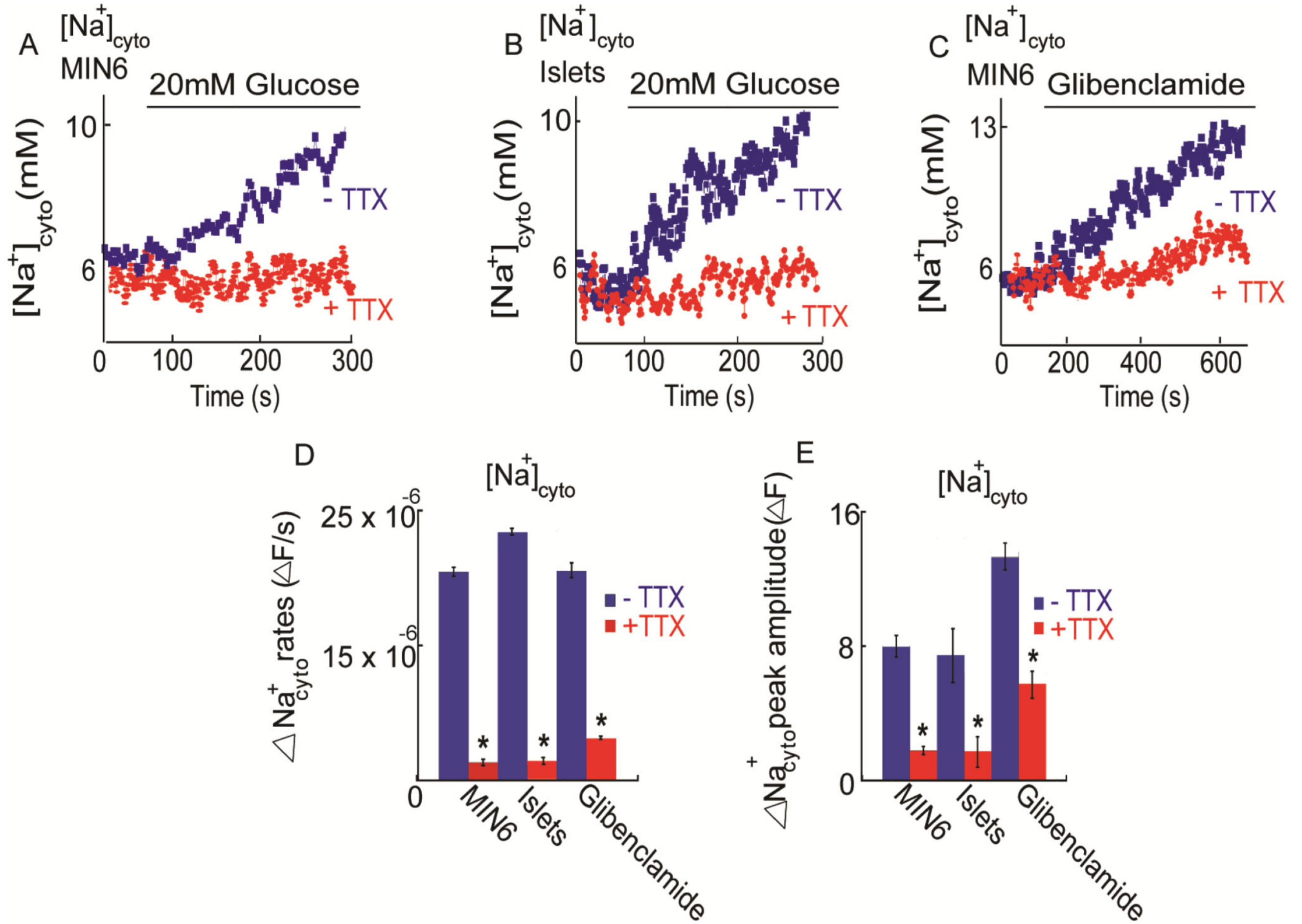

Figure 1. Glucose triggers cytosolic Na^+ influx via TTX-sensitive Na^+ channels preceeded by K-ATP channel closure.

Fluorescent traces of cytosolic Na^+ responses in pancreatic β cells loaded with CoroNaGreen. **A.** MIN6 and **B.** pancreatic primary islets cells were superfused with low (3mM) glucose followed by high (20mM) glucose Ringer’s solution in the presence or absence of TTX. **C.** MIN6 cells were superfused with glibenclamide-containing Ringer's solution in the presence or absence of TTX (?nM). **D.** Mean rates of glucose-dependent cytosolic Na^+ rise of Fig. 1 A, B, C, n=6 (*P<0.05). **E.** Mean amplitudes of glucose-dependent cytosolic Na^+ rise from panels A, B, C, n=6 (*P<0.05).

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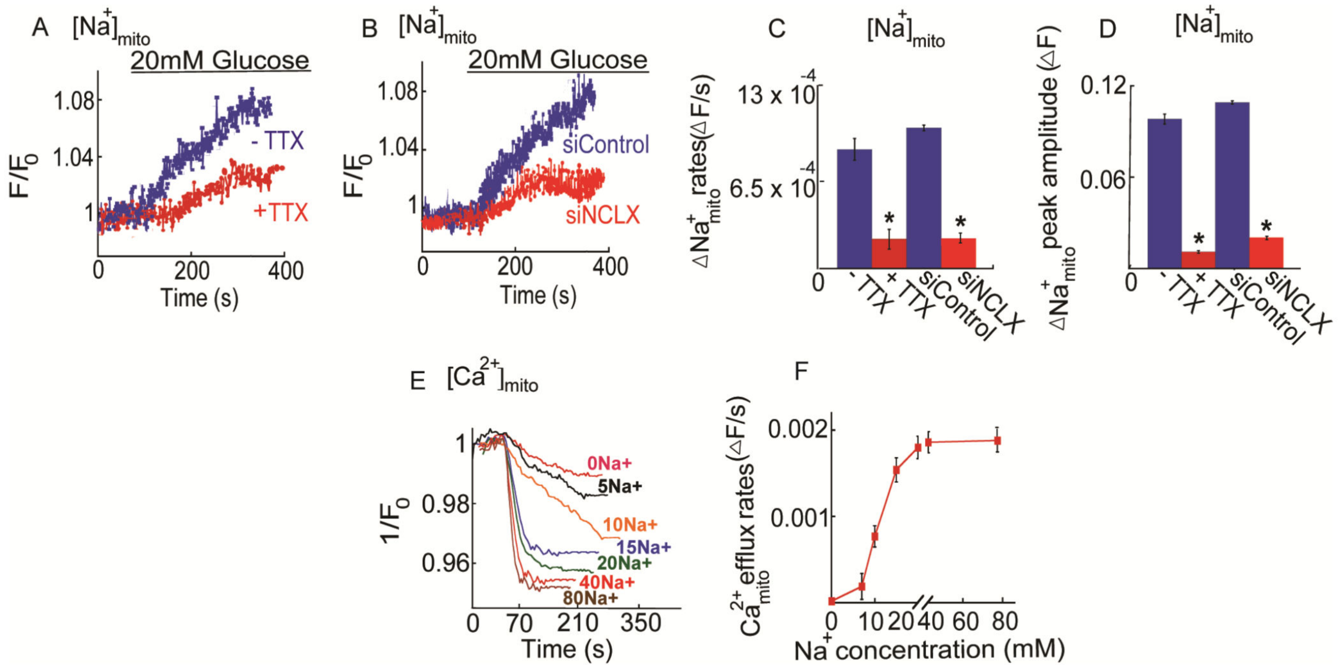

Figure 2. Glucose-dependent cytosolic Na^+ rises are followed by mitochondrial Na^+ uptake mediated by NCLX.

A. Fluorescent traces of mitochondrial Na^+ response in MIN6 cells loaded with CoroNa Red as described in Fig. 1A and superfused with or without TTX. **B.** Fluorescent traces of glucose dependent mitochondrial Na^+ responses in MIN6 cells transfected with siRNA NCLX vs. siRNA Control. **C.** Averaged rates of glucose dependent mitochondrial Na^+ influx of Fig. 2A, B, n=6 (*P<0.05). **D.** Averaged amplitudes of glucose dependent mitochondrial Na^+ influx of Fig. 2A, B, n=6 (*P<0.05). **E.** Affinity of NCLX to Na^+. Fluorescent traces of mitochondrial Ca^{2+} efflux in MIN6 cells transfected with mito-pericam, permeabilized by digitonin and superfused at the indicated Na^+ concentrations. **F.** Ca^{2+} efflux rates vs. Na^+ concentrations, from Fig. 2E. The fitted IC 50 value is 12 mM Na^+.

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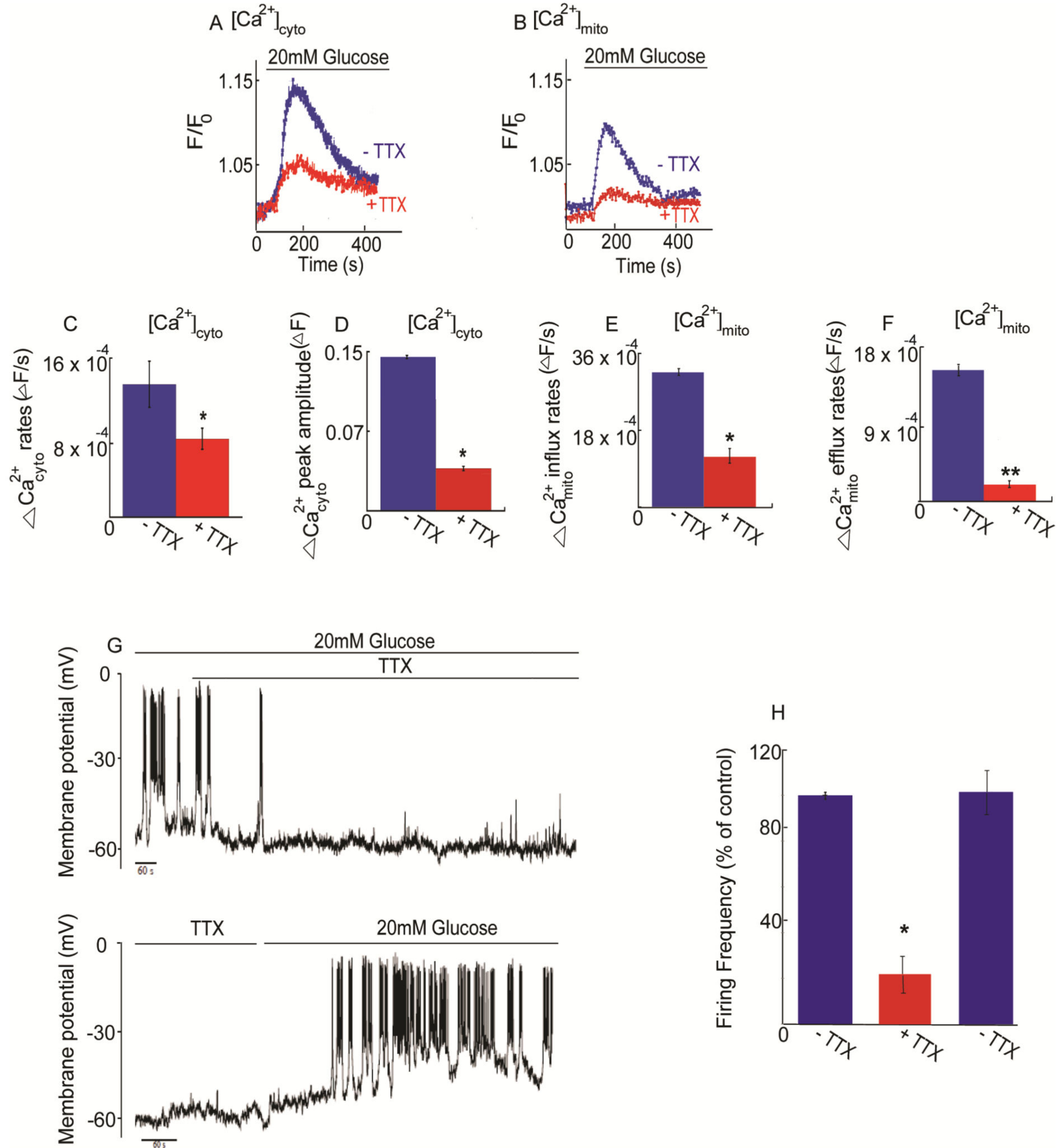

Figure 3. A TTX-sensitive Na^{+} pathway controls glucose-dependent cytosolic and mitochondrial Ca^{2+} responses.

A. Fluorescent traces of cytosolic Ca^{2+} responses in MIN6 cells loaded with Fura 2AM as described in Fig 1A, in the presence or absence of TTX. **B.** Fluorescent traces of mitochondrial Ca^{2+} responses in MIN6 cells transfected with mito-pericam and following the high glucose paradigm as described in Fig 1A, in the presence or absence of TTX **C.** Averaged rates of glucose dependent cytosolic Ca^{2+} response of Fig. 3A, n=7 (*P>0.05). **D.** Averaged amplitudes of glucose dependent cytosolic Ca^{2+} response of Fig. 3A, n=8

(*P>0.05). **E.** Averaged rates of glucose dependent mitochondrial Ca^{2+} influx of Fig. 3B, n = 7 (*P>0.05). **F.** Averaged rates of glucose dependent mitochondrial Ca^{2+} efflux of Fig. 3B, n = 7 (*P>0.05).**G.** The action of TTX on glucose-induced changes in membrane potential and electrical activity in primary mouse β cells. In an isolated β cell, substantial slowing of glucose-induced activity was produced by bath application of TTX (500 nM). A maximal inhibitory effect on membrane potential was achieved after prolonged application of the drug (>8 min). Electrical activity was restored within 2-3 min upon washout of TTX. **H.** Averaged rates of firing frequency in pancreatic primary islets of Fig. 3G, n = 6 (*P>0.05).

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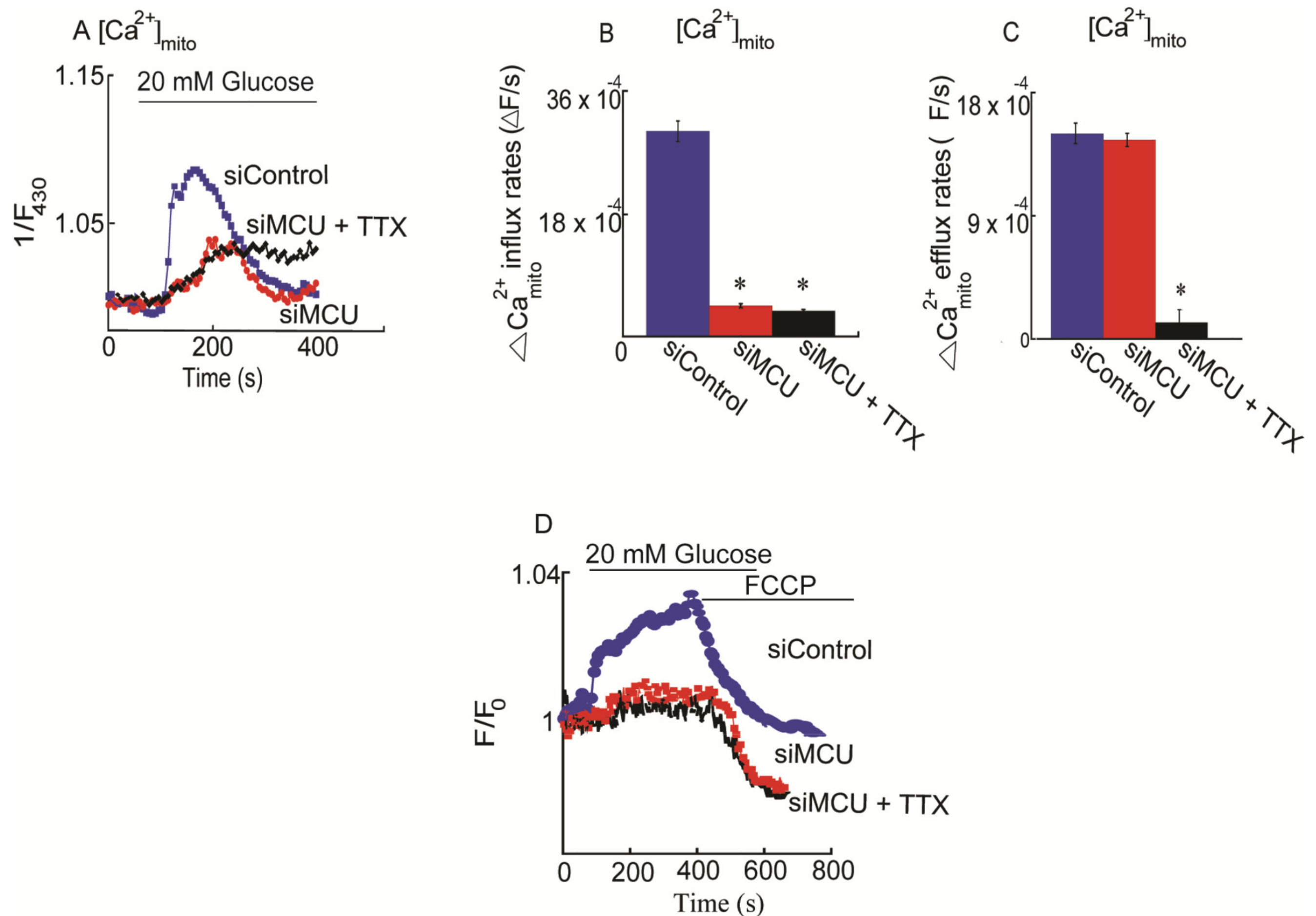

Figure 4. TTX sensitive Na$^+$ channels control the mitochondrial Ca^{2+} influx via MCU and Ca^{2+} efflux by the mitochondrial Na$^+$/Ca^{2+} exchanger.

A. Fluorescent traces of mitochondrial Ca^{2+} transients recorded in pancreatic β cells co-transfected with siRNA MCU vs. siRNA Control and mito-pericam, following the high glucose paradigm. **B.** Averaged rates of glucose dependent Ca^{2+} influx of Fig. 4A, n=8 (*P<0.05). **C.** Averaged rates of glucose dependent Ca^{2+} efflux of Fig. 4A, n=8 (*P<0.05). **D.** Fluorescent traces of ATP measurements in pancreatic β cells superfused with high glucose in presence or absesnce of TTX and co-transfected with siRNA MCU vs. siRNA Control and AT 1.03, ATP probe. The ATP measurments were calibrated by adding FCCP as indicated.

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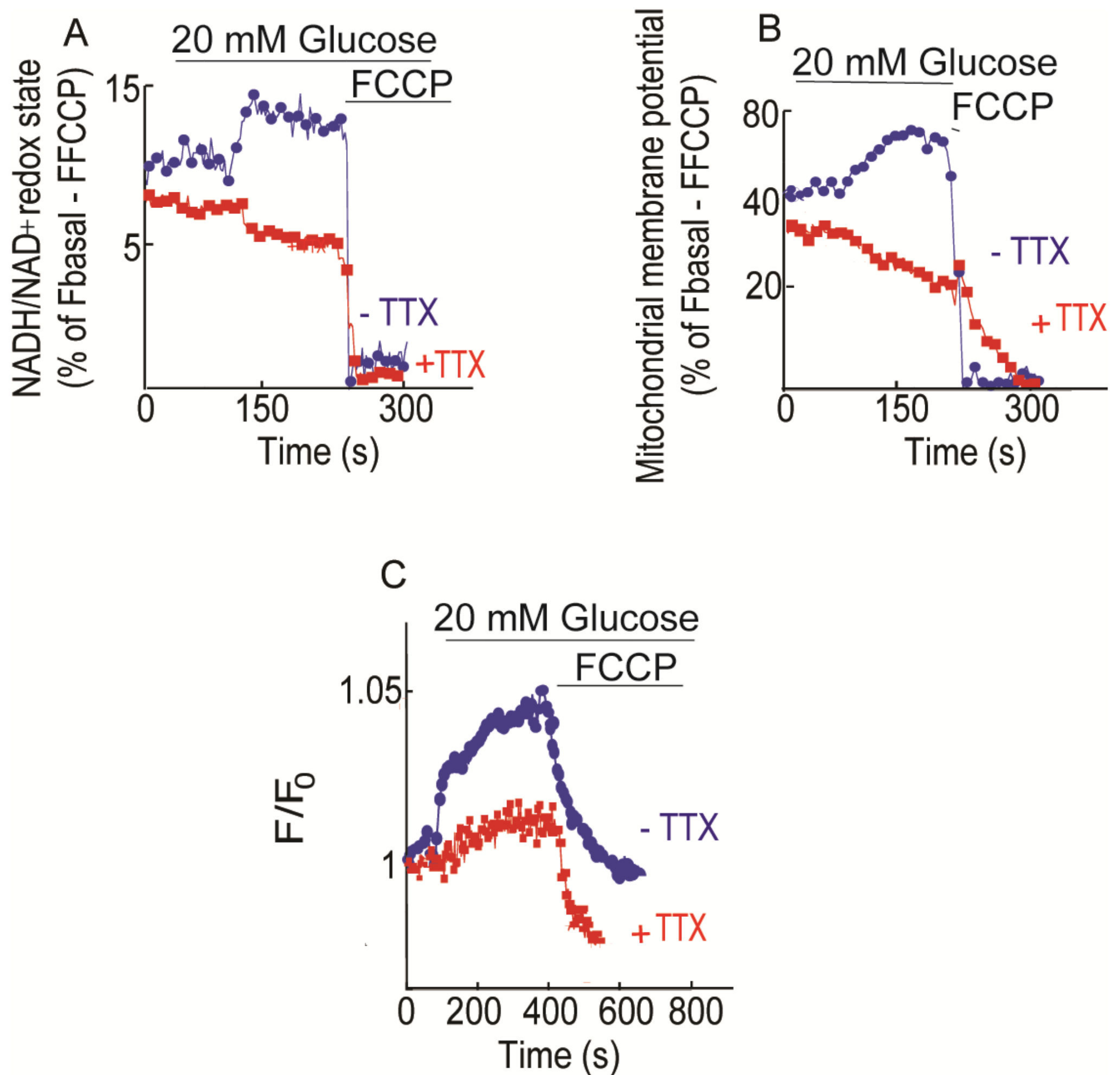

Figure 5. TTX significantly reduces the metabolic rate, mitochondrial membrane potential and ATP production.

A. Respiratory chain activity was determined by monitoring NAD(P)H intrinsic fluorescence in pancreatic primary islets cells. **B.** Fluorescent traces of mitochondrial membrane potential determined in MIN6 cells loaded with TMRM. The pancreatic β cells were superfused with high (20mM) glucose Na^+ full Ringer's solution in presence or absence of TTX. **C.** Fluorescent traces of ATP level monitored in pancreatic β cells transfected with AT 1.03. FCCP was added at the end of experiment as indicated.

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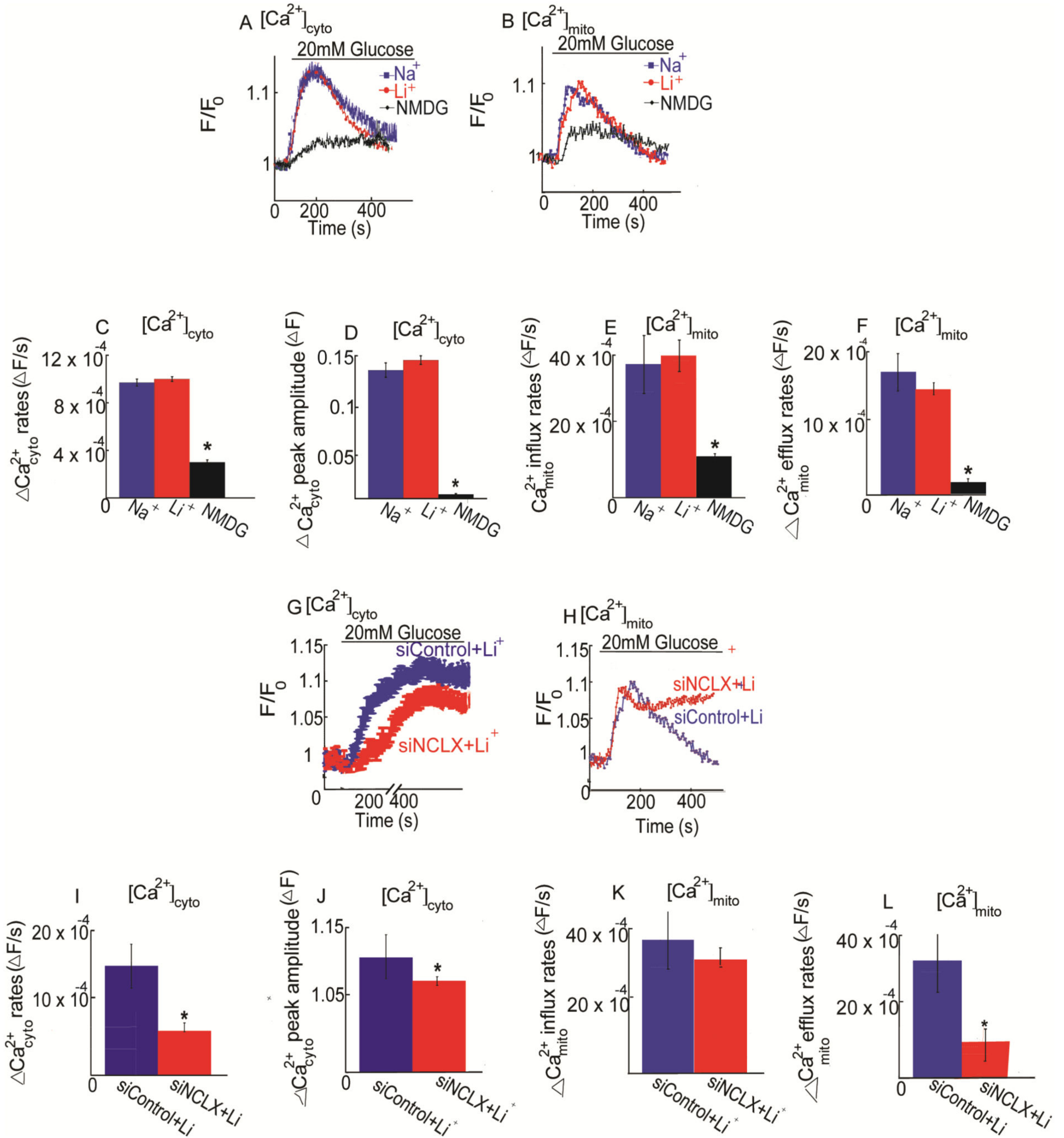

Figure 6. Li+ regulates the glucose dependent cytosolic and mitochondrial Ca^{2+} transients.

A. Fluorescent traces of Na^+, Li^+ or NMDG dependent cytosolic Ca^{2+} response in MIN6 cells loaded with Fura 2AM, following the same glucose paradigm described in Fig. 1A. **B.** Fluorescent traces of Na^+, Li^+ or NMDG dependent mitochondrial Ca^{2+} transients in MIN6 cells transfected with mito-pericam. **C.** Averaged rates of glucose dependent cytosolic Ca^{2+} response of Fig. 6A, n=8 (*P<0.05). **D.** Averaged amplitudes of glucose dependent cytosolic Ca^{2+} response of Fig. 6A, n=8 (*P<0.05). **E.** Averaged rates of glucose dependent Ca^{2+} influx of Fig. 6B, n=10 (*P<0.05). **F.** Averaged rates of glucose dependent Ca^{2+} efflux of

Fig. 6B, n=10 (*P<0.05). **G.** Fluorescent traces of Li^+ dependent cytosolic Ca^{2+} response in MIN6 cells loaded with Fura 2AM and transfected with siRNA NCLX vs. siRNA Control. **H.** Fluorescent traces of Li^+ dependent mitochondrial Ca^{2+} transients in MIN6 cells co-transfected with mito - pericam and siRNA NCLX vs. siRNA Control following application of high (20mM) glucose Ringer's solution. **I.** Averaged rates of glucose dependent cytosolic Ca^{2+} response of Fig. 6G, n=6 (*P<0.05). **J.** Averaged amplitudes of glucose dependent cytosolic Ca^{2+} response of Fig. 6G, n=6 (*P<0.05). **K.** Averaged rates of glucose dependent mitochondrial Ca^{2+} influx of Fig. 6H, n=10 (*P<0.05). **L.** Averaged rates of glucose dependent mitochondrial Ca^{2+} efflux of Fig. 6H, n=10 (*P<0.05).

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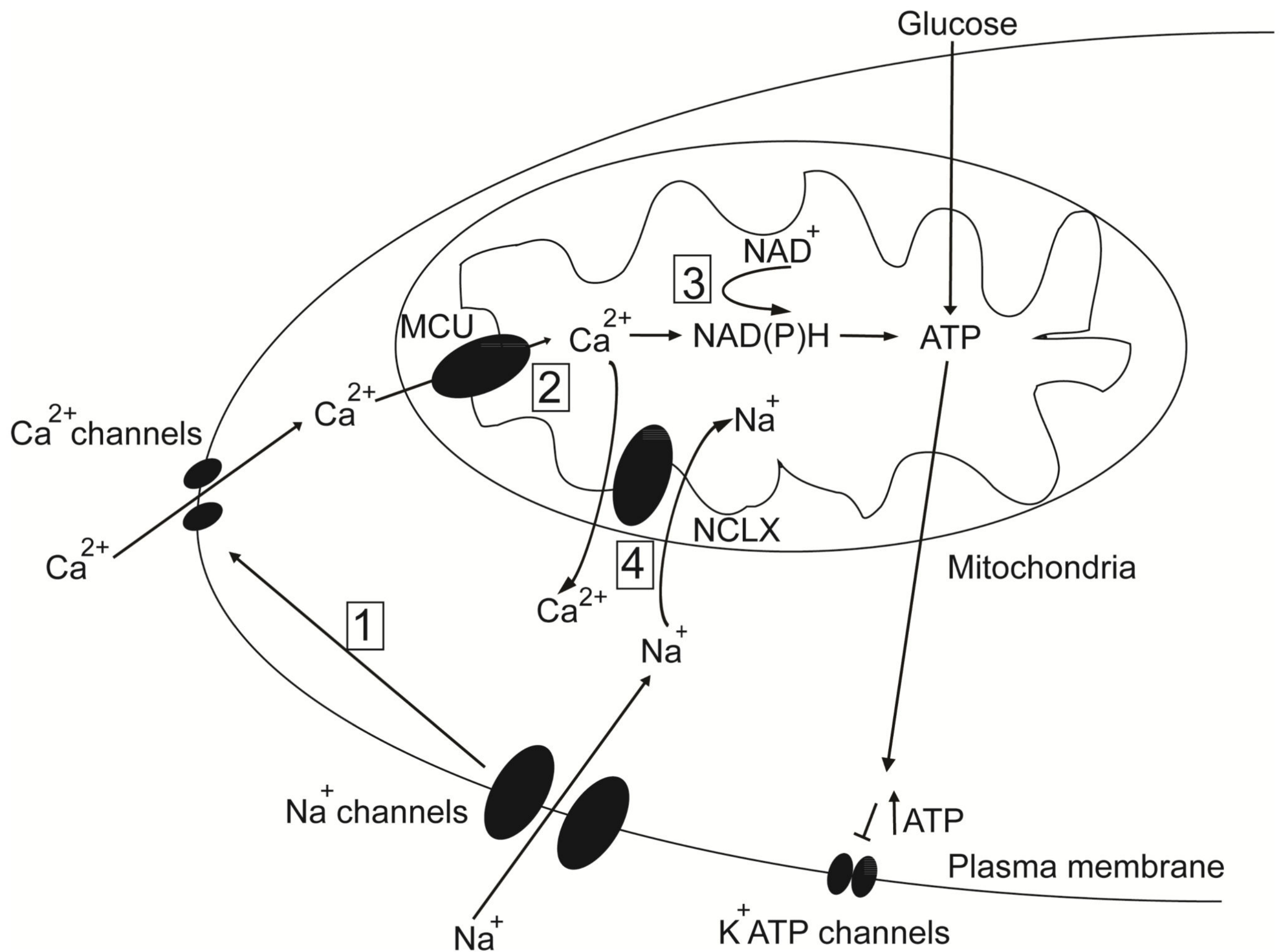

Scheme 1.

Cross talk between Na^+ channels and mitochondria in β cells. (1) The Na^+ flow through TTX Na^+ channels triggers further depolarization and rise in cytosolic Na^+ and enhance in Ca^{2+} responses. These Na^+ and Ca^{2+} responses are propagating to mitochondria. (2) Ca^{2+} enters through the Ca^{2+} uniporter MCU and (3) enhances metabolic rate while (4) Na^+ is required for mitochondrial Ca^{2+} efflux via NCLX.

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