Neuroscience 152 (2008) 40–49

INNERVATION AND ACTIVITY DEPENDENT DYNAMICS OF POSTSYNAPTIC OXIDATIVE METABOLISM

H. KAZAMA,[a1] A. ICHIKAWA,[a] H. KOHSAKA,[a] T. MORIMOTO-TANIFUJI[a1] AND A. NOSE[a,b*]

[a]*Department of Physics, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

[b]*Department of Complexity Science and Engineering, Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8561, Japan*

Abstract—Despite extensive investigations into the mechanisms of aerobic respiration in mitochondria, the spontaneous metabolic activity of individual cells within a whole animal has not been observed in real time. Consequently, little is known about whether and how the level of mitochondrial energy metabolism is regulated in a cell during development of intact systems. Here we studied the dynamics of postsynaptic oxidative metabolism by monitoring the redox state of mitochondrial flavoproteins, an established indicator of energy metabolism, at the developing *Drosophila* neuromuscular junction. We detected transient and spatially synchronized flavoprotein autofluorescence signals in postsynaptic muscle cells. These signals were dependent on the energy substrates and coupled to changes in mitochondrial membrane potential and Ca^{2+} concentration. Notably, the rate of autofluorescence signals increased during synapse formation through contact with the motoneuronal axon. This rate was also influenced by the magnitude of synaptic inputs. Thus, presynaptic cells tightly regulate postsynaptic energy metabolism presumably to maintain an energetic balance during neuromuscular synaptogenesis. Our results suggest that flavoprotein autofluorescence imaging should allow us to begin assessing the progress of synapse formation from a metabolic perspective.

Key words: metabolism, mitochondria, flavoprotein, autofluorescence, imaging, synaptogenesis.

Production of energy through aerobic respiration in mitochondria is essential for cellular activities in eukaryotes. While the function of mitochondria has been documented in great detail, much of this information has been obtained from studies on purified enzymes, isolated mitochondria, and dissociated cells (McCormack et al., 1990; Hansford and Zorov, 1998; Brookes et al., 2004; Kann and Kovacs, 2007). Recent development in pharmacological tools and imaging techniques has facilitated the examination of mitochondrial function in an intact tissue and *in vivo* (Foster et al., 2006). However, emphasis has been placed on characterizing the mitochondrial responses to exogenously applied stimuli. How spontaneous metabolic activity is dynamically regulated in a cell embedded in an intact animal is an important unanswered question.

To sustain cellular activities, the supply of energy must be adjusted to meet the varying demands of the cell. In nervous systems, for instance, it has been shown that mitochondrial function is coupled to cellular activity (Schwartz et al., 1979; Mata et al., 1980; Wong-Riley, 1989; Erecinska et al., 2004; Kann and Kovacs, 2007). This relationship is beneficial for neurons since their dominant energy-consuming task is the repolarization of the membrane potential following excitation (Wong-Riley, 1989). However, direct evidence is lacking as to whether this relationship holds during development, when cells receive novel inputs from surrounding environment. Again, this is due to the difficulty of observing the spontaneous metabolic activity of individual cells in real time, particularly at different developmental stages.

Flavoprotein autofluorescence imaging is a suitable technique to monitor the dynamics of oxidative metabolism. Flavoproteins containing flavin adenine dinucleotide and flavin mononucleotide are concentrated in mitochondria where they act as components of both the Krebs cycle and the respiratory complexes necessary for oxidative phosphorylation (McCormack et al., 1990). During oxidative phosphorylation, flavoproteins undergo oxidation and reduction. Because they emit green autofluorescence in blue light when oxidized and become non-fluorescent when reduced (Benson et al., 1979), their autofluorescence has long been used as an indicator of oxidative metabolism (Hassinen and Chance, 1968; Scholz et al., 1969; Masters, 1984; Kunz and Kunz, 1985; Duchen, 1992; Kann et al., 2003).

In this study, we examined whether and how the level of oxidative metabolism in the postsynaptic cell changes during synapse formation, by applying flavoprotein autofluorescence imaging to the *Drosophila* neuromuscular junction (NMJ). The *Drosophila* NMJ is an ideal model system to investigate the morphological and physiological events in postsynaptic cells during synapse formation. First, the postsynaptic muscle cells are large enough to perform imaging with single cell resolution. Second, since every

[1] Present address: Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA (H. Kazama); Laboratory of Cellular Neurobiology, School of Life Science, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan (T. Morimoto-Tanifuji).

*Correspondence to: A. Nose, Department of Complexity Science and Engineering, Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8561, Japan. Tel: +81-4-7136-3919; fax: +81-4-7136-3919.
E-mail address: nose@k.u-tokyo.ac.jp (A. Nose).

Abbreviations: AEL, after egg laying; ANOVA, analysis of variance; CV, coefficient of variation; DMSO, dimethylsulfoxide; DPI, diphenyleneiodonium; *mhc*, *myosin heavy chain*; M6, muscle 6; M7, muscle 7; NMJ, neuromuscular junction; *pros*, *prospero*; *syx*, *syntaxin*; TMRE, tetramethylrhodamine ethyl ester; TTX, tetrodotoxin; wt, wild type.

0306-4522/08$32.00+0.00 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2007.12.020

muscle cell is identifiable, a specific type of cell could be repetitively studied across animals (Keshishian et al., 1996). Finally, an extensive collection of mutants allows examination of biological processes under various molecular and cellular environments *in vivo*.

Here we report the dynamics of spontaneous flavoprotein autofluorescence in the muscle fiber interacting with presynaptic motoneurons during synaptogenesis. Autofluorescence signals were dependent on energy substrate and coupled to changes in mitochondrial membrane potential and Ca^{2+} concentration. Autofluorescence signals were cell autonomous, but the frequency of their occurrence was dependent on innervation and synaptic inputs. Our results suggest that presynaptic cells closely regulate the level of postsynaptic energy metabolism presumably to balance the demand and supply of energy during the development of neuromuscular synapses.

EXPERIMENTAL PROCEDURES

Flies and preparation of embryos

Flies were reared at room temperature (25 °C). *y*, *w* was used as wild type (wt); *prospero* (*pros*^{M4}) is a mutant in which the arrival of axons to muscle cells delays substantially (Vaessin et al., 1991). A mutant of *syntaxin-1A* (*syx*^{L371}) is defective in synaptic vesicular fusion, and thus is unable to support neuromuscular synaptic transmission (Broadie et al., 1995). *DGluRIIA&IIB*^{SP22} deletes the entire coding regions of glutamate receptor subunits DGluRIIA and IIB (DiAntonio et al., 1999). *Df(2L)*^{clh4} is a large deletion covering loci of *DGluRIIA* and *IIB*. A mutant of *myosin heavy chain* (*mhc*^{1}) is defective in muscle contraction (Mogami et al., 1986), but the morphology of NMJ and properties of synaptic currents in *mhc*^{1} are similar to those in wt (Yoshihara et al., 2000). Devitellinized embryos at 13 and 14 h after egg laying (AEL) were attached to glass slides and cut along the dorsal midline with a sharp glass needle. The gastrointestinal tracts were then removed, exposing the ventral nervous system, peripheral nervous system, and the somatic musculature. The external solution contained (in mM): 140 NaCl, 2 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 5 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid–NaOH, and 36 sucrose (pH 7.1). Sucrose at 36 mM was replaced with different concentrations of sucrose, trehalose, or glucose in experiments characterizing the sugar dependency of autofluorescence signals. Animal care was carried out in accordance with guidelines approved by the University of Tokyo. Every effort was made to minimize the number of animals used and their suffering.

Immunohistochemistry

Embryos were fixed with Bouin's fixative for 30 min at room temperature. Bouin's fixative contained 75 ml of saturated picric acid, 25 ml of 37% formaldehyde, and 5 ml of glacial acetic acid. After thorough washing, preparations were blocked with 5% donkey serum in 0.1 M phosphate buffer [pH 7.2] containing 0.2% Triton X-100 for at least 30 min. Next, goat anti–horseradish peroxidase (Jackson, West Grove, PA, USA, 1:4000) was added and incubated overnight at 4 °C. The washed preparations were then incubated in donkey anti-goat IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA, 1:300) for another 24 h at 4 °C. Confocal images were acquired with LSM 510 microscope (Zeiss, Oberkochen, Germany).

Live imaging

Autofluorescence was detected by a system comprising a cooled charge-coupled device camera (OrcaER, Hamamatsu Photonics, Hamamatsu, Japan), a mercury lamp (100 W), and an upright microscope equipped with ×63 (numerical aperture 0.9) water objective lens (Axioscope2, Zeiss) controlled by IPLab v3.6 software (BD Biosciences, San Jose, CA, USA). The intensity of the excitation light was reduced to 1.5% of maximum with neutral density filters. In a single session, binned (4×4) images were acquired at 2 Hz for 200 s using a 450–490 nm excitation filter and a >515 nm long pass emission filter. Generally, only one session was performed per segment to minimize the damage from photoillumination. Although the signal was detected over 1 h after dissection, imaging was performed within 30 min after dissection except for experiments with thapsigargin. The magnitude of autofluorescence signals was described as relative fluorescence change $\Delta F/F_0=(F-F_0)/F_0$, where F_0 is the dark current-corrected average fluorescence of the first 10 frames and ΔF is the deviation of the signal from F_0. A fluorescence rise whose amplitude is larger than three times the standard deviation of the baseline fluorescence fluctuation was recognized as a signal. $\Delta F/F_0$, rise time, half-decay time, and frequency of signals were analyzed with a custom program written in Matlab 6.5 (MathWorks, Natick, MA, USA). Images were processed with ImageJ (NIH, Bethesda, MD, USA). Images showing $\Delta F/F_0$ in pseudo-colors were filtered with a Gaussian kernel (standard deviation of the kernel=one pixel). To image the mitochondrial membrane potential, preparations were incubated with 2 nM tetramethylrhodamine ethyl ester (TMRE, Invitrogen) for 5 min. TMRE was dissolved in external solution with 0.0002% dimethylsulfoxide (DMSO, Sigma, St. Louis, MO, USA). For mitochondrial Ca^{2+} imaging, the preparation was incubated with 10 μM acetoxymethyl ester form of rhod-2 (Dojindo, Kumamoto, Japan) for 15–30 min. Rhod-2 was dissolved in external solution with 0.04% pluronic acid (Invitrogen) and 1% DMSO. TMRE and rhod-2 were excited with the argon/krypton laser using 543 nm excitation and >590 nm emission filters. For dual imaging of autofluorescence and TMRE or rhod-2 signals as well as for the characterization of wavelength-dependence of flavoprotein autofluorescence, images were captured with a FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). Dual imaging was performed in a sequential scanning mode to reduce the bleed-through of fluorescence (frame rate=0.5 Hz). Excitation and emission wavelengths are indicated in the figures. All experiments were performed at room temperature (25 °C). Statistical analyses were done by one-way analysis of variance (ANOVA), two-way ANOVA, post hoc Tukey test, and Student's *t*-test.

Pharmacology and the staining of mitochondria

Diphenyleneiodonium (DPI, Sigma), an irreversible inhibitor of flavoprotein, was used at 10 μM concentration. Thapsigargin (Sigma), an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase, was used at 5 μM concentration. To block the generation of action potentials, preparations were incubated with 3 μM tetrodotoxin (TTX). To label functional mitochondria, preparations were incubated in 100 nM MitoTracker Red (Invitrogen) for 20 min.

RESULTS

Spontaneous autofluorescence signals in embryonic *Drosophila* muscle cells

We started by examining whether there is any heterogeneity or fluctuation in the levels of cellular autofluorescence at developing NMJs of *Drosophila* embryo. Embryos were imaged at 14 h AEL, approximately 30 min after motoneuronal axons and muscle cells make initial synaptic contact. We excited these embryos with blue light (450–490 nm) and collected green emission light (>515 nm) for analysis. At this stage of development, nerve terminals and muscles showed low levels of baseline autofluorescence. However,