

Changes in FAD and NADH Fluorescence in Neurosecretory Terminals Are Triggered by Calcium Entry and by ADP Production

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Abstract. We measured changes in the intrinsic fluorescence (*IF*) of the neurosecretory terminals of the mouse neurohypophysis during brief (1–2 s) trains of stimuli. With fluorescence excitation at either 350 ± 20 or 450 ± 50 nm, and with emission measured, respectively, at 450 ± 50 or ≥ 520 nm, $\Delta F/F_0$ was ~ 5 – 8% for a 2 s train of 30 action potentials. The *IF* changes lagged the onset of stimulation by ~ 100 ms and were eliminated by $1 \mu\text{M}$ tetrodotoxin (TTX). The signals were partially inhibited by $500 \mu\text{M}$ Cd^{2+} , by substitution of Mg^{2+} for Ca^{2+} , by Ca^{2+} -free Ringer's with 0.5 mM EGTA, and by $50 \mu\text{M}$ ouabain. The *IF* signals were also sensitive to the mitochondrial metabolic inhibitors CCCP ($0.3 \mu\text{M}$), FCCP ($0.3 \mu\text{M}$), and NaN_3 (0.3 mM), and their amplitude reflected the partial pressure of oxygen (pO_2) in the bath. Resting fluorescence at both 350 nm and 450 nm exhibited significant bleaching. Flavin adenine dinucleotide (FAD) is fluorescent, while its reduced form FADH_2 is relatively non-fluorescent; conversely, NADH is fluorescent, while its oxidized form NAD is non-fluorescent. Thus, our experiments suggest that the stimulus-coupled rise in $[\text{Ca}^{2+}]_i$ triggers an increase in FAD and NAD as FADH_2 and NADH are oxidized, but that elevation of $[\text{Ca}^{2+}]_i$ alone cannot account for the totality of changes in intrinsic fluorescence.

Key words: Autofluorescence — Flavoproteins, NADH — Neurosecretory terminals — Intrinsic optical changes

Introduction

Neuropeptide and neurotransmitter release are tightly regulated processes that culminate in the calcium-dependent fusion of dense core granules and synaptic vesicles and the exocytosis of their contents (Douglas & Poisner, 1964; Katz & Miledi, 1969). Because both dense core granules and synaptic vesicles undergo an obligatory maturation process that requires ATP (Baker & Knight, 1981; Morita et al., 1988; Banerjee et al., 1996; Hosaka & Sudhof, 1998), it may be expected that mitochondrial activity will be coupled to excitation and secretion at nerve terminals. Indeed, there are multiple examples of coupling between neuronal activity and metabolic changes. Positron emission tomography (PET) studies have revealed non-oxidative glucose consumption during local neuronal activity (Fox et al., 1988) and *in vivo* reflectance measurements in cat cortex (Malonek & Grinvald, 1996) have demonstrated optical correlates of oxidative metabolism that are driven by neuronal activity. More than four decades ago, Britton Chance and colleagues showed that the intrinsic fluorescence of β -nicotinamide adenine dinucleotide (NADH) is an indicator of oxidative (Chance & Baltscheffsky, 1958; Chance & Williams, 1955a, b, c) as well as glycolytic (Aubert, Chance & Keynes, 1964) energy metabolism and demonstrated, shortly thereafter, that changes in the redox state of tissue could be monitored by following the fluorescence of flavoproteins (Chance, 1966; Chance, Mela & Wong, 1968). These observations were later extended to brain (Austin, Chance & Barlow, 1978), with the introduction of a variety of powerful new techniques (Chance, 1991). Since NADH is fluorescent, while its oxidized form NAD is not, and conversely, flavin adenine dinucleotide (FAD) is significantly more fluorescent than its reduced form FADH_2 , pyridine

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nucleotide-derived and flavoprotein-derived fluorescence changes tend to be opposite in sign.

More recently, stimulus-evoked changes in NADH fluorescence were observed from both neurons and glia in hippocampal slice preparations using 2-photon excitation of the pyridine nucleotides (Kasischke et al., 2004)¹. These experiments suggested that the initial response to neuronal activity is oxidative metabolism, with a delayed activation of astrocytic glycolysis. *In vivo* measurements of flavoprotein autofluorescence in mouse cerebellum (Reinert et al., 2004) as well, have shown clearly that the intrinsic fluorescence signals in brain are mitochondrial in origin and probably reflect the oxidation and subsequent reduction of NADH and FADH₂ (Benson et al., 1979; Duchen, 1992). In the report by Reinert et al. (2004), the flavoprotein signal is attributed to mitochondrial events in the Purkinje cells and cerebellar interneurons that are postsynaptic to the stimulated beam of parallel fibers. At virtually the same time, it was shown that biphasic stimulus-evoked changes in NADH fluorescence (as well as oppositely directed flavoprotein fluorescence signals) could be used to monitor neuronal activation in area CA1 of murine hippocampal slices (Shuttleworth, Brennan & Connor, 2003), and that, similarly, these intrinsic signals reflected postsynaptic rather than presynaptic activity. In disagreement with observations in isolated dorsal root ganglion neurons (Duchen, 1992), these authors concluded that the changes in NADH fluorescence do not depend upon intracellular Ca²⁺ dynamics; “even protracted removal of extracellular Ca²⁺” (and consequent block of intracellular Ca²⁺ transients) did not diminish the NADH transients (Shuttleworth et al., 2003).

In none of these recent studies was the intracellular localization of the activated mitochondria identified. Thus, it was with great interest that we began to examine the intrinsic fluorescence signals that we had discovered, independently, in the mouse neurohypophysis, a neurosecretory organ that comprises, primarily, nerve terminals and secretory swellings. The mammalian neurohypophysis is a system in which intrinsic optical signals, e.g., changes in large angle light scattering, are easily measured (Salzberg, Obaid & Gainer, 1985). Here the relative homogeneity of the preparation, and the enormous proliferation of

magnocellular neuron terminals, undoubtedly contribute to the generation of these relatively large optical changes. Magnocellular neurons, located in the hypothalamus, project their axons as bundles of fibers through the median eminence and infundibular stalk to terminate in the neurohypophysis, where neuropeptides and proteins are secreted into the local circulation. In the rat, for example, some 18,000 magnocellular neurons give rise to ~40,000,000 terminals in the neurohypophysis, and a very high proportion of the excitable membrane belongs to terminals or secretory swellings (Nordmann, 1977). At the same time, the precise control of the timing of the action potential in a large, synchronously activated population of nerve terminals, and the attendant large IF signals, make the neurohypophysis a particularly favorable system for understanding any intrinsic optical changes in neurons and their relation to secretion of neuropeptides and neurotransmitters. Preliminary results have been reported in abstract form (Kosterin et al., 2005; Kim et al., 2006).

Materials and Methods

PREPARATION AND APPARATUS

Details of the preparation and apparatus have been reported previously (Salzberg et al., 1983; Salzberg et al., 1985; Gainer et al., 1986; Obaid, Flores & Salzberg, 1989; Parsons, Obaid & Salzberg, 1992; Obaid et al., 1995; Muschol & Salzberg, 2000). Typically, 30–60 day old CD-1 female mice (Jackson Laboratories, Bar Harbor, ME) were sacrificed using CO₂ asphyxiation followed by decapitation. 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. The neurohypophysis was mounted in an optical recording chamber and superfused with physiological saline (normal mouse Ringer's or NMR) containing (in mM): NaCl 155, KCl 5.6, CaCl₂ 2.2, MgCl₂ 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 tissue. Trains of stimuli were created with a Master-8 pulse generator (A.M.P.I. Jerusalem, Israel) driving a stimulus-isolator (SIO-102, Warner Instruments, Inc., Hamden, CT). Two platinumiridium (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 ± 2°C) and all chemicals used were from Sigma (St. Louis, MO) unless otherwise indicated.

OPTICAL MEASUREMENTS OF CHANGES IN CYTOSOLIC CALCIUM, TRANS-MEMBRANE VOLTAGE, AND INTRINSIC FLUORESCENCE

All fluorescence measurements were performed on a UEM upright microscope (Zeiss, Oberkochen, Germany) using either a 20 ×, 0.5 numerical aperture, water-immersion objective (Leica Micro-systems, Bensheim, Germany) or, in the case of NADH measurements, a 20 ×, 0.65 numerical aperture quartz objective (Partec GmbH, Munster, Germany). Either a 300 W tungsten

¹Here and elsewhere, we refer to reduced pyridine nucleotide exclusively as NADH, since its identity was established definitively by analytical biochemical measurements of the rapid response of NADH and the lack of response of NADPH to mitochondrial redox state (Chance, B., Schoener, B., Krejci, K., Russmann, H., Wesemann, W., Schnitger, H., Bucher, T. 1965. Kinetics of Fluorescence and Metabolite Changes in Rat Liver During a Cycle of Ischemia. *Biochem Zeit* 341:325–333).

halogen lamp or a 150W or 250W Xenon short arc was used as the epi-illumination source.

For calcium measurements, the preparation was incubated for 90 min in a Ringer's solution containing 10–20 μM of the low-affinity ($K_{D,\text{Ca}} = 22 \mu\text{M}$) calcium indicator dye Mag-Fluo-4/AM (M14206, Molecular Probes (Invitrogen), Eugene, OR), 0.9% dimethyl sulfoxide, and 0.1% Pluronic F-127 (BASF, Wyandotte, MI). The filter set comprised a $470 \pm 20 \text{ nm}$ excitation filter, 500 nm dichroic mirror (Chroma Technology, Brattleboro, VT) and 515 nm long-pass filter (OG 515, Schott Optical Glass Co. Duryea, PA).

For optical measurement of transmembrane voltage, the neurohypophysis was incubated for 40 min in a Ringer's solution containing 50 μM of the fast-response potentiometric naphthylstyryl-pyridinium dye Di-4-ANEPPDHQ (JPW-5029) (Obaid et al., 2004) in a Ringer's solution containing 0.25% (v/v) ethanol. The filter set used for Di-4-ANEPPDHQ comprised a $530 \pm 45 \text{ nm}$ excitation filter (Corion, Holliston MA), a 585 nm dichroic mirror (Chroma Technology, Brattleboro, VT) and a 610 nm long-pass filter (RG 610, Schott Optical Glass Co). Changes in light scattering (Salzberg et al., 1985) were monitored with the same UEM upright microscope (Zeiss, Oberkochen, Germany), using a 740 nm LED (Roithner LaserTechnik, Vienna, Austria) as the transillumination source (Rumyantsev et al., 2004; Salzberg et al., 2005). For intrinsic fluorescence measurements, the filter sets consisted of either a $450 \pm 50 \text{ nm}$ excitation filter (Chroma Technology, Brattleboro, VT), a 515 nm dichroic mirror (Chroma Technology) and a Hi-Q 520 nm long-pass filter (Chroma Technology), or a $350 \pm 20 \text{ nm}$ (Chroma Technology) excitation filter, a 395 nm dichroic mirror (Chroma Technology), and a $450 \pm 50 \text{ nm}$ (Chroma Technology) band-pass emission filter.

PHOTODIODE MEASUREMENTS

Tissue-averaged measurements of optical signals were obtained with a large area photodiode (PV-444, Perkin-Elmer Optoelectronics, Vaudreuil, Canada). The photocurrent was converted to a voltage signal with either a commercial current-to-voltage converter (DLPCA 200, Femto Messtechnik, Berlin Germany) or 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 / data sampling pairs were 500 Hz / 1 kHz for calcium changes, 1 kHz / 2 kHz for voltage recordings, and 20–50 Hz / 1 kHz for intrinsic fluorescence (*IF*) signals.

DATA ANALYSIS

All data analysis was performed using the IGOR data analysis software (Wavemetrics, Lake Oswego, OR). Intrinsic fluorescence (*IF*) traces were bleach-corrected by fitting (to $\geq 95\%$ confidence level) a single exponential to the baseline 6–8 s prior to stimulation and subtracting the extrapolated bleaching curve from the record. $\Delta F/F_0$ was calculated using the extrapolated value of F_0 at the peak of the fluorescence change. The ΔCa signal (see Fig. 1B) was bleach-corrected by subtraction of the linear extrapolation of a 1 s baseline segment at the beginning of the recording. Voltage-sensitive dye records and light scattering signals required no baseline correction.

Results

Figure 1 illustrates the variety of extrinsic and intrinsic optical signals that can be recorded from the

neurosecretory terminals of the mouse neurohypophysis *without signal averaging*. Panel A shows an optical recording of a train of seven action potentials (15 Hz) obtained by monitoring the fluorescence of the neurohypophysis following staining with the potentiometric naphthylstyryl-pyridinium dye Di-4-ANEPPDHQ (Obaid et al., 2004). The rising phase of the action potentials is faithfully recorded, but the after-hyperpolarization is replaced with an apparent after-depolarization that actually reflects the response of glial cells (pituicytes) to an increase in $[\text{K}^+]_o$. This interpretation is confirmed by experiments in which the terminals were stained from the *inside* with an impermeant voltage-sensitive dye (by backfilling the terminals from the cut end of the infundibular stalk) (Muschol et al., 2003) so that there was no staining of pituicytes. As expected under those conditions, the action potentials were inverted and they were followed by a clear after-hyperpolarization. Panel B shows a measurement of $[\text{Ca}^{2+}]_i$ in neurohypophysial neurosecretory terminals using the low-affinity (22 μM) Ca-indicator dye Mag-Fluo-4 AM (Muschol & Salzberg, 2000). Records of this type, with their extraordinary S:N ratio, provide important information concerning $[\text{Ca}^{2+}]_i$ transients, build-up of residual $[\text{Ca}^{2+}]_i$, and $[\text{Ca}^{2+}]_i$ clearance (Muschol & Salzberg, 2000). Panel C, by way of contrast, illustrates an *intrinsic* optical signal, large-angle light-scattering, that can be recorded from the nerve terminals of mammalian (but not amphibian) neurohypophysis (Salzberg et al., 1985; Obaid et al., 1989; Parsons et al., 1992). This signal comprises at least two separate components. The initial sharp decreases in transmitted light intensity (*E*-waves; increased large-angle scattering) at $675 \pm 50 \text{ nm}$ report the arrival of the action potential (and action currents) in the terminals, and the slow, sustained increases in transmitted light intensity (decrease in light scattering) are related to neuropeptide secretion (Salzberg et al., 1985; Obaid et al., 1989; Parsons et al., 1992).

Because relatively large and very rapid changes in light scattering could be recorded from the mouse neurohypophysis, we decided to examine the response to electrical stimulation of another intrinsic optical property, the neurosecretory terminal autofluorescence. Panel D shows, on a slower time-base, the change in intrinsic fluorescence (*IF*) following 40 stimuli delivered at 40 Hz. Excitation was at $450 \pm 50 \text{ nm}$, with emission recorded with a Hi-Q 520 long-pass filter (HQLP520, Chroma Technology, Brattleboro, VT). The *IF* signal is long-lasting and dramatically biphasic. Given the large *IF* signal excited at a wavelength appropriate to flavoprotein (FAD) absorption, we thought it would be interesting to look for an *IF* signal at 350 nm, near the peak of reduced pyridine nucleotide (NADH) absorption. Panel E shows the response to 40 stimuli (40 Hz)

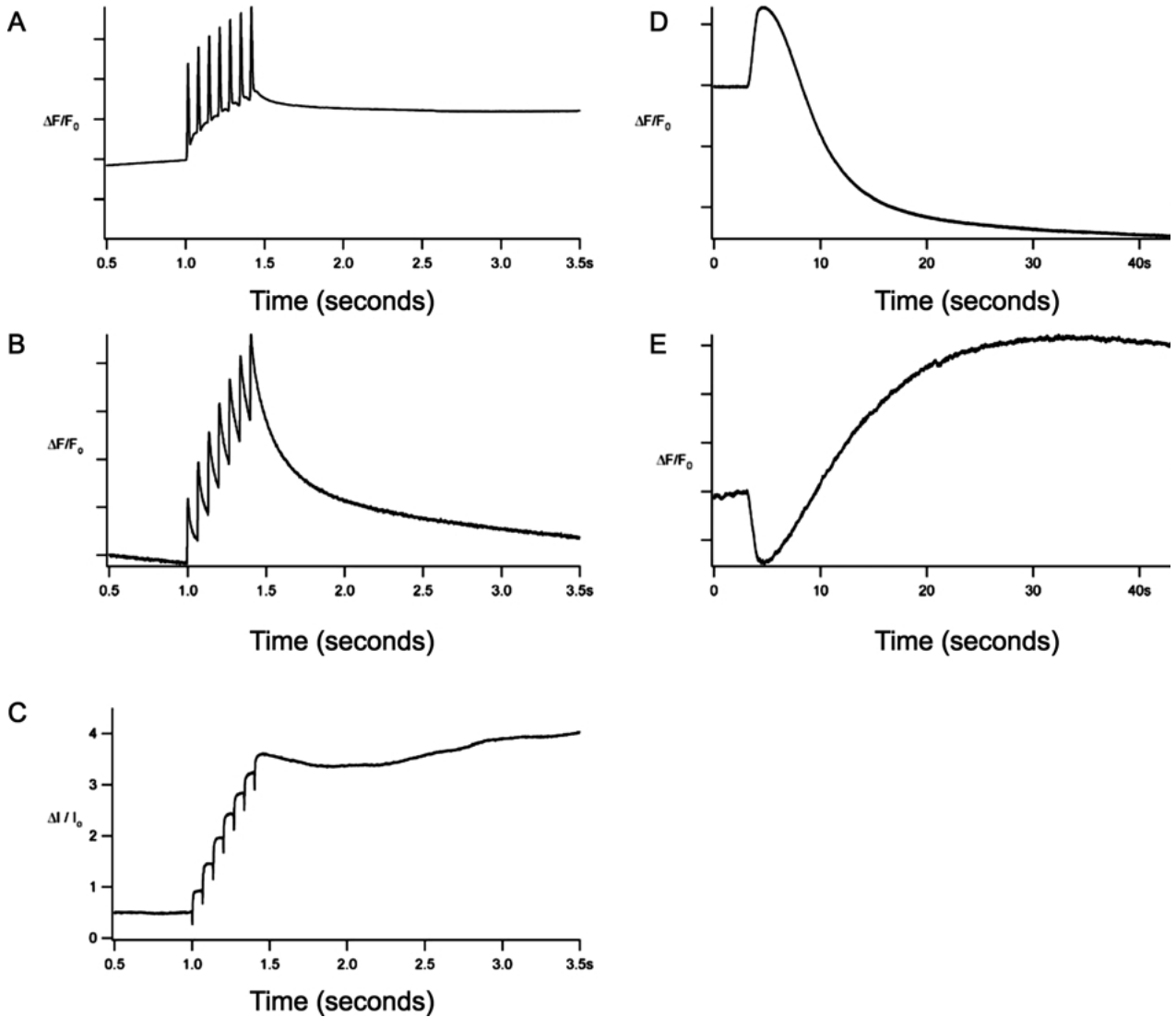


Fig. 1. Comparison of extrinsic and intrinsic optical changes recorded from nerve terminals of the mouse neurohypophysis. (A) Optical recording of membrane potential changes in response to a train of seven stimuli at 15 Hz following staining with the naphthylstyryl-pyridinium potentiometric dye Di-4-ANEPPDHQ. Stimulation was initiated at $t = 1$ s. Filters were (excitation / dichroic / emission): 530 ± 45 nm (with Schott KG1 heat filter) / 585 nm / Schott RG 610 glass. 300 W tungsten-halogen lamp. Feedback resistor 10 M Ω ; 2nd stage gain $\times 100$; bandwidth 500 Hz; 1 kHz sampling frequency. (B) Optical recording of intracellular Ca^{2+} transients during a train of stimuli identical to that in A. 470 ± 20 nm (with Schott KG1 heat filter) / 500 nm / Schott OG 515 glass. 300 W tungsten-halogen lamp. Feedback resistor 1 G Ω ; bandwidth 500 Hz; 1 kHz sampling frequency. (C) Intrinsic optical changes (light scattering) at 675 ± 50 nm in response to the same stimulus train. Feedback resistor 1 M Ω ; 2nd stage gain $\times 150$; bandwidth 500 Hz; 1 kHz sampling frequency. (D) Intrinsic fluorescence (IF) signal, on a slower time base, recorded in response to a train of 40 stimuli at 40 Hz initiated at $t = 3$ s. 450 ± 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 50 Hz; 200 Hz sampling frequency. (E) Intrinsic fluorescence (IF) signal recorded in response to the same train of 40 stimuli as in panel D, but at 350 ± 20 nm / 395 nm / 450 ± 50 nm; 250 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 200 Hz sampling frequency. Temperature 23 $^{\circ}$ C. All traces in this and subsequent figures are single sweeps.

recorded at ≥ 520 nm with 350 ± 20 nm excitation. Once again, the IF change is strongly biphasic, with the early response a decrease in fluorescence, opposite to that measured with 450 ± 50 nm excitation. We have not attempted, here, to measure the kinetics of the intrinsic fluorescence signals. Reinert et al. (2004) reported that in the molecular layer of the cerebellum, the early rapid rise in flavoprotein (FAD) fluo-

rescence occurred within 50 ± 30 ms of stimulation, and this value is consistent with our observations, and with in vitro measurements (Chance & Erecinska, 1972) of a 70 ms time to half maximum at 25 $^{\circ}$ C (see Discussion).

It is reasonable to expect that signals that report the oxidation of either reduced flavoprotein (FADH₂) or pyridine nucleotide (NADH) should be

sensitive to the partial pressure of oxygen (pO_2) in the bath. Figure 2 illustrates these effects on flavoprotein fluorescence (Fig. 2A) and NADH fluorescence (Fig. 2B). All of the records shown in Fig. 2 were obtained in a Ringer's solution containing elevated (10 mM) $[Ca^{2+}]_o$. It is interesting to note the relatively large effect on the FAD fluorescence signal compared with that on the NADH signal, and also that it is the second phase of the NADH signal that is primarily affected by pO_2 , with a decrease in the magnitude of the IF in the presence of higher pO_2 .

The lipid-soluble weak acids FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone) and CCCP (carbonyl cyanide m-chloro phenyl hydrazone) are powerful mitochondrial uncouplers that specifically increase the proton permeability of the inner mitochondrial membrane and disconnect the electron transport chain from the formation of ATP. In this manner, they abolish the obligatory linkage between the respiratory chain and the phosphorylation system that is found in intact mitochondria. When the electron transport chain is blocked, all the intermediates on the substrate side of the block become more reduced, while all these on the oxygen side become more oxidized. We examined the effect of these two mitochondrial uncouplers on the IF signals that are triggered by neurosecretory terminal action potentials. Figs. 3 and 4 illustrate the suppression of the IF signals by 5 μ M FCCP and CCCP, respectively. In both instances, the effect of the uncoupler is more dramatic on the NADH-derived signal (350 nm, Figs. 3B and 4B) than it is on the FAD signal (450 nm, Figs. 3A and 4A). Neither the effects of FCCP nor CCCP could be reversed.

Sodium azide (NaN_3) is an inhibitor of the mitochondrial enzyme cytochrome C oxidase (Chance & Williams, 1956). At 0.3 mM, NaN_3 completely eliminates the IF signals at 450 nm (FAD) and 350 nm (NADH). This is seen clearly in Figs. 5A and 5B. Furthermore, in contrast with the uncouplers FCCP and CCCP, the effects of NaN_3 are fully reversible (Figs. 5A and B). Note that, exclusively in Fig. 5B, the records are corrected for bleaching by subtracting traces without stimulation, since these records were too noisy to yield good exponential fits to the 3 s pre-stimulus baseline.

Given the prominent role that mitochondria play in cellular Ca^{2+} homeostasis (Chance, 1965; Duchon, 1992, 1999; Landolfi et al., 1998; Lee & Tse, 2005), it was of interest to determine whether, and to what degree, the Ca^{2+} that enters neurohypophysial terminals through voltage-gated Ca^{2+} channels during the action potential is responsible for the coupling between excitation and the intrinsic fluorescence signals. Figure 6 illustrates the effect of blocking Ca^{2+} entry using a high (1.0 mM) concentration of Cd^{2+} . Evidently, both the FAD signal (at 450 nm) (panel A) and the NADH signal (at 350 nm) (panel B) are

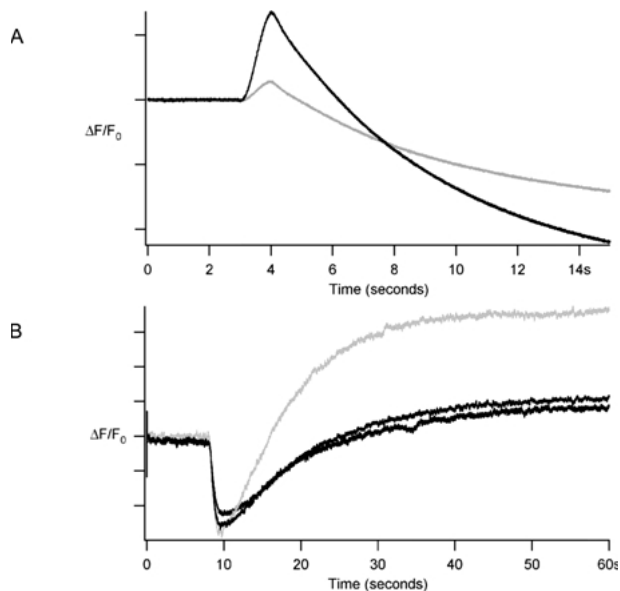


Fig. 2. The effect of pO_2 on the intrinsic fluorescence (IF) signals from mammalian nerve terminals. (A) The effect of pO_2 on the FAD fluorescence signal in response to a train of 40 stimuli at 40 Hz initiated at $t = 3$ s. 450 ± 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 50 Hz; 200 Hz sampling frequency. Grey trace was obtained in non-oxygenated, 10 mM Ca^{2+} Ringer's solution; black trace illustrates the effect of increasing the pO_2 of the Ringer's solution by bubbling for ~ 20 minutes with 95% O_2 / 5% CO_2 . (B) The effect of pO_2 on the NADH fluorescence signal in response to the same stimulus initiated at 8 s. 350 ± 20 nm / 395 nm / 450 ± 50 nm. 250 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. Grey trace, non-oxygenated 10 mM Ca^{2+} Ringer's solution; black traces were recorded after increasing pO_2 , by bubbling the Ringer's solution for 30 and 60 minutes with 95% O_2 / 5% CO_2 . Temperature 23°C.

dramatically reduced in amplitude, with substantial increases in the times-to-peak. It is interesting to note that the second (delayed) phase of both signals seems to be eliminated entirely. Since the dominant voltage-activated calcium channel (VACC) in mouse neurohypophysial terminals is N-type (Obaid et al., 1989), we were not surprised to observe that ω -conotoxin GVIA also reduced the amplitude of both FAD- and NADH-associated fluorescence signals (*data not shown.*)

The effect of $[Ca^{2+}]_o$ on the IF signals is illustrated in Fig. 7. Figures 7A and B demonstrate that complete removal of Ca^{2+} from the extracellular medium, with 0.5 mM EGTA added, substantially reduces the amplitudes of both the FAD and the NADH fluorescence signals. The kinetics are slowed and, it appears, the late phase is either delayed or eliminated. Nonetheless, in the complete absence of extracellular Ca^{2+} , significant IF changes remain and these are coupled to excitation. Figures 7C and D demonstrate, that simply lowering $[Ca^{2+}]_o$ from 2.2

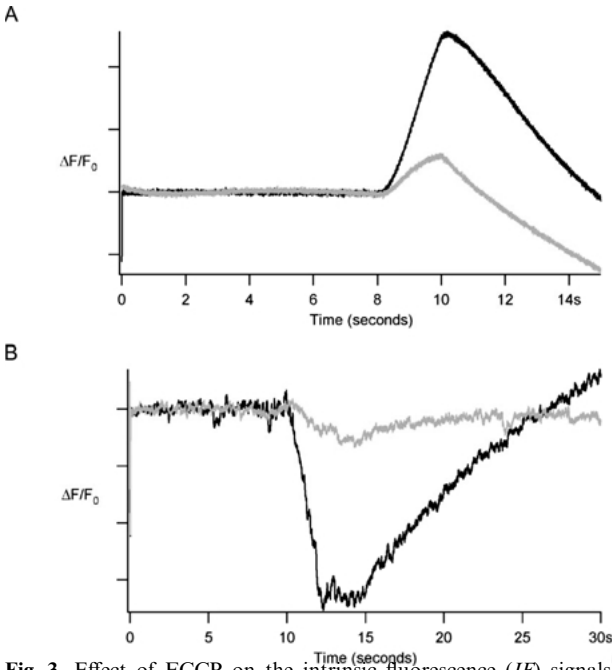


Fig. 3. Effect of FCCP on the intrinsic fluorescence (*IF*) signals from mammalian nerve terminals. (A) Effect of 5 μM FCCP on the FAD fluorescence signal in response to a train of 30 stimuli at 15 Hz initiated at $t = 8$ s. 450 ± 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 50 Hz; 200 Hz sampling frequency. Black trace, normal mouse Ringer's (NMR); grey trace, 40 min in 5 μM FCCP. (B) Effect of 5 μM FCCP on the NADH fluorescence signal in response to the same stimulus used in A, initiated at $t = 10$ s. 350 ± 20 nm / 395 nm / 450 ± 50 nm. 250 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. Black trace, normal mouse Ringer's (NMR); grey trace, 40 min in 5 μM FCCP. Temperature 23°C.

mM (NMR) to 1.0 mM, with Mg^{2+} substitution, is enough to reduce the size of the FAD fluorescence signal, although the effect upon the NADH signal is negligible, except for a slight slowing of the kinetics. Conversely, raising the $[\text{Ca}^{2+}]_o$ from 2.2 mM to 10 mM (Figs. 7E and F) increased the amplitude of both IF signals by $\sim 20\%$. The observations made using 10 mM Ca^{2+} were somewhat puzzling, however. Reproducibly, the IF signals at 350 nm and 450 nm measured 10 minutes after changing the Ringer's solution from 2.2 mM Ca^{2+} (NMR) to one containing 10 mM Ca^{2+} were substantially larger than those illustrated in Figs. 7E and F. However, after 20 minutes in 10 mM Ca^{2+} the signals decreased in amplitude to the size shown, where they remained stable.

Figure 8 illustrates the approximate $[\text{Ca}^{2+}]_o$ -dependence of the FAD and NADH *IF* signals under the condition in which the neurosecretory terminals are stimulated at 15 Hz for two seconds (30 stimuli). The variability from experiment to experiment was quite large, but, nonetheless, it appears that the half-

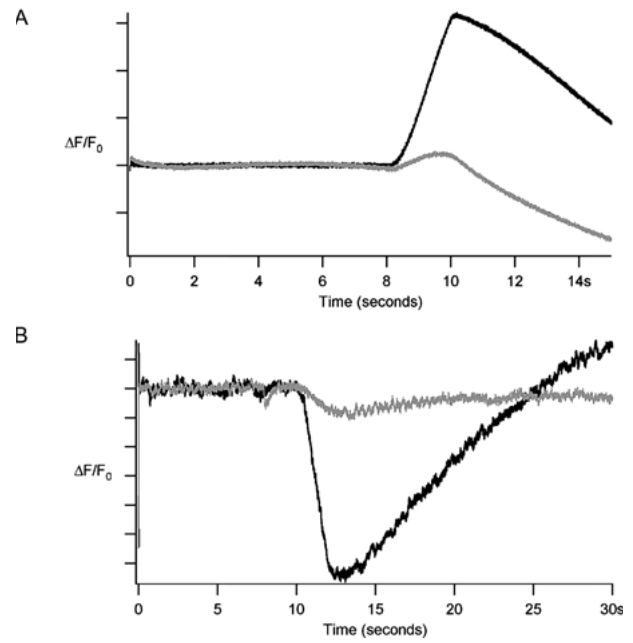


Fig. 4. Effect of CCCP on the intrinsic fluorescence (*IF*) signals from mammalian nerve terminals. (A) The effect of 5 μM CCCP on the FAD fluorescence signal in response to a train of 30 stimuli at 15 Hz initiated at $t = 8$ s. 450 ± 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 50 Hz; 1 kHz sampling frequency. Black trace, normal mouse Ringer's (NMR); grey trace, 40 min in 5 μM CCCP. (B) Effect of 5 μM CCCP on the NADH fluorescence signal in response to the same stimulus initiated at $t = 10$ s. 350 ± 20 nm // 395 nm // 450 ± 50 nm. 250 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. Black trace, normal mouse Ringer's (NMR); grey trace, 30 min in 5 μM CCCP. Temperature 23°C.

maximal Ca^{2+} -concentration is higher for the FAD (1.0 mM) than for the NADH (0.4 mM) intrinsic fluorescence signal. However, we believe that the data do not support the attribution of a $K_{D,\text{Ca}}$.

The persistence of residual *IF* signals, albeit smaller and slower ones, in the complete absence of $[\text{Ca}^{2+}]_o$ requires explanation. Two possible mediators of the information transfer from the terminal plasma membrane to the mitochondria, besides Ca^{2+} , suggest themselves. The first is $[\text{Na}^+]_i$, possibly acting through a mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (see Discussion). The second is ADP, whose concentration is expected to rise as the Na^+/K^+ ATPase in the plasma membrane hydrolyzes ATP to restore the ionic concentration gradients that begin to dissipate with repetitive stimulation of the small neurosecretory terminals. To examine the possible role of the Na^+/K^+ ATPase, and of ADP, we blocked the pump with ouabain, after replacing $[\text{Ca}^{2+}]_o$ in the Ringer's solution with 2.2 mM Mg^{2+} and adding 0.5 mM EGTA. The results are shown in Fig. 9. The dark grey traces illustrate the effects of eliminating

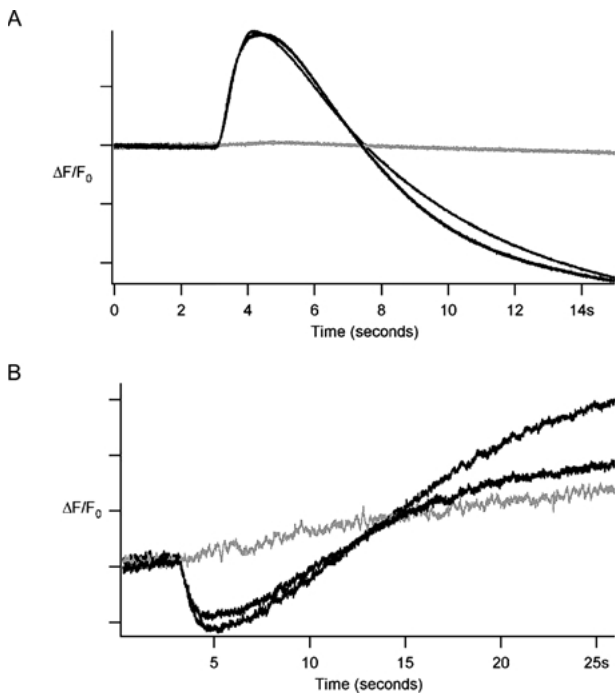


Fig. 5. Effect of sodium azide on the intrinsic fluorescence (*IF*) signals from mammalian nerve terminals. (A) Effect of 0.3 mM NaN_3 on the FAD fluorescence signal in response to a train of 40 stimuli at 40 Hz initiated at $t = 3$ s. 450 ± 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 50 Hz; 200 Hz sampling frequency. Black traces, normal mouse Ringer's (NMR) before, and 60 minutes after washing out the NaN_3 ; grey trace, 70 min in 0.3 mM NaN_3 . (B) Effect of 0.3 mM NaN_3 on the NADH fluorescence signal in response to the same stimulus initiated at $t = 3$ s. 350 ± 20 nm / 395 nm / 450 ± 50 nm. 250 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 200 Hz sampling frequency. Black traces, obtained in NMR before, and 37 minutes after washing out 0.3 mM NaN_3 ; grey trace, 15 min in 0.3 mM NaN_3 . Temperature 23°C.

$[\text{Ca}^{2+}]_o$, on the *IF* signals that reflect changes in FAD (A) and NADH (B), and the light grey traces demonstrate the *additional* effects of 200 μM ouabain. Both the FAD and NADH fluorescence changes were eliminated entirely in 0 $[\text{Ca}^{2+}]_o$ / ouabain, while excitability was not compromised, as confirmed by the unaltered appearance (*not shown*) of the *E*-wave of the light scattering signal at 740 nm (Salzberg et al., 1985).

Discussion

Changes in the autofluorescence (*IF*) of neurohypophysial secretory terminals provide a fascinating window into the stimulus-coupled response of nerve terminal mitochondria and, more generally, into the linkage between neuronal function and metabolic activity. The discovery of NADH and flavoproteins as the predominant indicators (fluorochromes) of bio-

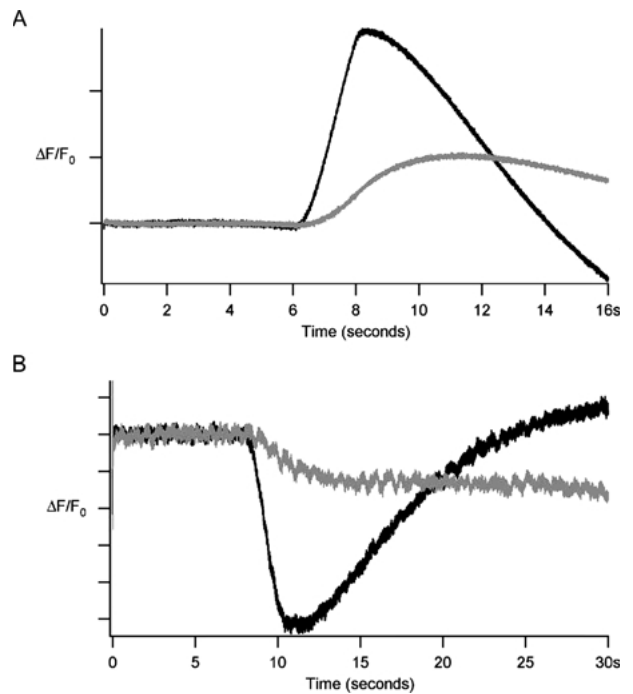


Fig. 6. Effect of Cd^{2+} on the intrinsic fluorescence (*IF*) signals from mammalian nerve terminals. (A) Effect of 1.0 mM Cd^{2+} on the FAD fluorescence signal in response to a train of 36 stimuli at 15 Hz initiated at $t = 6$ s. 450 ± 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 50 Hz; 1 kHz sampling frequency. Black trace, NMR; grey trace, 10 minutes in 1.0 mM Cd^{2+} . (B) Effect of 1.0 mM Cd^{2+} on the NADH fluorescence signal in response to the same stimulus initiated at $t = 8$ s. 350 ± 20 nm / 395 nm / 450 ± 50 nm. 150 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. Black traces, NMR; grey trace, 20 minutes in 1.0 mM Cd^{2+} . Temperature 23°C.

energetic activity in mitochondria (Chance & Williams, 1955b; Chance & Baltscheffsky, 1958) suggested that these two optically detectible components of the Keilin-Warburg respiratory chain of mitochondria may serve as intrinsic monitors of neuronal activity. It was not until very recently, however (Shibuki et al., 2003; Shuttleworth et al., 2003; Kasischke et al., 2004; Reinert et al., 2004; Kosterin et al., 2005; Kim et al., 2006), that autofluorescence changes associated with oxidation and reduction of mitochondrial fluorochromes were observed in response to physiological stimulation in neuronal systems. The relatively large (± 5 –8 %) intrinsic fluorescence changes reported here are clearly associated with mitochondrial activation in response to action potential firing in the neurosecretory terminals and swellings of the mouse neurohypophysis.

Mitochondria may be viewed as autonomous intracellular organs and, as such, have very similar characteristics regardless of the tissue of origin. Thus,

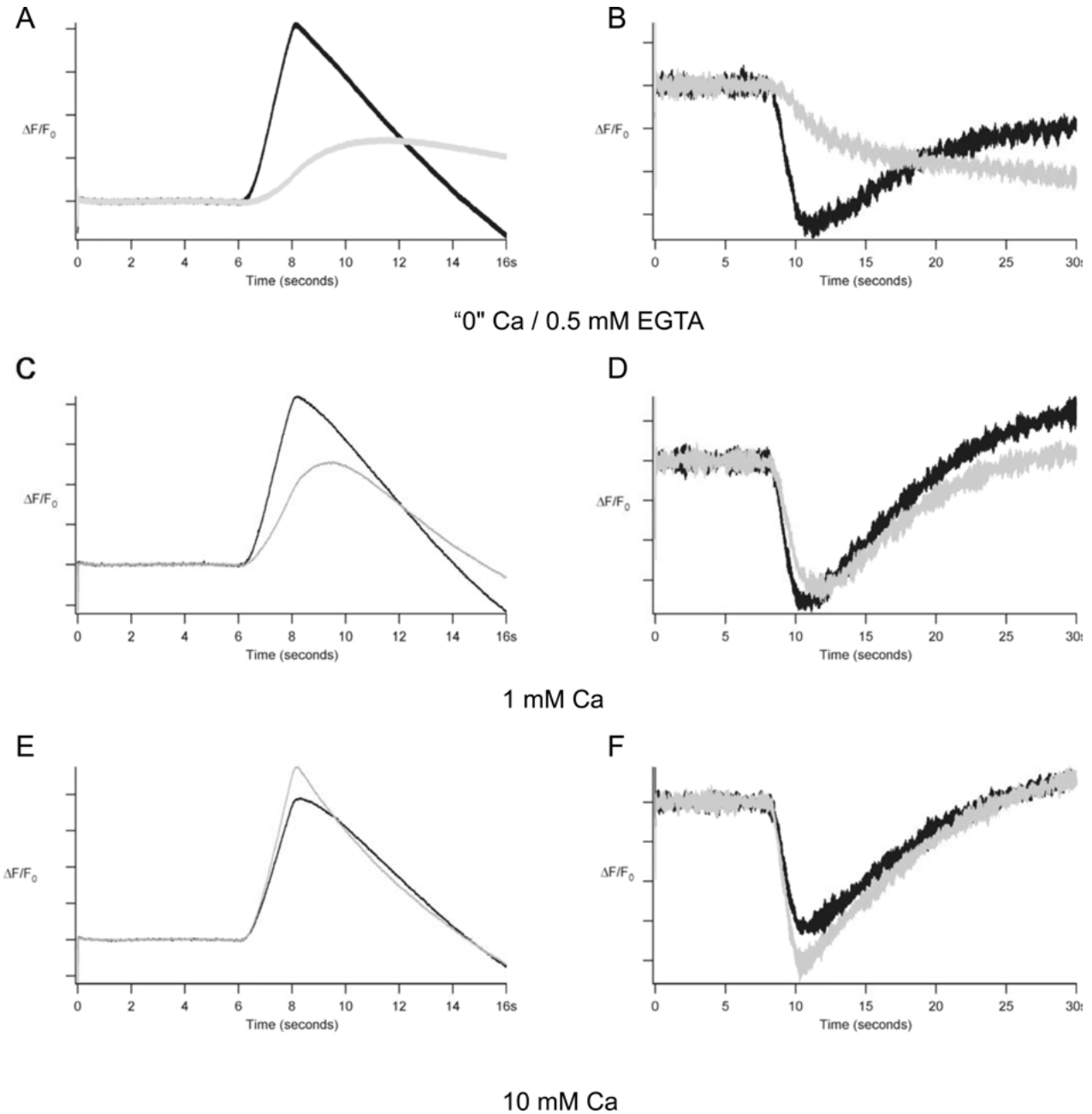


Fig. 7. Effects of $[Ca^{2+}]_o$ on the intrinsic fluorescence signals from neurohypophysis. (*A*, *C*, and *E*) Effects, respectively, of "0" Ca^{2+} / 0.5 mM EGTA (*A*), 1.0 mM Ca^{2+} (*B*), and 10.0 mM Ca^{2+} (*C*), on the FAD fluorescence signal in response to trains of 30 stimuli at 15 Hz initiated at $t = 6$ s. 450 ± 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. *Black traces*, NMR; *grey traces*, 19 minutes in the stated $[Ca^{2+}]_o$. (*B*, *D*, and *F*) Effects, respectively, of "0" Ca^{2+} / 0.5 mM EGTA (*B*), 1.0 mM Ca^{2+} (*D*), and 10.0 mM Ca^{2+} (*F*), on the NADH fluorescence signal in response to the same stimulus initiated at $t = 8$ s. 350 ± 20 nm / 395 nm / 450 ± 50 nm. 150 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. *Black traces*, NMR; *grey traces*, 19 minutes in the stated $[Ca^{2+}]_o$. Temperature 23°C.

in the neurosecretory terminals of the mouse neurohypophysis, as in other tissues, it seems clear that it is the redox state of the mitochondria that is the principal determinant of the autofluorescence that we observe. Figures 2–5 provide strong evidence that the nerve terminal *IF* signals are mitochondrial in origin. The

clear pO_2 dependence of both signals (Fig. 2), and the high oxygen affinity of mitochondria, suggest oxidation; and pharmacological interventions (Figs. 3–5) that complement those used by Reinert et al. (2004) and Shuttleworth et al. (2003) further implicate mitochondrial flavoproteins and nicotinamide dinu-

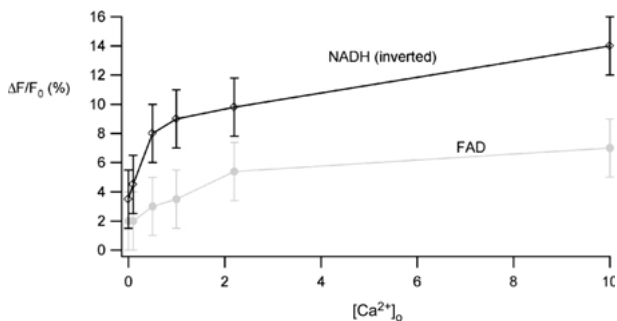


Fig. 8. $[Ca^{2+}]_o$ -dependence of the two *IF* signals. Summary of $[Ca^{2+}]_o$ data for the two intrinsic fluorescence signals, indicating the greater sensitivity of the NADH fluorescence change to extracellular Ca^{2+} $[Ca^{2+}]_o$ in mM.

cleotides. In general, one may expect that in the neuronal response to stimulation, there will be a displacement of the steady state, and an accumulation of NAD, FAD, and, *in vivo*, deoxygenated hemoglobin. Blocking the electron transport chain is a clear test of whether the intrinsic signals are mitochondrial in origin. When the electron transport chain is blocked, e.g., by CCCP, the accumulation of electrons serves to reduce maximally all components of the chain, including the flavoproteins. This has the effect of blocking any stimulus-dependent changes in the redox state, and, hence, changes in fluorescence.

As suggested earlier, the cellular localization of the intrinsic fluorescence signals, whether flavoprotein or NADH, has been difficult to identify with certainty in neuronal tissue. For this reason, the neurohypophysis is a particularly favorable preparation in which to study these signals; the relative *homogeneity* of the preparation, compared with brain slices (Shuttleworth et al., 2003) or intact cerebellum (Reinert et al., 2004), and the precise control of the timing of the action potential in a large, synchronously activated population of nerve terminals (Salzberg et al., 1983; Salzberg et al., 1985), with no confounding postsynaptic targets, renders the interpretation of the *IF* signals somewhat simpler than in other regions of the brain. Only the presence of glial elements, the pituicytes, complicates the issue in the neurohypophysis, and their numbers are relatively small compared with axons, terminals, and secretory swellings (Nordmann, 1977). Reinert et al. (2004) concluded that the majority of their autofluorescence signal could be attributed to activation of the postsynaptic targets of parallel fibers (Purkinje cells and cerebellar interneurons) in the mouse cerebellum, but they could not rule out contributions from parallel fibers or glia (Pellerin & Magistretti, 1994). This is in contrast with our experiments where, as indicated, there are no postsynaptic targets and the *IF* signal clearly arises, primarily, from nerve terminals. The peak amplitude of their FAD signal was $\sim 1.57\%$ ($\Delta F/F$) and the oppositely directed NADH signal was

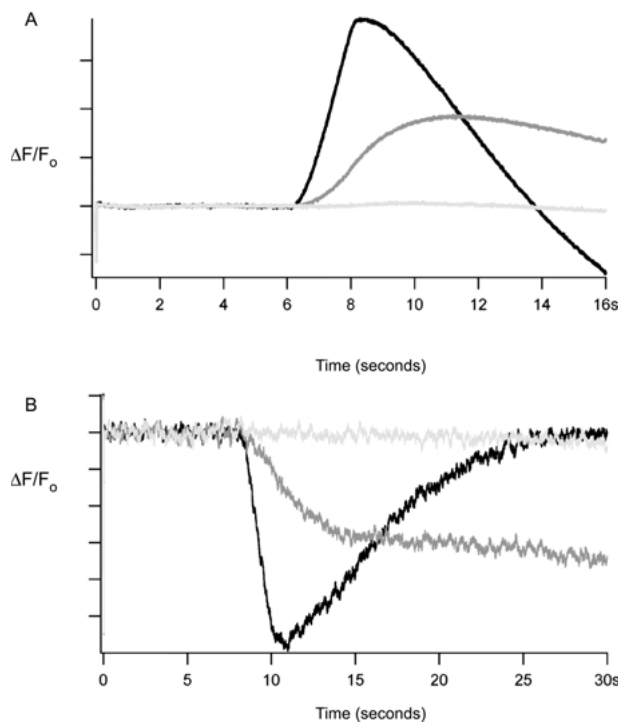


Fig. 9. Removal of $[Ca^{2+}]_o$ and block of the plasma membrane Na^+/K^+ ATPase are, together, sufficient to eliminate both the FAD and the NADH *IF* signals. Additive effects of "0" Ca^{2+} / 0.5 mM EGTA and 200 μM ouabain on the *IF* signal from mitochondrial flavoprotein (FAD) and from NADH. (A) The black trace shows the FAD signal in NMR in response to a train of 30 stimuli at 15 Hz initiated at $t = 6$ s. The dark grey trace shows the effect of 9 minutes exposure to "0" Ca^{2+} / 0.5 mM EGTA Ringer's solution (with Mg^{2+} substitution for Ca^{2+}). The light grey trace demonstrates the complete block of the flavoprotein *IF* signal following 19 minutes exposure to "0" Ca^{2+} / 0.5 mM EGTA plus 200 μM ouabain. 450 \pm 50 nm / 515 nm / Hi-Q 520 nm long pass. 300 W tungsten-halogen lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. Temperature 23°C. (B) The black trace shows the NADH signal in NMR in response to a train of 30 stimuli at 15 Hz initiated at $t = 8$ s. The dark grey trace shows the effect of 9 minutes exposure to "0" Ca^{2+} / 0.5 mM EGTA Ringer's solution (with Mg^{2+} substitution for Ca^{2+}). The light grey trace demonstrates the complete elimination of the NADH *IF* signal following 19 minutes exposure to "0" Ca^{2+} / 0.5 mM EGTA plus 200 μM ouabain. 350 \pm 20 nm / 395 nm / 450 \pm 50 nm. 150 W Xe-short arc lamp. Feedback resistor 100 M Ω ; 2nd stage gain $\times 100$; bandwidth 20 Hz; 1 kHz sampling frequency. Temperature 23°C.

$\sim -0.22\%$ ($\Delta F/F$) (Reinert et al., 2004). It is not entirely clear why the amplitudes of the FAD and NADH signals were smaller in the cerebellum than in the neurohypophysis, but the explanation most likely lies in the homogeneity and uniform activation of the latter preparation. Also, the relative distribution of mitochondrial and cytoplasmic NADH may differ in the two tissues and this may be a factor.

In the hippocampal slice preparation of Shuttleworth et al. (2003), the NADH- and flavoprotein-derived fluorescence changes are also de-

scribed as having primarily a postsynaptic origin although, in this tissue, the magnitude of the signals is closer to what we observe. A significant difference between the *IF* signals reported here and some of those described above (Shuttleworth et al., 2003) is the greater sensitivity to $[Ca^{2+}]_o$ of our *IF* changes. There are good reasons why Ca^{2+} might act as the principal mediator of signaling between plasma membrane excitation and any metabolic response. Shibuki and colleagues (Shibuki et al., 2003) have demonstrated that the Ca^{2+} increase precedes any autofluorescence signal and our data (Figs. 1B, D and E) confirm this. The increase in $[Ca^{2+}]_i$ is partially absorbed by mitochondria through a uniporter driven by the very high -190 mV potential across the inner mitochondrial membrane (Gunter et al., 1998; Rizzuto, Bernardi & Pozzan, 2000). This uptake of Ca^{2+} depolarizes the mitochondria and induces calcium cycling; both of these factors reduce the proton gradient across the inner mitochondrial membrane, resulting in a short-term oxidation of the respiratory chain as it restores the gradient. After the Ca^{2+} enters the mitochondrial matrix, it activates the pyruvate dehydrogenase complex and other dehydrogenases associated with the tricarboxylic acid cycle, resulting in a delayed increase in NADH along with reduction of the respiratory chain. This oxidation and reduction seem to be responsible for the biphasic character of the autofluorescence signal (Reinert et al., 2004), and, in particular, the oxidation and reduction of flavoproteins, particularly FAD. The opposite sign of the NADH signal in our preparation, as well as in the observations of Reinert and Shuttleworth and their colleagues (Reinert et al., 2004; Shuttleworth et al., 2003) supports this analysis. It should be noted, in this context, that numerous studies (Chance, 1965; Duchen, 1992, 1999; Duchen & Biscoe, 1992; Pralong et al., 1992; Duchen, Smith & Ashcroft, 1993; Pitter et al., 2002; Voronina et al., 2002) have shown that mitochondrial Ca^{2+} dynamics can regulate mitochondrial NADH levels.

Shuttleworth et al. (2003) found that in 0 Ca^{2+} / 0.5 mM BAPTA, there was no effect on the amplitude of the NADH signal. These authors concluded that the changes in NADH fluorescence do not depend upon intracellular Ca^{2+} dynamics, since preventing any rise in intracellular Ca^{2+} did not diminish the NADH transients. However, they did find that ouabain (30 μ M) abolished both components of the NADH signal. They concluded that the activity of the Na^+/K^+ ATPase and the consequent increase in the ADP/ATP ratio during neuronal activity is a significant contributor to the late phase of the NADH signal, but not to the early phase (see below).

Closer to our findings, Reinert et al. (2004) determined that their autofluorescence signal was only partially blocked in a 0 Ca^{2+} Ringer's solution. Our observation that total elimination of $[Ca^{2+}]_o$ did not

block either of the *IF* signals raises the important question of the identity of the agent that carries the information about electrical excitation of the terminals to the mitochondria. It could be that in the absence of extracellular Ca^{2+} the action potentials trigger a rise in intracellular Ca^{2+} by means of release from intracellular Ca^{2+} stores. That this cannot be the case in neurohypophysial nerve terminals is clear. We have measured $[Ca^{2+}]_i$ during trains of 40 action potentials using both high- and low-affinity Ca^{2+} indicator dyes, Fluo-4 AM ($K_{D,Ca} = 345$ nM) and Mag-Fluo-4 AM ($K_{D,Ca} = 22$ μ M), both in 1.0 mM Cd^{2+} and in 0 Ca^{2+} with 0.5 mM EGTA added, and no detectable changes in intraterminal Ca^{2+} were observed under either of these conditions (*data not shown*).

Another obvious candidate for transmitting the signal is Na^+ ion, possibly acting through a mitochondrial Na^+/Ca^{2+} exchanger. To try to identify a role for such a transporter we used a mitochondrial Na^+/Ca^{2+} exchanger antagonist, CGP 37157, in conjunction with a " 0 " Ca^{2+} / 0.5 mM EGTA Ringer's solution. With 100 μ M CGP 37157, we did not observe any inhibition of the *IF* signal beyond that found using " 0 " Ca^{2+} / 0.5 mM EGTA alone. We also attempted to examine the possibility that Na^+ ion acts as the intracellular messenger by substituting LiCl for all of the NaCl in the Ringer's solution. (Since Li^+ is capable of replacing Na^+ in the generation of the action potential, but not in any of the known active transport systems, this intervention might have been able to rule out a role for Na^+ in the information transfer.) Unfortunately, in the mouse neurohypophysis, a Ringer's solution containing Li^+ and K^+ as the only monovalent cations did not support action potential production (*data not shown*). In as much as this effect may reflect some hitherto unreported toxicity of Li^+ in the neurohypophysis, we cannot rule out a role for Na^+ in the mediation of the coupling between electrical activity and mitochondrial metabolism in neurosecretory terminals.

Yet another possibility (Magistretti et al., 1999; Shuttleworth et al., 2003; Reinert et al., 2004) is that the induction of mitochondrial metabolism also depends upon the depletion of ATP and the production of ADP by the Na^+/K^+ pump that restores ionic gradients across the nerve terminal plasma membrane. This increase in the ADP/ATP ratio, with the added ADP translocated across the inner mitochondrial membrane, both increases the activity of ATP synthase and reduces the proton gradient. This has the effect of an immediate oxidation of the components of the respiratory chain (Nicholls & Ferguson, 2002). The increase in ADP also activates other key enzymes in glycolysis and the tricarboxylic acid cycle, resulting in increased NADH and reduction of the respiratory chain. Thus, both increases in intracellular Ca^{2+} and ATP depletion could generate the biphasic time courses of both *IF* signals. We

concur with Reinert et al. (2004) that the induction of mitochondrial metabolism and the concomitant *IF* signals probably depend upon a combination of Ca^{2+} increase and ATP depletion. In fact, the data in Fig. 9 strongly support the conclusion that it is ADP together with Ca^{2+} that drives both the mitochondrial *IF* signals.

The possibility of highly synchronous activation makes the neurohypophysis an excellent preparation in which to investigate the detailed kinetics of the *IF* signals. In particular, the Ca^{2+} - and ADP-dependent components of the FAD and NADH signals that follow action potentials can be dissected, and their individual phases compared, internally, and with *in vitro* measurements of mitochondrial dynamics (Chance & Erecinska, 1972). We have not reported those results here, because experiments that address these issues are ongoing.

Finally, it should be observed that the neuron / astrocyte model of Magistretti (Pellerin & Magistretti, 1994) may have a parallel in the nerve terminal / pituicyte system in the mammalian neurohypophysis. While the *IF* signals reported here are too slow and too loosely coupled to serve as a spike-by-spike monitor of electrical activity, these signals may yet be extremely useful as a probe of global neuronal activation as well as brain metabolism. Neuropeptide secretion and mitochondrial activation are clearly coupled, as strongly suggested by the FAD *IF* signal, which is most likely related to ketoglutarate and pyruvate dehydrogenases of the mitochondrial oxidative system. Thus, the observations reported here confirm and extend the known links between neuronal activity and bioenergetics.

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