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Review

Use of NAD(P)H and flavoprotein autofluorescence transients to probe neuron and astrocyte responses to synaptic activation

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ABSTRACT

Synaptic stimulation in brain slices is accompanied by changes in tissue autofluorescence, which are a consequence of changes in tissue metabolism. Autofluorescence excited by ultraviolet light has been most extensively studied, and is due to reduced pyridine nucleotides (NADH and NADPH, collectively termed NAD(P)H). Stimulation generates a characteristic compound NAD(P)H response, comprising an initial fluorescence decrease and then an overshooting increase that slowly recovers to baseline levels. Evoked NAD(P)H transients are relatively easy to record, do not require the addition of exogenous indicators and have good signal–noise ratios. These characteristics make NAD(P)H imaging methods very useful for tracking the spread of neuronal activity in complex brain tissues, however the cellular basis of synaptically-evoked autofluorescence transients has been the subject of recent debate. Of particular importance is the question of whether signals are due primarily to changes in neuronal mitochondrial function, and/or whether astrocyte metabolism triggered by glutamate uptake may be a significant contributor to the overshooting NAD(P)H fluorescence increases. This mini-review addresses the subcellular origins of NAD(P)H autofluorescence and the evidence for mitochondrial and glycolytic contributions to compound transients. It is concluded that there is no direct evidence for a contribution to NAD(P)H signals from glycolysis in astrocytes following synaptic glutamate uptake. In contrast, multiple lines of evidence, including from complimentary flavoprotein autofluorescence signals, imply that mitochondrial NADH dynamics in neurons dominate compound evoked NAD(P)H transients. These signals are thus appropriate for studies of mitochondrial function and dysfunction in brain slices, in addition to providing robust maps of postsynaptic neuronal activation following physiological activation.

1. Introduction

The pioneering work of Britton Chance and colleagues in the 1950s and 1960s established that when live tissues are illuminated with near UV wavelength light, a large portion of autofluorescence generated is due to reduced nicotinamide adenine dinucleotide (NADH) and furthermore that changes in metabolic state could be readily monitored by changes in autofluorescence intensity (Chance, 2004). Over 50 years of work has established that NADH autofluorescence signals can be effectively used to monitor neuronal activation in isolated neurons, brain slices and intact brain. The history and application of these and related autofluorescence approaches have been extensively reviewed (Anderson and Meyer, 2002; Chance, 2004; Kann and Kovacs, 2007; Mayevsky and Chance, 2007; Mayevsky and Rogatsky, 2007; Reinert et al., 2007; Tohmi et al., 2009). The present review concentrates on issues concerning the cellular basis of NADH autofluorescence transients, and mechanisms underlying their generation following synaptic stimulation. Over the years, there have developed two views on the origins of NADH changes that accompany electrical activity in brain slices: one, that changes in neuronal mitochondrial function are primarily responsible, and two, that glycolysis triggered by glutamate transport into astrocytes is a major contributor. Understanding the contributions of these two different mechanisms is important, particularly since the autofluorescence approach is being increasingly used to investigate mechanisms of neuronal and astrocyte metabolism in complex brain preparations, including questions of metabolic coupling between these cell types.

2. NADH fluorescence

Redox transitions between NADH and NAD^+ are required for a multitude of metabolic processes, including many central to neuronal function. Within mitochondria, NADH serves as a principal electron donor in the electron transport chain. Oxidation

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of NADH to NAD^+ occurs at complex I, and subsequent electron transfer is responsible for generation of the proton gradient across the inner mitochondrial membrane, which in turn is required for ATP synthesis (Nicholls and Ferguson, 2002). NADH required for this important function is obtained from reduction of NAD^+ by tricarboxylic acid (TCA) cycle activity, and can also be transferred from the cytosol via shuttle systems (McKenna et al., 2006; Satrustegui et al., 2007). Glycolysis depends on NAD^+ as a cofactor for glyceraldehyde phosphate dehydrogenase activity, and oxidation of cytosolic NADH by lactate dehydrogenase can maintain cytosolic NAD^+ requirements required for anaerobic glycolysis.

Optical measurements of NADH/NAD^+ transitions are relatively straightforward, because NADH is strongly fluorescent (particularly in mitochondrial compartments, see below) while NAD^+ is non-fluorescent (Chance et al., 1962). The excitation and emission spectra of purified NADH are relatively broad (peak excitation in the near UV ~340–360 nm; emission in the blue range ~430–450 nm (Chance et al., 1962, 1979; Aubin, 1979)) and early studies confirmed that the fluorescence spectra of a range of neuronal tissues (including brain, single neurons and axons) are similar to pure reduced NADH (Chance et al., 1962; Terzuolo et al., 1966; Doane, 1967). NADH fluorescence can be effectively excited with either standard wide-field epifluorescence illumination or confocal microscopes equipped with a standard UV laser source. Recent work has demonstrated effective 2 photon excitation, which can offer improved spatial resolution and penetration into thick tissues (Patterson et al., 2000; Huang et al., 2002; Zipfel et al., 2003). Autofluorescence detected with photomultiplier devices or CCD cameras is generally quite robust and stable, as long as measures are taken to minimize photobleaching and tissue damage due to excessive UV exposures. Generally, improving detection sensitivity with high NA objectives and cooled CCD cameras, and minimizing exposure rates and/or durations is helpful for long-term recordings of neuronal activity (Duchen et al., 2003). With these considerations, toxicity and bleaching can be avoided over recording periods of hours, as measured from the reproducibility of synaptically-evoked responses. Alternatively, deliberate bleaching of NADH with very high intensity UV flashes can be useful for kinetic studies of tissue NADH generation, as the enzymatic regeneration of NADH within tissues can be monitored (Combs and Balaban, 2001, 2004; Joubert et al., 2004).

From early studies of a range of tissues, it was concluded that the intensity of cellular NADH fluorescence was not uniform, but was strongly increased in the mitochondrial compartment, and quenched when located in the cytosol (Chance and Baltscheffsky, 1958). The reason(s) for enhanced mitochondrial fluorescence was not immediately established, but association with matrix proteins was considered a major contributor (Chance and Baltscheffsky, 1958; Avi-Dor et al., 1962; Estabrook, 1962). Jobsis and colleagues concluded that NADH fluorescence measured in intact cat cortex was primarily mitochondrial, and that enhancement of fluorescence was due to mitochondrial dehydrogenases (Jobsis et al., 1971). Recent work utilizing isolated mitochondria has shown that the NADH fluorescence lifetime is greatly enhanced in mitochondria and appears that this is due to binding to complex I (Blinova et al., 2005, 2008). Substantially increased NADH fluorescence in the mitochondrial environment may be one reason why mitochondrial signals seem to dominate measured responses in many cases, despite the fact that NADH is involved in a multitude of extra-mitochondrial reactions (including glycolysis) occurring simultaneously throughout a cell.

3. NADPH and NAD(P)H autofluorescence

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and NADH have very similar optical properties, and therefore is expected that NADPH also contributes to some extent to total autofluorescence signals. NADPH levels in brain tissue have long been recognized to be much lower than NADH in brain tissue (Chance et al., 1962) and more recent HPLC studies have shown that NADPH/NADP levels are in the order of 10-fold lower than NADH/NAD^+ levels throughout the mouse brain (midbrain, hippocampus, striatum and cortex) (Klaidman et al., 1995). In addition to lower levels, NADPH fluorescence is enhanced much less than NADH in mitochondria (Avi-Dor et al., 1962), further contributing to the dominance of NADH fluorescence in tissue measurements. In contrast to NADH, NADPH oxidation does not directly contribute to mitochondrial electron transport, and would be expected to normally make little contribution to measured autofluorescence transients during synaptic activity. While this assumption is likely to be valid for most physiological studies, changes in NADPH levels may become significant under some conditions, for example under pathologic conditions where NADPH oxidase activity may be greatly elevated (Suh et al., 2007). These considerations have led a number of groups to refer to "NAD(P)H" auofluorecence, to acknowledge the possible contribution from both reduced pyridine nucleotides (e.g. Duchen, 1992; Barbour et al., 1993; Patterson et al., 2000; Schuchmann et al., 2001; Huang et al., 2002; Kunz et al., 2002; Kann et al., 2003; Shuttleworth et al., 2003; Galeffi et al., 2007; Kahraman and Fiskum, 2007) and this terminology will be used in the following discussion of responses to nerve stimulation.

4. NAD(P)H autofluorescence transients evoked by neuronal stimulation

An early study from Lipton (1973) used a dual wavelength fluorimeter to record NAD(P)H autofluorescence transients following electrical stimulation of guinea-pig neocortical slices. Quite intense periods of stimulation were utilized, and resulted in an initial fluorescence decrease, followed by a longer lasting increase which gradually recovered to pre-stimulus levels (Lipton, 1973). Very similar responses can be collected with CCD-based imaging systems, and Fig. 1 shows an example following stimulation of a mouse hippocampal slice. In this example, a short train of stimuli was applied to Schaffer collateral inputs (50 Hz, 500 ms), and fluorescence changes were extracted from a region of interest in CA1 stratum radiatum. A neuronal basis of the signal (rather than an artifactual response to strong electrical stimulation) was verified by tetrodotoxin block, and the response to this brief stimulus was also abolished by block of ionotropic glutamate receptors, implying a dependence on postsynaptic depolarization of CA1 neurons (Shuttleworth et al., 2003). It is useful to note that the signal shown here is the response to a single stimulus trial, and that averaging of multiple trials was not required to achieve a good signal/noise. The amplitude of signals (in the order of a few percent change in total fluorescence) is much greater than is usually observed with intrinsic optical signal (detected by changes in light transmission) and initial fluorescence decreases can be resolved within 100 ms of the onset of the stimulus.

Very similar compound NAD(P)H responses following synaptic stimulation in hippocampal slices have been described by a number of groups using wide-field epifluorescence systems (e.g. Schuchmann et al., 2001; Foster et al., 2005). A 2 photon study in rat hippocampal slices also generated very similar signals, with an initial NADH fluorescence decrease followed by a long-lasting overshoot response (Kasischke et al., 2004). There are multiple cell types in the brain slice preparation, including postsynaptic dendritic processes, presynaptic terminals and astrocytes. While a number of recent imaging studies have emphasized a mitochondrial and neuronal basis for these signals (Schuchmann et al., 2001; Brennan et al., 2006), it has also been speculated that astrocytic