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Positive Feedback Amplifies the Response of Mitochondrial Membrane Potential to Glucose Concentration in Clonal Pancreatic Beta Cells☆

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ABSTRACT

Analysis of the cellular mechanisms of metabolic disorders, including type 2 diabetes mellitus, is complicated by the large number of reactions and interactions in metabolic networks. Metabolic control analysis with appropriate modularization is a powerful method for simplifying and analyzing these networks. To analyze control of cellular energy metabolism in adherent cell cultures of the INS-1 832/13 pancreatic β-cell model we adapted our microscopy assay of absolute mitochondrial membrane potential (ΔψM) to a fluorescence microplate reader format, and applied it in conjunction with cell respirometry. In these cells the sensitive response of ΔψM to extracellular glucose concentration drives glucose-stimulated insulin secretion. Using metabolic control analysis we identified the control properties that generate this sensitive response. Force-flux relationships between ΔψM and respiration were used to calculate kinetic responses to ΔψM of processes both upstream (glucose oxidation) and downstream (proton leak and ATP turnover) of ΔψM. The analysis revealed that glucose-evoked ΔψM hyperpolarization is amplified by increased glucose oxidation activity caused by factors downstream of ΔψM. At high glucose, the hyperpolarized ΔψM is stabilized almost completely by the action of glucose oxidation, whereas proton leak also contributes to the homeostatic control of ΔψM at low glucose. These findings suggest a strong positive feedback loop in the regulation of β-cell energetics, and a possible regulatory role of proton leak in the fasting state. Analysis of islet bioenergetics from published cases of type 2 diabetes suggests that disruption of this feedback can explain the damaged bioenergetic response of β-cells to glucose. This article is part of a Special Issue entitled: Oxidative Stress and Mitochondrial Quality in Diabetes/Obesity and Critical Illness Spectrum of Diseases - edited by P. Hemachandra Reddy.

1. Introduction

Mitochondrial metabolism of glycolytic pyruvate plays a central role in insulin secretion [1]. The canonical pathway of glucose-stimulated insulin secretion (GSIS) relies on hyperpolarization of mitochondrial membrane potential (ΔψM) (more strictly, hyperpolarization of the protonmotive force) leading to increased mitochondrial production of ATP, and is largely responsible for the first phase of insulin secretion [2–7]. Both type 1 and type 2 diabetes (T2D) in humans are characterized by early impairment of this phase [8–14], suggesting a possible role of disturbed cellular energy metabolism. While rare mitochondrial defects can cause diabetes [15], it is possible that more subtle derangements in cellular energy metabolism contribute more generally to β-cell impairment in diabetes. In support of this idea, the dampened response of ΔψM to glucose in T2D human β-cells may reflect a subtle bioenergetic supply-demand dysfunction [16].

Glucose stimulation of the β-cell changes virtually all variables of cellular energy metabolism, making it non-trivial to define which processes drive observed changes and which processes are responsible for deficiencies observed in disease. This is complicated by the large number of reactions in metabolic networks and the multiple interactions between them. Metabolic control analysis [17–19] with appropriate modularization is a powerful method for simplifying and analyzing these systems. Metabolic control analysis is a mathematical formalism to describe the control and regulation of metabolic systems; in particular,

Abbreviations: a_R', activity coefficient ratio; CDC, complete depolarization cocktail; ΔψM, mitochondrial membrane potential; ΔψP, plasma membrane potential; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; GSIS, glucose-stimulated insulin secretion; K_{ATP}, ATP-sensitive K^+-channels; MDC, mitochondrial depolarization cocktail; OCR, oxygen consumption rate; PM, potentiometric medium; PMK, K^+-based potentiometric medium; PMPI, ΔψP indicator; SE, standard error; T2D, type 2 diabetes mellitus; TMRM, tetramethylrhodamine methyl ester; TPB, tetraphenylborate; V_F, mitochondria:cell volume fraction; F.U., fluorescence units; V_{FM}, matrix:mitochondria volume fraction.

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it can quantify how a change in a steady state is caused by changes in the activities of individual components of the system. Here, we reduce the complexity of β-cell energy metabolism to a simple modular system in which the modules of glucose oxidation, phosphorylation plus ATP turnover, and proton leak are linked by ΔψM as their common intermediate. Using this simplified system, the only measurements needed to describe the internal control structure and the regulation by external glucose are the fluxes through each module and the level of ΔψM.

Two complementary technologies make possible the measurement of these bioenergetic variables in adherent cell cultures. First, flux through each of the modules can be determined by measuring the extracellular oxygen consumption rate, where respiration is restricted by different combinations of mitochondrial inhibitors to report on a given module [20]. To this end, we used the Seahorse Extracellular Flux Analyzer, which utilizes our calibration algorithm [21], in conjunction with whole-well cell counting by fluorescence microscopy to normalize the measured rates. Second, we previously developed a method for determining absolute values of ΔψM in intact cells using fluorescence microscopy, allowing comparison between adherent cells in which plasma membrane potential (ΔψP) and morphological factors may differ [16,22]. To enable measurement of ΔψM in populations of cells in microplate samples, comparable to the populations used for cell respirometry, we introduce here a microplate reader-based adaptation of this method. Together, these technical advances allow measurement all of the variables required for metabolic control analysis of energy metabolism in adherent cells. This is a major advance; though control analysis of mitochondrial energy metabolism has been applied previously, it was limited to isolated mitochondria [17,20] and, because of the need for bulk suspensions for Clark electrode-based respirometry and radioisotope-distribution based ΔψM determinations, to suspensions of cells such as hepatocytes [18], precluding its application to adherent cells.

Using these approaches and analyses, we report two major findings. First, our data indicate a strong positive feedback loop in β-cell energy metabolism by ATP/ADP or other factors downstream of ΔψM. In other words, glucose stimulates its own metabolism, and this stimulation requires mitochondrial ATP synthesis. Second, the analysis suggests that the putative role of proton leak in regulating β-cell energetics is constrained to the fasting state of the cell, implying that physiological levels of uncoupling proteins are expected not to limit ATP/ADP at high glucose concentrations. We propose that the operation of the feedback mechanism and its defect in T2D explains recent bioenergetic findings on human primary β-cells [16] and islets [23].

2. Materials and Methods

2.1. Materials

The ΔψP indicator (PMPI; #R8042 FLIPR Membrane Potential Assay Explorer Kit) was from Molecular Devices (Sunnyvale, CA); tetramethylrhodamine methyl ester (TMRM) and Hoechst 33342 were from Life Technologies (Carlsbad, CA); zosuquidar was from MedKoo Biosciences (Chapel Hill, NC), and other fine chemicals were from Sigma-Aldrich (St. Louis, MO) or Santa Cruz Biotechnology (Dallas, TX) unless otherwise noted.

2.2. Insulinoma cell line

INS-1 832/13 cells [24] were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1 mM Na-pyruvate, 0.05 mM β-mercaptoethanol, 10 mM HEPES, and 10 v/v% fetal bovine serum. An INS-1 832/13 line stably transfected with non-targeted pSilencer 4.1 CMV-puro vector was used, and 2 μg/ml puromycin was added during cell culture and plating. INS-1 832/13 cells were plated 48–72 h prior to the experiment in Seahorse V7 PS Flux plates and in alternating columns of Corning 3340 96-well plates (both prepared by coating with a 1:15,000 dilution of polyethylimine) at 4×10^4 cells per well in 100 μl growth medium. For fluorescence microscopy 8-well LabTek coverglass-bottomed dishes were used, coated as described above.

2.3. Fluorescence microscopy assay of ΔψP and ΔψM

Absolute calibrated assays of ΔψP and ΔψM using wide field fluorescence microscopic time lapse imaging were performed as described in [25], in conditions similar to those described below for the microplate reader assay. The ΔψM assay is based on recording of time courses of the fluorescence of the cationic dye TMRM and an anionic bis-oxonol ΔψP indicator (PMPI). The calibration relies on a biophysical model of lipophilic potentiometric probe distribution to back-calculate potentials that cause changes in fluorescence intensities of the two probes [22]. Absolute values of potentials in millivolts are calculated from paired TMRM and PMPI fluorescence intensity values and their rates of change, using a set of calibration parameters (see Supplemental Material Appendix A). The prototypical experiment in Fig. 1 was designed to allow computation of these calibration parameters, and therefore ΔψP and ΔψM, from a single fluorescence microscopy recording [22]. The calibration of ΔψP is a prerequisite for the calculation of ΔψM, because it allows separation of the effects of ΔψP and ΔψM on TMRM fluorescence and therefore calculation of ΔψM. In Fig. 1, ΔψP was calibrated by manipulation of extracellular K^+ in the presence of the K^+-ionophore valinomycin to ensure that the K^+ equilibrium potential dominates ΔψP [22]. ΔψM was then calculated from the decay characteristics of TMRM fluorescence when mitochondria were completely depolarized at a maintained ΔψP. Both calibrations require fluorescence intensity measurement at 0 mV. These steps were combined in the single-run, or "complete" calibration (Fig. 1). After a recording of interest (e.g. in the presence of 30 mM glucose in Fig. 1 or a "challenge time course" in general) the mitochondrial inner membrane was depolarized using a mitochondrial depolarization cocktail (MDC; see compositions in Suppl. Table 1) triggering a characteristic decay of TMRM fluorescence intensity. Next, extracellular $[K^+]$ was elevated stepwise in the presence of valinomycin (a component of the MDC). The experiment was concluded by complete cellular depolarization using an ionophore cocktail (complete depolarization cocktail, CDC; Suppl. Table 1; [22]). Fluorescence image analysis and calculations of potentials were performed using standard image processing pipelines and the "Membrane Potential Calibration Wizard" in Image Analyst MKII (Image Analyst Software, Novato, CA).

2.4. Microplate reader assay of ΔψP and ΔψM

90 min prior to experiment, growth medium was exchanged for potentiometric medium (PM) containing 120 mM NaCl, 3.5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 20 mM TES (N-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid), 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 2 mM D-glucose, TMRM (10 nM), PMPI (1:200), tetraphenylborate (TPB; 1 μM) and zosuquidar (1 μM [26]). PM was made from a 2× stock (2 × PM) containing all components except NaCl, and diluted to final volume using 240 mM NaCl (or 240 mM KCl, resulting in PMK). K^+ was varied by partial replacement of PM with PMK over the specimen.

Fluorescence intensity time courses were recorded using a Pherastar FS (BMG Labtech Inc., Cary, NC) fluorescence microplate reader capable of focusing on the cell monolayer in bottom fluorescence readout mode and simultaneous detection of TMRM and PMPI fluorescence emission with a custom dual-excitation, dual-emission filter set. The filter set (Semrock, Rochester, NY) comprised a 503/572-nm dual bandpass exciter, a 444/520/590-nm multi-edge excitation beamsplitter, a 562-nm emission beamsplitter, a 537/26-nm emission filter for PMPI (custom matched to the exciter to avoid bleedthrough) and a 641/75-nm emission filter for TMRM. Assays were performed at 37 °C under air. All media and reservoir plates were held at 37 °C in an air incubator. Fluorescence measurements of the cell monolayer were optimized by focusing at the appropriate *z*-offset for the cell monolayer, determined