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A new mitochondrial pH biosensor for quantitative assessment of pancreatic β-cell function

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ABSTRACT

Mitochondrial pH is a key determinant of mitochondrial energy metabolism. We have developed a new fluorescence-based ratiometric pH biosensor using a chloride-insensitive and hydrogen-sensitive probe for direct, quantitative and bleaching-free measurement in a living cell. Fusing this biosensor with a mitochondrial localization signal (MTpHGV) allowed us to determine mitochondrial pH. This new system was applied to measure mitochondrial pH in pancreatic β-cells, in which mitochondrial function plays a pivotal role in insulin secretion. Rat INS1 cells and mouse MIN6 cells are transfected with MTpHGV stably to monitor mitochondrial pH. While carbonyl cyanide 3-chlorophenylhydrazone (CCCP) treatment rapidly decreased mitochondrial pH in cultured rat MTpHGV-INS-1 cells, MTpHGV-MIN6 cells showed a rapid increase. These data suggest that MTpHGV probe exist in matrix side in INS-1 cells, but on the outer side of mitochondrial inner membrane in MIN6 cells. Moreover, while MTpHGV-INS-1 cells showed a rapid increase of pH by glucose stimulation, mitochondrial pH decreased quickly by glucose stimulation in all MTpHGV-MIN6 cells examined and recovered smoothly. Perfusion study of glucose load in MTpHGV-MIN6 cells under aminooxyacetate (AOA) or 100 μM diazoxide showed that this mitochondrial pH acidification was dependent on nicotinamide adenine dinucleotide (NADH) shuttle, but independent from KATP channel. This new system for measuring mitochondrial pH is sensitive across the range of physiologic conditions and may be a useful tool for evaluating mitochondrial function in living cells.

1. Introduction

Mitochondrial pH reflects the functional viability of the organelle [1,2]. Non-ratiometric pH monitoring methods do not allow precise quantitative pH measurement due to artifacts caused by poor expression, cell movement and optical focusing. A ratiometric measurement using the coexpression of pH-sensitive and -insensitive green fluorescent protein (GFP) mutants should be useful [3]. However, problems remain with a dual-probe system including variable expression levels, poor targeting or uneven distribution of two probes. To overcome such limitations, we have generated a novel ratiometric pH indicator by linking a pH-sensitive yellow fluorescent protein (YFP) mutant and a less pH-sensitive GFP mutant in a single probe that allows both dual excitation/single emission and single excitation/dual emission measurements.

We have previously reported a pH sensor system using YFP and GFP that indicates pH of intercellular organelles [4]. The accuracy of measuring pH in this system, however, is compromised due to its chloride sensitivity. The Venus is a derivative of YFP, whose emission is not influenced by chloride or hydrogenions [5]. We therefore constructed a series of mutant proteins based on the Venus and identified H148 V that was hydrogen ion-sensitive with a pKa of pH 7.2–8.4. The H148 V mutational venus was fused to GFPuv, a GFP variant, which reported to be hydrogen insensitive, to generate a new pH biosensor, pHGV. Using this new pH biosensor, accurate and long time pH monitoring of the mitochoncria is made possible. Here we show the application of this probe (MTpHGV) to measure mitochondrial pH in insulin-secreting β-cell lines under various physiologic conditions.

2. Materials and methods

2.1. MTpHGV construct

A mutation was introduced at H148 of the Venus fluorescent protein (Invitrogen, Carlsbad, CA), and the selected mutant, H148 V was fused to GFPuv (ClontechTakaraBio, Mountain View, CA) with a cytochrome-C subclass IV localization signaling

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molecule (MTpHGV), then stably transfected into Chinese hamster ovary (CHO) cells (Invitrogen) INS-1 [6] and MIN6 cells [7] using Lipofectamine (Invitrogen).

2.2. Spectroscopy and pH titration in vitro

A pGEX-4T fused fluorescent protein probe vector was made for in vitro fluorescence spectra analysis. Fluorescence spectra of purified cell-free pGEX–Venus or pGEX–pHGV were recorded using a spectrophotometer (F-2500 Hitachi Tokyo Japan). Purified protein probes (3–10 mg/ml) were dissolved in solution A containing (in mM): 120 KCl, 5 NaCl, 0.5 CaCl2, 0.5 MgSO4, 10 MES, 10 MOPS, 10 citrate, pH 8.0.

2.3. Cultured-cell lines

MIN6 cells were first selected based on Ca^{2+} activity in response to glucose-induced insulin secretion at 25 mM Glucose. Then the selected colony was expanded and divided for experiments described below. COS-7 (Riken BRC, Ibaraki Japan), CHO-K1, and INS-1 cells cultured on glass coverslips were transfected with MTpHGV using Fu-Gene6 (Roche Diagnostics, Indianapolis, IN). MIN6 cells were transfected with MTpHGV using the Nucleofectom system (Amaxa Co Ltd., Koeln, Germany) and cultured on glass coverslips. COS-7 cells expressing MTpHGV were used for experiments 2 to 4 days later. CHO-K1 (MTpHGV-CHO), INS-1 (MTpHGV-INS-1), and MIN6 (MTpHGV-MIN6) cells that stably expressed MTpHGV were selected using blastocydin S (Gibco BRL, Rockville, MD) and cultured in dishes for 2 months.

2.4. Fluorescent imaging

Fluorescence images of transient MTpHGV-transfected COS-7 cells and stably transfected CHO-K1, INS-1 and MIN6 cells were obtained using an LSM510 confocal microscope under excitation wavelengths of 380 nm and 488 nm. Mitochondrial pH was quantified serially in accordance with a standard curve for the purified biosensor during the stimulation of insulin secretion using glucose or arginine.

2.5. Mitochondrial morphology and immunoelectron microscopy

Subcellular localization of MTpHGV in transfected INS-1 and MIN6 cells was determined incubated with 10 nM Mito Tracker Red (Invitrogen), a specific mitochondrial dye, for 30 min at 37 °C. Mitochondrial morphology was observed with a digital imaging system using a confocal microscope with an excitation wavelength of 602 nm.

2.6. Immunoelectron microscopy

INS-1 cells stably transfected with MTpHGV were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 50 mM sodium carbohydrate buffer (pH 7.2). Cells were incubated with 1 ng/ml anti-GFP polyclonal antibody (Medical & Biologic Laboratories Co. Ltd., Tokyo, Japan) overnight at 4 °C. Samples were then stained with 2% uranyl acetate and antibodyanti-IgG(H + L)goat, rabbit-polyclonal antibody (Gold 10 nm, EM:Seracare Life Sciences, Milford, MA) for 2 h at 4 °C.

2.7. Measurement of mitochondrial pH, cytoplasm pH and Ca^{2+}signals

Fluorescent signals were observed using a 60× objective with a microscope (IX 71, Olympus, Tokyo, Japan), equipped with an image intensifier charged-coupled device camera (ORCA-ER, Hamamatsu, Shizuoka, Japan). Interference filters (Omega Optical, Brattleboro, VT) of 380 ± 10 and 480 ± 10 nm were used for dual excitation and 520 ± 20 nm for single emission. A dichroic mirror used was 505 DCLP (Omega Optical). Using Meta Flow, an image processor (Meta Flow, Tokyo Japan), regions of interest were manually selected and pixel intensities were spatially averaged after background subtraction. The fluorescence ratio was calculated in a pixel-to-pixel manner. In some experiments, cells were observed using a laser scanning confocal microscope (FV1000-D, Olympus, Tokyo, Japan). MTpHGV-INS1 and MTpHGV-MIN6 cells were incubated with 2 μM Fura-2AM (Dojindo Co. Ltd., Kumamoto, Japan) and Ca^{2+} signals were calculated as previously reported [8]. MTpHGV-MIN6 cells were incubated for 45 min with 1 μM BCECF-AM (Invitrogen) with 0.0025% F127 added at room temperature. Cytoplasmic pH was assessed based on the fluorescence ratio at 440 and 490 nm. Since a part of the fluorescence spectrum of BCECF overlapped with that of MTpHGV, BCECF fluorescence was measured in portions of the nucleus or cytoplasm without MTpHGV fluorescence.

3. Results

3.1. Generation of a biosensor MTpHGV

We have generated a biosensor that accurately monitors mitochondrial pH in β cell lines, INS-1 and MIN6 cells. Since mitochondrial pH in these cells is slightly acidic, it requires a probe with a lower pKa than those currently available [3]. We first constructed various variants of the Venus fluorescent protein by introducing mutations at H148 to identify a protein with a pKa between 7.0 and 8.0. We found that H148 V the most efficiently fit into the criteria. Changes in the relative fluorescence intensity along with pH for single amino acid mutations of H148H for each purified protein are shown in Fig. 1A. Venus-H148 V has a pKa around 7.0 and thus we fused this protein with GFP_{UV} for MTpHGV (Fig. 1B). Since fusing two fluorescence proteins is prone to introducing mutations, we added a silence mutation to the GFP_{UV} and fused with mutated Venus fluorescence protein. The probe can monitor mitochondrial pH (MTpHGV) by the use of a cytochrome C subunit IV localization signal of MtAlpHi [9]. Fluorescence intensity of MitpHGV-CHO has two distinct peaks with excitation at 380 nm and 490 nm. The peak induced by 400 nm excitation, which is near the peak excitation wavelength for GFP_{UV},is constant for pH 6.0–8.0. On the other hand, the peak induced by 490 nm excitation, which is near the peak excitation wavelength for Venus, shows a stepwise change of fluorescence intensity that was dependent on the pH of the medium. (Fig. 1B)

3.2. MTpHGV is localized in the inner mitochondrial membrane in transfected cells

MTpHGV was transfected to INS-1 and MIN6 cells. Expression of MTpHGV showed an essentially identical pattern comparable to mitochondria stained with Mito Tracker Red (Fig. 1C and D). Immunoelectron microscopy of transfected INS-1 cells using an anti-GFP antibody labeled the mitochondrial membrane, especially the cristae (Fig. 1E).

3.3. Fluorescence ratio from MTpHGV reports pH of the mitochondrial membrane

Ratios of fluorescence intensity obtained at 380 nm and 490 nm (F490/F380) were used to calculate mitochondrial pH based on responses of a purified biosensor (Fig. 1F). Basal mitochondrial pH was about 7.8 in MTpHGV-CHO cells ($n = 20$). Application of 1 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) induced

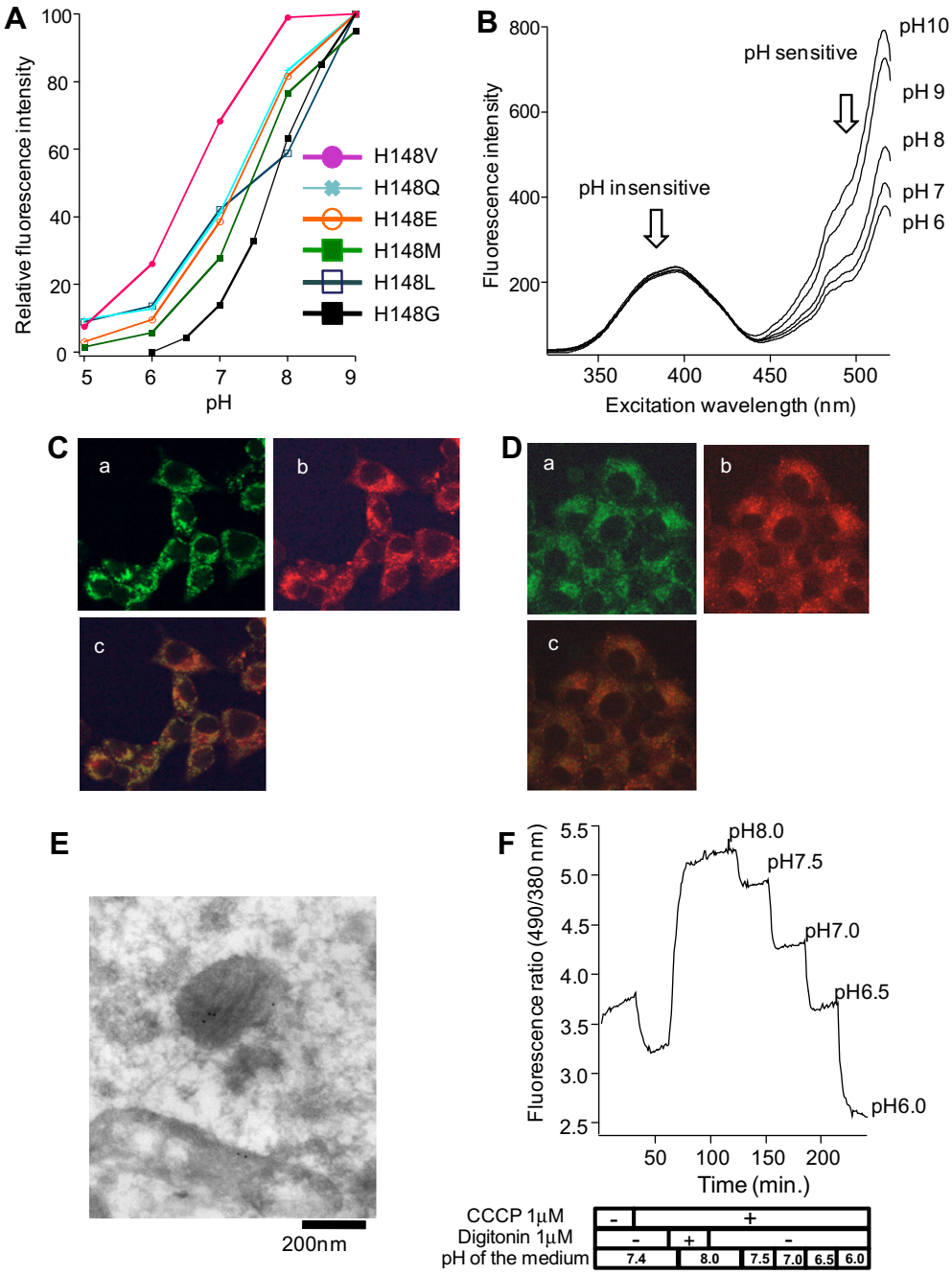

Fig. 1. Generation of a biosensor MTpHGV. (A) Relative fluorescence intensity change by pH due to 148 histidine amino acid mutations of Venus in the vicinity of a chromophore. (B) Fluorescence intensity by dual-emission two-excitation result of the H148 V amino acid Venus mutation fused with GFP_{UV}-transfected CHO cells (pHGV-CHO). (C–E) Subcellular localization of MTpHGV. (C-a) Confocal images of INS-1 cells stably transfected with MTpHGV. (b) MitoTrackerRed labeling. (c) An overlay of images in figure C-a and C-b. (D-a) Confocal images of MIN6 cells stably transfected with MTpHGV. (b) MitoTrackerRed labeling. (c) An overlay of figure D-a and D-b. (E) Immunoelectron microscopic image of MTpHGV labeled with a GFP antibody in MTpHGV-INS1 cells. (F) Fluorescence ratio of fluorescent intensity 490 nm and 380 nm change by pH of the medium, which was varied from 6.0 to 8.0 after CCCP and digitonin treatment in MTpHGV-INS1 cells.

a rapid decrease in mitochondrial pH in MTpHGV–INS1 cells by CCCP (Fig. 2A). In contrast, MTpHGV-MIN6 cells showed a rapid increase of mitochondrial pH by 1 μM CCCP (Fig. 2B). Digitonin treatment induced a stepwise change in pH that was dependent on pH of the medium, which varied from 7.4 to 8.0 (Fig. 1F) in MTpHGV-INS1 cells. Ratio changes in the control experiments were dependent on a stepwise change of pH of the medium, therefore we were able to convert the fluorescence ratio to mitochondrial pH in each MTpHGV-transfected cell. Intensity changes induced by CCCP and digitonin were consistent with MTpHGV being localized on the inner mitochondrial membrane.

3.4. Mitochondrial pH,cytoplasmic pH, and Ca^{2+} reaction in cultured cells according to stimulated insulin secretion

Cytoplasmic pH was increased in MTpHGV-MIN6 cells loaded with BCECF following glucose challenge, as reported previously (Fig. 2C) [10]. Basal mitochondrial pH (without stimulation) was lower in MTpHGV-INS1 and MTpHGV-MIN6 cells (mean of pH7.4 and 6.7 respectively in medium without glucose) than in MTpHGV-CHO cells (pH 7.8). Mitochondrial pH in INS-1 and MIN6 cells varied from dish to dish; mean pH under basal conditions in medium without glucose was 7.4 ± 0.4 in INS-1 and

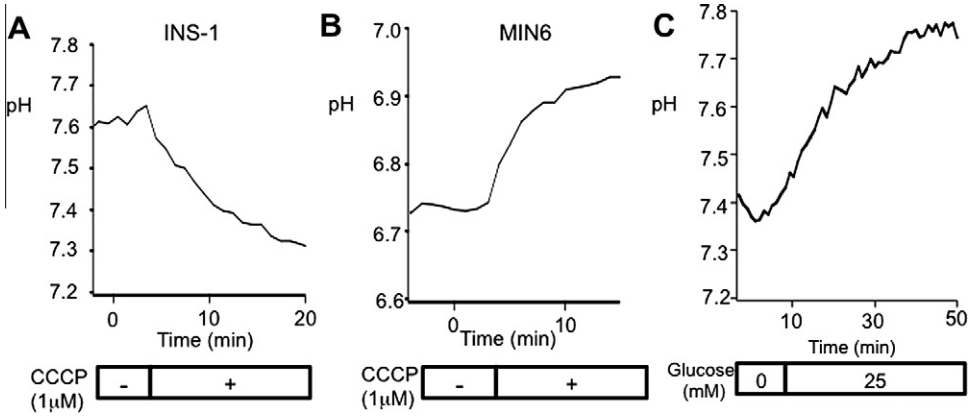

Fig. 2. Sensitivity of MTpHGV transfected cells to uncoupler, and cytoplasm pH change by glucose load. (A) 1 μM CCCP effect on calculated mitochondrial pH in MTpHGV-INS-1 cells. (B) 1 μM CCCP effect on calculated mitochondrial pH in MTpHGV-MIN6 cells. (C) Change of cytoplasm pH by glucose load calculated by BCECF in MTpHGV-MIN6 cells.

6.7 ± 0.4 in MIN6 (12–14 cells/dish and 8 dishes/experiment; mean ± SD). In MTpHGV-CHO cells, glucose stimulation did not change mitochondrial pH (data not shown). We calculated that the mitochondrial pH was increased by 0.25 ± 0.05 (n = 6 cells) in MTpHGV-INS1 cells (Fig. 3A.a) and decreased by 0.33 ± 0.11 units (12–14 cells/dish and 8 dishes) in MTpHVG-MIN-6 cells (Fig. 3B.a and c) along with glucose load. In MTpHGV-INS-1 cells this response was observed only in 6/39 cells and all cells showed Ca^{2+} response (Fig. 3A.a and b), and almost all cells showed response to glucose load in MTpHGV-MIN6 cells (Fig. 3B). Considering that CCCP treatment of MTpHGV-CHO INS-1 cells rapidly decreased fluorescence intensity at both excitation wavelengths (Fig. 2A), and response to glucose load was resembles to the previous report by MtAlpH in INS-1 cells [9,11], the calculated pH in MTpHGV-INS-1 cell is a mitochondrial matrix pH. Note the calculated mitochondrial pH in MTpHGV-MIN6 cells in response to CCCP load as well as to glucose load are opposite in MTpHGV-INS-1 cells (Fig. 2B and 3B). Taken together, the MTpHGV probe should be localized in matrix side of the inner-membrane in MTpHGV-INS-1 cells and outside of the inner membrane in MTpHGV-MIN6 cell.

Since, steady response was observed in MTpHGV-MIN6 cells, further experiment was carried out using MTpHGV-MIN6cells. The stepwise glucose stimulation from 0 mM to1.5 mM glucose loading in MtpHGV-MIN6 cells induced partial and weak acidification. The condition of preloading high glucose medium (25 mM) induced greater acidification in all cells tested, although Ca^{2+} response was not observed with 1.5 mM glucose in MTpHGV-MIN6 cells (Fig. 3B-a,b). On the other hands, the same stepwise glucose stimulation with preloading of 1.5 mM glucose 3hours showed a marked decrease of mitochondrial pH in 1.5 mM glucose stimulation to almost the same degree of 25 mM glucose stimulation, although Ca^{2+} response was observed in some cells in 1.5 mM and all cell in 25 mM glucose (Fig. 3B-c,d). These findings indicate that it is unlikely that Ca^{2+} response directly change mitochondrial pH response. To clarify the relationship between Ca^{2+} response and mitochondrial pH, we examined the effect of diazoxide, a KATP opener, during glucose load. While diazoxide (100 μM) abolished intracellular Ca^{2+} response evoked by 25 mM glucose, it did not affect mitochondrial pH (Fig. 4A,B). Thus mitochondrial pH change by glucose load in MTpHGV-MIN6 cells is independent to Ca^{2+} response. NADH shuttle is another system to regulate mitochondrial membrane potential in vital cells and the main gateway for insulin secretion by glucose stimulation [12]. To clarify the effect of NADH shuttle on mitochondria membrane pH, perfusion study of glucose load in MTpHGV-MIN6 cells under aminooxyacetate (AOA) was carried out. Mitochondrial pH acidification was abolished by 5 μM AOA (Fig. 4C). A slow decrease in the mitochondrial inner membrane pH by arginine was observed in MTpHGV-MIN6 cells, which stimulation did not affect directly to mitochondria on insulin secretion (Fig. 4D).

4. Discussion

We have developed a new biosensor, MTpHGV to measure mitochondrial membrane pH, which is selectively targeted to the mitochondrial matrix and is not toxic nor interferes with normal cellular functions. A ratiometric modification of an alkaline pH indicator, such as MtAlpHi [9], has resolved problems associated with using two probes, which often results in inconsistent expression levels and uneven distribution, and thus it is useful for real time assessment of mitochondrial pH in perfusion studies. GFP and YFP are hydrogen and chloride sensitive [13]. Chloride is a factor that determines pH of the inner-cells. $Chloride^{-}$–HCO_3^- exchanger has a role for adjusts the cytosolic pH, and chloride is also reported that the one of the important factors on insulin secretion in β cells [14,15]. So chloride ion must be change by glucose load. The hydrogen sensitive but chloride insensitive probe enabled us to obtain reliable pH measurement.

Immunohistochemistry and immunoelectron microscopy revealed that the MTpHGV probe was expressed in the mitochondrial inner membrane. Estimated mitochondrial pH on loading CCCP or glucose indicates that the MTpHGV probe exists in the matrix side of the mitochondria inner membrane in MTpHGV-INS-1 cells, and outer side of the mitochondria inner membrane in MTpHGV-MIN6 cells. MTpHGV-INS-1cells show alkalization of mitochondrial pH as reported previously by using MtAlpHi fused with EYFP [11]. Interestingly, although the mitochondrial pH alkalization by glucose was observed in some cells (n = 6/39), Ca^{2+} spike reaction was observed in almost all the cells in MTpHGV-INS-1 cells by glucose load (Fig. 3A.b) [16]. Also in MtpHGV-MIN6 cells, it seems that the degree of the mitochondrial membrane pH acidification by glucose was not changed in conjunction with Ca^{2+} response. Although the lag time of Ca^{2+} response is reported to be inversely correlated with mitochondrial mass [17], the converse effect of Ca^{2+} response to mitochondrial reaction is not clear. According to our results, at least the threshold of Ca^{2+} response is different from mitochondrial pH change of the inner membrane in cultured β cell. The findings that mitochondrial membrane acidification by glucose is not blocked by diazoxide but blocked by AOA indicate that response of mitochondrial internal membrane pH to glucose load is mainly depend on NADH shuttle, not on KATP channel in MIN6 cells. It may be due to intrinsic characteristics of MIN6 cells that this cell line utilizes NADH shuttle more than human β cells. Nonetheless

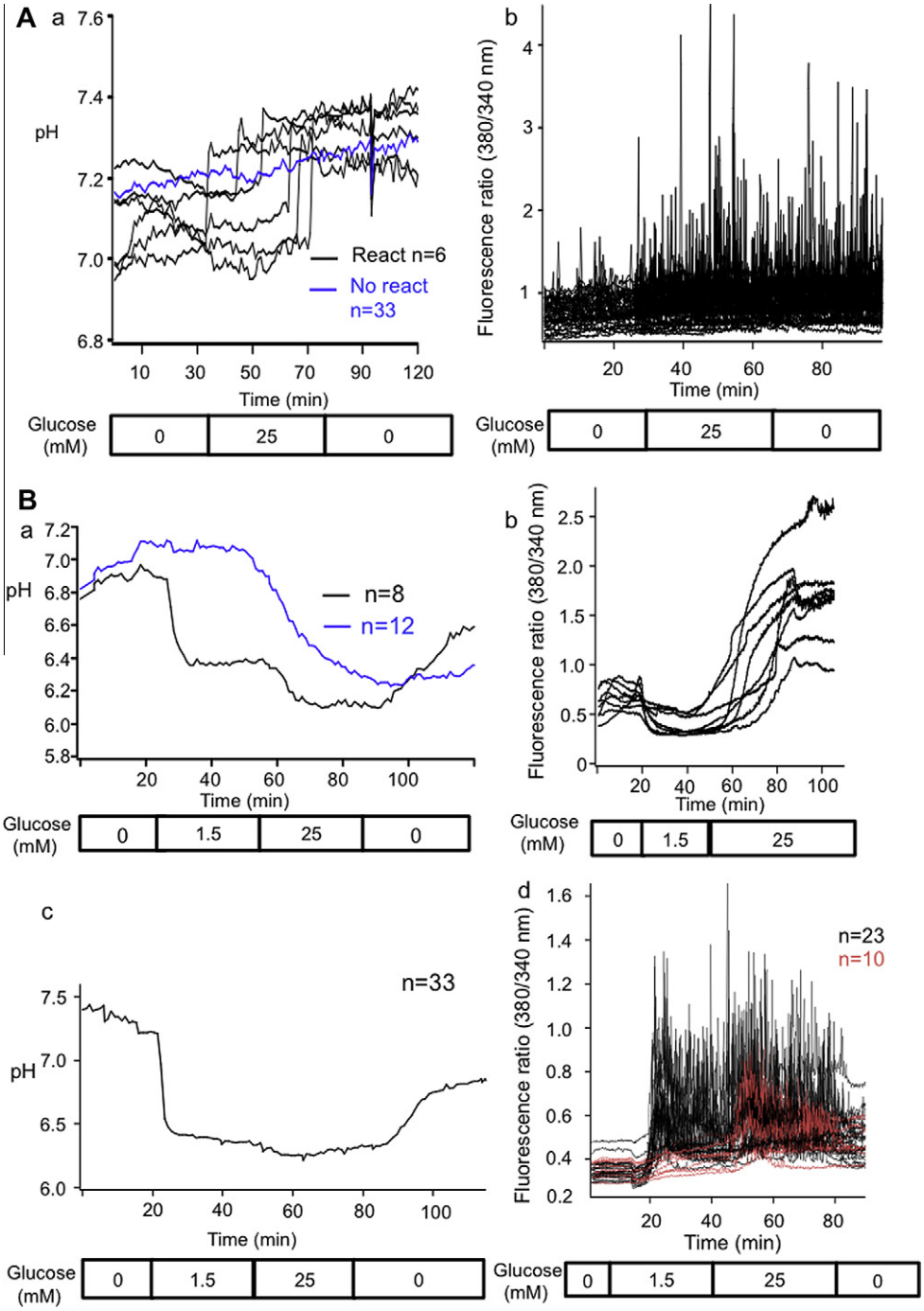

Fig. 3. Effects of glucose on mitochondrial pH and intracellular Ca^{2+} in MTpHGV-INS-1cells and MIN6 cells. (A-a) Mitochondrial pH change by 25 mM glucose load in MTpHGV-INS-1 cells. (b) Ca^{2+} response to glucose load in MTpHGV-INS-1 cells. (B-a) Mitochondrial pH change by 1.5 mM and 25 mM glucose load in MtpHGV-MIN6 cells after 25 mM glucose medium preloading. Black line shows the result of the cells that responded to 1.5 mM glucose load (n = 12). Blue line shows the result of the cells that did not respond 1.5 mM glucose. (b) Ca^{2+} response to glucose load in MTpHGV-MIN6 cells after 25 mM glucose preload. (c) Mitochondrial pH change by 1.5 mM and 25 mM glucose load in MtHGV-MIN6 cells after preloading of 1.5 mM glucose 3 h. (d) Ca^{2+} response to 1.5 mM and 25 mM glucose load in MTpHGV-MIN6 cells after preloading of 1.5 mM glucose for 3 h. Black line shows the result of the cells that responded to 1.5 mM glucose load (n = 23). Blue line shows the result of the cells that did not respond 1.5 mM glucose (n = 10).

it is clear that NADH shuttle activity is one of the determinant of mitochondrial inner membrane pH of cultured β cell. The result is consistent with a previous report [12]. A slow decrease in the mitochondrial inner membrane pH by arginine was oberserved in MTpHGV-MIN6 cells. Arginine stimulates insulin secretion without passing through mitochondria in β cell. Therefore it is possible that KATP channel also affect the mitochondrial inner membrane pH to some extent. Our result is consistent with a previous report that mitochondrial membrane potential is independent of Ca^{2+} concentration in cytoplasm in a single β cell [16].

The present study demonstrated that MTpHGV transfected cultured cells should be useful tool for adjective evaluation of the mitochondrial membrane response to glucose load. Alterations in mitochondrial function have been reported in high-fat dieted mice [18], diabetic rats [19], as well as patients with type 2 diabetes [20]. As pancreatic β cells are excitatory cells in which insulin

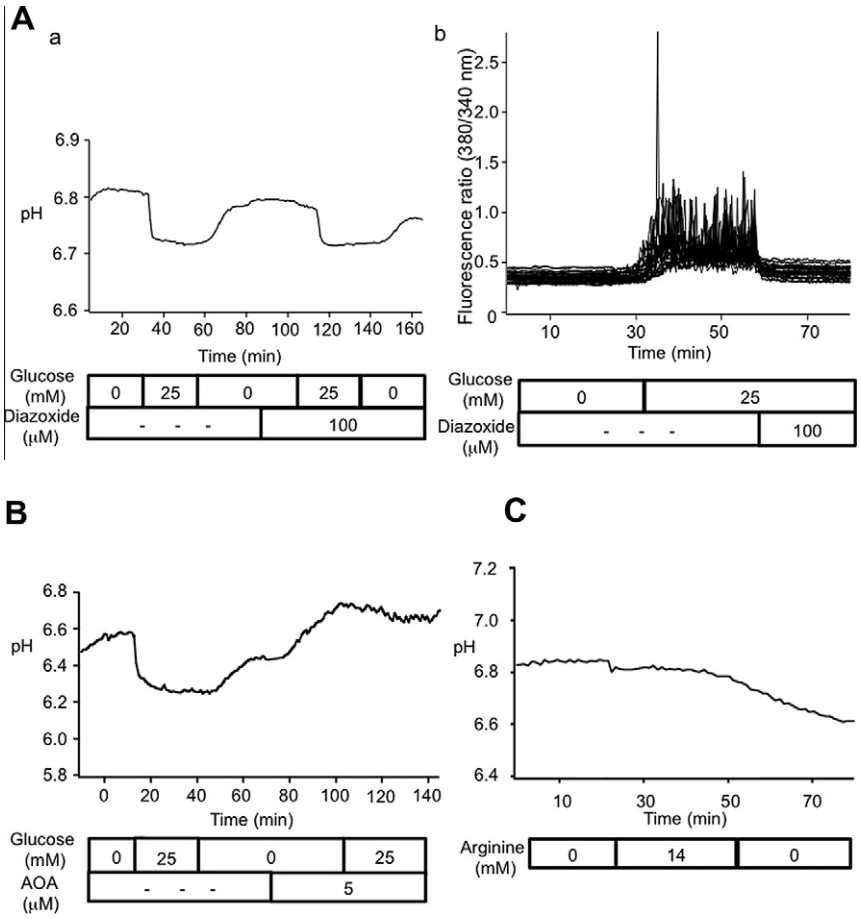

Fig. 4. Effect of KATP opener and malate-aspartate shuttle inhibitor in MTpHGV-MIN6 cells. (A-a) Mitochondrial pH change by 25 mM glucose load with or without 100 μM diazoxide in MTpHGV-MIN6 cells. (b) The effect of 100 μM diazoxide to intracellular Ca^{2+} mobilization on 25 mM glucose load in MTpHGV-MIN6 cells. (B) Mitochondrial pH change by 25 mM glucose load with or without 5 μM AOA in MTpHGV-MIN6 cells. (C) Mitochondrial pH change by a14 mM arginine load in all MTpHGVMIN6 cell.

secretion is initiated by the closure of KATP channels, energy metabolism in the mitochondria ultimately regulates the ability of a given β-cell to secrete insulin. Understanding the function of mitochondria, particularly the mechanisms of pH control may lead to developing new therapeutics to treat diabetes.

Acknowledgments

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