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INTRINSIC FLUORESCENCE CHANGES IN NERVE TERMINALS, EVOKED BY ACTION POTENTIALS, ARE MODULATED BY KREBS CYCLE SUBSTRATES*

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Electrical stimulation of the mammalian neurohypophysial infundibular stalk evokes the entry of Na^+ and Ca^{2+} into the neurosecretory terminals during the action potential. These events, in turn, increase intracellular Ca^{2+} and activate NaK- and Ca-ATPases, prompting the mitochondria to increase oxidative phosphorylation which can be monitored by recording the changes in FAD and NADH fluorescence. This paper reflects our efforts to determine whether or not modulating the capacity of mitochondria to produce ATP, by changing the concentrations of two important substrates of the Krebs cycle of the nerve terminal mitochondria, pyruvate and glucose, has an effect on the intrinsic fluorescence changes triggered by action potential stimulation.

Keywords: Fluorescence; FAD; NADH; action potential; metabolism.

1. Introduction

In a series of landmark papers sixty years ago,[1−9] Britton Chance and colleagues developed instruments for continuous measurement of the fluorescence of the β-nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide intrinsic to mitochondria and cells. These were shown to be highly responsive to changes in energy metabolism, serving as indicators of both the glycolytic and oxidative ATP production pathways. This became, and remained, one of several leitmotivs of Chance's work. He recognized, and made use of, the fact that flavin adenine dinucleotide (FAD) is quite fluorescent when excited at 450 nm, while its reduced form $FADH_2$ is only weakly so; in contrast, NADH is fluorescent when excited at 350 nm, while its oxidized form NAD is not. Thus, flavoprotein-derived and pyridine nucleotide-derived fluorescence changes tend to be opposite in sign. We[10] took advantage of that difference and showed that changes in FAD and NADH fluorescence emission in mammalian neurosecretory terminals, when FAD and NADH are excited at 450 and 350 nm respectively, are triggered by calcium entry, and by ADP production. In particular, the signal to the mitochondria to increase oxidative phosphorylation[10] comes from the increase

*This article is dedicated to the memory of Britton Chance, friend and teacher.

in the ADP/ATP ratio that results from turning on the nerve terminal NaK-ATPase, along with some Ca^{2+}-entry during the action potential.[10] While others had found that autofluorescence changed with stimulation,[11−14] our result in the neurosecretory terminals of the mouse neurohypophysis is the only one that identified the intracellular localization of the activated mitochondria. Mitochondria make ATP and supply ionic pumps with the source of energy required for the restoration of ionic gradients altered during synaptic transmission, and, in nerve terminals in particular, in response to the requirement that secretory granules become fusion-competent. It was, therefore, of considerable interest for us to determine whether modulating the capacity of mitochondria to produce ATP, by changing the concentrations of two important substrates of the Krebs cycle of the nerve terminal mitochondria, pyruvate and glucose, would have an effect on the intrinsic fluorescence changes triggered by action potential stimulation. Our expectation was that changes in either pyruvate or glucose concentration would alter the intrinsic fluorescence response to action potential stimulation.

2. Materials and Methods

2.1. *Preparation and apparatus*

Details of the preparation and apparatus have been reported previously.[15−20] Typically, 30−60-day-old CD-1 female mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized by CO_2 inhalation and then decapitated. The pituitary gland was removed and the intact neurohypophysis, together with part of the infundibular stalk and the *pars intermedia*, were separated from the anterior pituitary (*pars anterior*). The neurointermediate lobe (neurohypophysis and *pars intermedia*) was mounted in an optical recording chamber and superfused with normal mouse Ringer's (NMR) containing (in mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1, glucose 10, HEPES 20, adjusted to pH 7.4. The preparation was excited by direct field stimulation of the axons just as they branch into the body of the neurohypophysis. Trains of stimuli were provided by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel) driving a stimulus isolator (SIO-102, Warner Instruments, Inc., Hamden, CT). Two platinum/iridium (90%/10%) electrodes delivered balanced bipolar stimuli of 1 ms total duration (500 μs each) to the tissue. The voltage drop across the preparation was approximately 10 V, and remained constant throughout the train. This stimulation protocol avoided electrode polarization artifacts and solution electrolysis. All experiments were carried out at room temperature ($22 \pm 2°C$) and all chemicals used were from Sigma (St. Louis, MO) unless otherwise indicated.

2.2. *Optical measurements of intrinsic fluorescence*

All fluorescence measurements were performed on a UEM upright microscope (Zeiss, Oberkochen, Germany) using a 20×, 0.5 numerical aperture, water immersion objective (Leica Micro-systems, Bensheim, Germany) for FAD fluorescence, or a 20×, 0.45 numerical aperture mirror objective (Spiegelobjektiv, Carl Zeiss, Jena, Germany) for NADH fluorescence. A 250-W tungsten-halogen lamp was used as the epi-illumination source for 450 nm light, and a PhotoFluor metal halide lamp equipped with a liquid light guide (Chroma Technology, Bellows Falls, VT) was used for 350 nm illumination. The filter sets (both from Chroma Technology, Bellows Falls, VT) comprised a 450 ± 50 nm excitation filter, a 515 nm dichroic mirror, and a Hi-Q 420 nm long-pass filter for measuring FAD fluorescence changes, and a 350 ± 20 nm excitation filter, a 395 nm dichroic mirror, and a 450 ± 50 nm band-pass emission filter for measuring NADH fluorescence changes.

2.3. *Photodiode measurements*

Tissue-averaged measurements of intrinsic fluorescence were obtained with a large area photodiode (PV-444, Perkin-Elmer Optoelectronics, Vaudreuil, Canada). The photocurrent was converted to a voltage signal using a custom-built sample-and-hold amplifier (Cellular and Molecular Physiology Electronics Shop, Yale University School of Medicine, New Haven, CT). The resulting voltage traces were low-pass filtered with an 8-pole Bessel filter (Model 410, Brownlee Precision, Santa Clara, CA) and digitized at 16 bit resolution using a data acquisition board (AT-MIO-16XE-50, National Instruments, Austin, TX). Typical low-pass filter/sampling pairs were 20 Hz/1 kHz.

2.4. *Data analysis*

All data analysis was performed using the IGOR Pro data analysis software (Wavemetrics, Lake

Oswego, OR). The intrinsic fluorescence (IF) traces were bleach-corrected by fitting (to $\geq$95% confidence level) a single exponential to the baseline 5 s prior to stimulation, and then subtracting the extrapolated bleaching curve from the record. Ordinate scales are given as $\Delta F/F_0$, in arbitrary units, because steady state flavoprotein levels are uncertain and bleaching effects are significant. Fractional fluorescence changes in response to 30 stimuli are on the order of one to five percent. The figures show results from individual experiments. However, the results are a typical representation of those observed in a series of 11 experiments that measured the effects of pyruvate and/or glucose on stimulation-induced FAD fluorescence changes, and a series of 14 other experiments that measured the effects of pyruvate and/or glucose on stimulation-induced NADH fluorescence changes.

3. Results

Because ATP production is essential for the action potential−dependent secretion of arginine-vasopressin and oxytocin from the neurohypophysial terminals,[10] and ATP production is accompanied by the oxidation of $FADH_2$ to FAD, we examined the changes of FAD fluorescence in response to action potential stimulation as a function of glucose concentration in the bath. The results are illustrated in Fig. 1. The black trace shows the fluorescence change in response to a train of 30 action potentials at 15 Hz, from a neurohypophysis which had been kept for two hours in a medium containing 0 mM glucose. The solution was then changed to a NMR containing 10 mM glucose, and the red trace shows that the response of the preparation to the same stimulation paradigm, after exposure to the glucose-rich medium for 25 min, is much more robust. After this run the medium was replaced once again by the glucose-free solution, and three more records were obtained (blue traces) at (a) 30, (b) 60, and (c) 90 min respectively. The declining size of these responses as the preparation is being gradually depleted once again of the substrate is worth noting. Figure 2 shows another, very similar, experiment that examines the effect of glucose on the stimulation-induced changes in NADH fluorescence excited at 350 ± 20 nm. Again, the black trace represents the NADH fluorescence response to 30 stimuli delivered at 15 Hz for two seconds after a two-hour exposure of the preparation

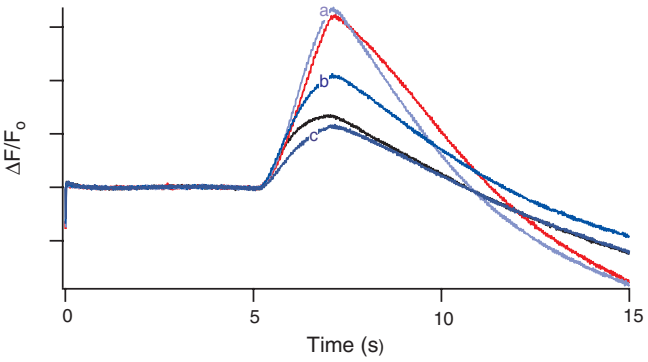

Fig. 1. Changes in FAD fluorescence in response to action potential stimulation as a function of glucose concentration in the bath. Electrical stimulation consisted of a train of 30 action potential stimuli delivered at 15 Hz, 5 s after the beginning of the traces. The black trace is the response after a two-hour exposure to glucose-free mouse Ringer's solution. The red trace shows the response after 25-min exposure to 10 mM glucose (NMR). The blue traces show the effect of returning to glucose-free mouse Ringer's solution for 30 min (largest response, light blue, (a)), 60 min (medium size response, darker blue, (b)), and 90 min (smallest response, darkest blue, (c)). The fluorescence excitation was at 450 ± 50 nm and the emission wavelength was longer than 515 nm. Room temperature (22 ± 2°C).

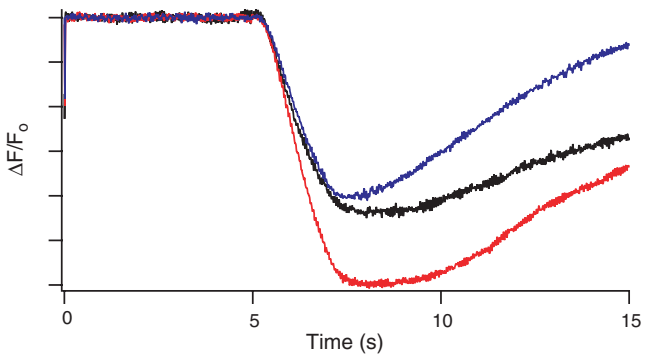

Fig. 2. Changes in NADH fluorescence in response to action potential stimulation as a function of glucose concentration in the bath. NADH fluorescence responses to a train of 30 stimuli delivered at 15 Hz, 5 s after the beginning of the traces. The black trace is the evoked response after a two-hour exposure to glucose-free mouse Ringer's solution. The red trace shows the response after a 25-min exposure to 10 mM glucose (NMR). The blue trace illustrates the recovery after 25 min back to the glucose-free mouse Ringer's solution. The fluorescence excitation was at 350 ± 20 nm and the emission wavelength was 450 ± 50 nm. Room temperature (22 ± 2°C).

to a glucose-free medium. The red trace illustrates the change in NADH fluorescence evoked by identical stimulation after a 25-min exposure to NMR (10 mM glucose) for 25 min. The blue trace shows the

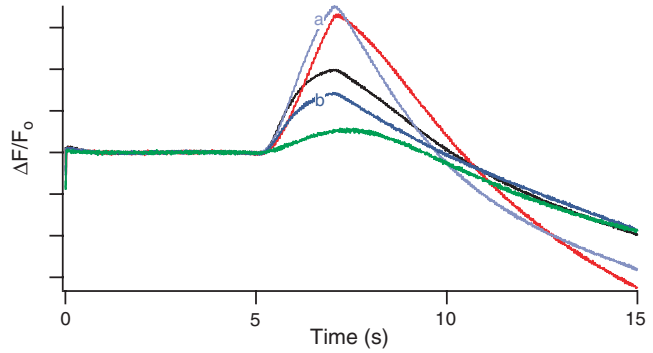

Fig. 3. FAD fluorescence responses, as a function of sodium pyruvate concentration, to a train of 30 action potential stimuli delivered at 15 Hz, 5 s after the beginning of the traces. The black trace is the control, taken after a two-hour exposure to glucose-free mouse Ringer's solution, with 0 mM sodium pyruvate. The red trace illustrates the effect of 30-min exposure to a glucose-free mouse Ringer's solution containing 1 mM sodium pyruvate. The green trace shows the effect of a 30-min exposure to a glucose-free mouse Ringer's solution containing 10 mM sodium pyruvate. The blue traces show, respectively, the effects of washing out the sodium pyruvate for 30 min (upper blue trace, (a)) and 60 min (lower blue trace, (b)). The fluorescence excitation was at 450 ± 50 nm and the emission wavelength was longer than 515 nm. Room temperature (22 ± 2°C).

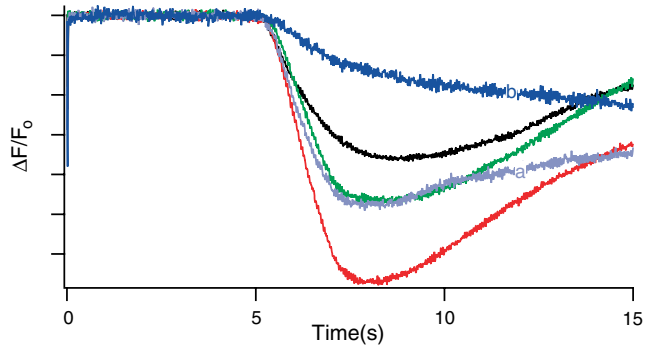

Fig. 4. NADH fluorescence responses, as a function of sodium pyruvate concentration, to a train of 30 action potential stimuli delivered at 15 Hz, 5 s after the beginning of the traces. The black trace is the control, taken after bathing the preparation for two hours in glucose-free mouse Ringer's solution containing 0 mM sodium pyruvate. The red trace illustrates the effect of a 30-min exposure to a glucose-free mouse Ringer's solution containing 1 mM sodium pyruvate. The green trace shows the effect of a 20-min exposure to a glucose-free mouse Ringer's solution containing 10 mM sodium pyruvate. The blue traces show the responses upon return to the glucose-free mouse Ringer's solution for 30 min (larger response, lower trace, (a)) and 50 min (smaller response, upper trace, (b)), respectively. The fluorescence excitation was at 350 ± 20 nm and the emission wavelength was 450 ± 50 nm. Room temperature (22 ± 2°C).

fluorescence change evoked by the same stimulation after 25 min back in the original medium containing 0 mM glucose.

We next examined the effect of pyruvate on the action potential–stimulated FAD fluorescence change in a background of glucose-free NMR. Figure 3 illustrates the results. The black trace is the control response to a train of 30 stimuli at 15 Hz, after the preparation had been incubated for two hours in the glucose-free medium. The red trace illustrates the effect of a 30-min exposure to 1 mM sodium pyruvate. The signal is substantially larger. Thirty minutes after increasing the concentration of sodium pyruvate to 10 mM, the fluorescence signal evoked by electrical stimulation is greatly depressed (green trace). Thirty minutes after washing out the pyruvate, and always in the glucose-free background, the same stimulation yields the large fluorescence signal (upper blue trace), followed at 60 minutes, by the smaller blue trace which is similar to the original control (black trace).

While Fig. 3 measured changes in FAD fluorescence, Fig. 4 represents, in a complementary fashion, the action potential-stimulated changes in NADH fluorescence excited at 350 ± 20 nm under the same experimental conditions. The black trace illustrates the control, in glucose-free medium. The red trace shows the response 30 min after the addition of 1 mM sodium pyruvate to the glucose-free solution. The green trace shows the fluorescence change 20 min after increasing the sodium pyruvate concentration to 10 mM, and the blue traces show the responses to stimulation upon return to the pyruvate-free, glucose-free NMR for 30 min (lower, larger response) and 50 min (upper, smaller response), respectively. The latter, greatly depressed trace after a 50-min recovery, probably reflects the photodynamic damage induced by the ultraviolet excitation of the fluorescence at 350 ± 20 nm.

4. Discussion

The intrinsic fluorescence, or autofluorescence, changes that are coupled to action potential stimulation of the neurohypophysial terminals[10] clearly depend upon TCA-cycle substrate availability. This is because electrical activity imposes additional needs for ATP production. Indeed, the action potential–dependent secretion of neuropeptides in the neurosecretory terminals of the mammalian neurohypophysis demands augmented ATP production, as do the NaK-ATPase and other ion pumps. This is reflected in Figs. 1 and 2, where

changes in glucose concentration from 0 to 10 mM modulate the changes in FAD and NADH fluorescence evoked by action potential stimulation. In a glucose-free MR, low concentrations of pyruvate (in this case, 1 mM) can mimic the effect of 10 mM glucose by providing necessary substrate to the TCA-cycle in a depleted preparation. An excess of substrate (e.g., 10 mM pyruvate), however, dampens the effect of action potential stimulation because the additional substrate for the dehydrogenases increases the rate at which the oxidized NAD and FAD, generated by the electron transport chain, are again reduced by the TCA-cycle. This is illustrated in Figs. 3 and 4, where the green traces show that an excess of pyruvate results in a diminished extent of oxidation in response to action potential stimulation.

These observations confirm and extend the known links between neuronal activity and bioenergetics. The changes with neuronal activity of the intrinsic fluorescence (autofluorescence) may ultimately prove useful as a probe of global areas of neuronal activation as well as of brain metabolism.

Acknowledgments

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