Ca\textsuperscript{2+} - and Metabolism-Related Changes of Mitochondrial Potential in Voltage-Clamped CA1 Pyramidal Neurons In Situ

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Schuchmann, S., M. Lückermann, A. Kulik, U. Heinemann, and K. Ballanyi. Ca\textsuperscript{2+} - and metabolism-related changes of mitochondrial potential in voltage-clamped CA1 pyramidal neurons in situ. J. Neurophysiol. 83: 1710–1721, 2000. In hippocampal slices from rats, dialysis with rhodamine-123 (Rh-123) and/or fura-2 via the patch electrode allowed monitoring of mitochondrial potential (ΔΨ) changes and intracellular Ca\textsuperscript{2+} (\(\text{[Ca}^{2+}]_i\)) of CA1 pyramidal neurons. Plasmalemmal depolarization to 0 mV caused a mean [Ca\textsuperscript{2+}]_i rise of 300 nM and increased Rh-123 fluorescence signal (RFS) by ≤50% of control. The evoked RFS, indicating depolarization of ΔΨ, and the [Ca\textsuperscript{2+}]_i rise were abolished by Ca\textsuperscript{2+}-free superfusate or exposure of Ni\textsuperscript{2+}/Cd\textsuperscript{2+}. Simultaneous measurements of RFS and [Ca\textsuperscript{2+}]_i showed that the kinetics of both the Ca\textsuperscript{2+} rise and recovery were considerably faster than those of the ΔΨ depolarization. The plasmalemmal Ca\textsuperscript{2+}/H\textsuperscript{+} pump blocker eosin-B potentiated the peak of the depolarization-induced RFS and delayed recovery of both the RFS and [Ca\textsuperscript{2+}]_i. Transient. The evoked ΔΨ depolarization due to plasmalemmal depolarization is related to mitochondrial Ca\textsuperscript{2+} sequestration secondary to Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels. CN\textsuperscript{−} elevated [Ca\textsuperscript{2+}]_i by <50 nM but increased RFS by 221% as a result of extensive depolarization of ΔΨ. Oligomycin decreased RFS by 52% without affecting [Ca\textsuperscript{2+}]_i. In the presence of oligomycin, CN\textsuperscript{−} and p trifluoromethoxy-phenylhydrazone (FCCP) elevated [Ca\textsuperscript{2+}]_i, by <50 nM and increased RFS by 285 and 290%, respectively. Accordingly, the metabolism-related ΔΨ changes are independent of [Ca\textsuperscript{2+}]_i. Imaging techniques revealed that evoked [Ca\textsuperscript{2+}]_i rises are distributed uniformly over the soma and primary dendrites, whereas corresponding changes in RFS occur more localized in subregions within the soma. The results show that microfluorometric measurement of the relation between mitochondrial function and intracellular Ca\textsuperscript{2+} is feasible in whole cell recorded mammalian neurons in situ.

INTRODUCTION

In central neurons, particularly in hippocampal CA1 neurons, pharmacological approaches established that synaptically evoked local changes of the free concentration of intracellular Ca\textsuperscript{2+} (\(\text{[Ca}^{2+}]_i\)) play a key role in excitability and synaptic plasticity (Bliss and Collingridge 1993; Edwards 1995; Ito et al. 1995). For a decade, whole cell recording techniques have been used in combination with microfluorometric measurements of [Ca\textsuperscript{2+}]_i (Neher 1989) to analyze cellular mechanisms of synaptic processes in neurons of functionally intact slice preparations (Eilers et al. 1995; Regehr et al. 1989). Recently it was proposed that activity-evoked changes of energy metabolism may contribute to adaptive neuronal processes by redox modulation of ion channels (Kohr et al. 1994; Tang and Zucker 1997). A full test of this hypothesis requires simultaneous monitoring of metabolic activity with membrane properties and [Ca\textsuperscript{2+}]_i in neurons that are embedded in their natural environment.

Metabolic parameters such as uptake (Hubel et al. 1978) or catabolism (Sibson et al. 1998) of glucose or oxygen saturation of hemoglobin (Bonhoeffer et al. 1995) have been used to visualize neuronal activity in vivo. Mitochondrial membrane potential (ΔΨ) is a further measure of metabolism with a high temporal resolution (Duchen 1992, 1999; Gunter et al. 1994; McCormack et al. 1990). So far, microfluorometric measurements of relative changes in ΔΨ were done in acutely isolated or cultured neurons that were bulk-loaded with rhodamine-123 (Rh-123) (Duchen 1992, 1999; Duchen and Biscoe 1992; Nowicky and Duchen 1998; Schinder et al. 1996; Schuchmann et al. 1998; White and Reynolds 1996). However, synaptic integrity as maintained within brain slices is necessary to study the interaction of activity-related changes in metabolism and neuronal excitability. In this regard, it recently was demonstrated that mitochondrial function can be analyzed in Rh-123 bulk-loaded hippocampal slices (Bindokas et al. 1998). Under these conditions, the dye distributes nonselectively in diverse compartments of various cellular elements within the slice. Discrimination between pre- and postsynaptic processes as well as discrimination between neurons and different types of glial cells is rather difficult under these conditions. The latter technique does also not provide information on the temporal relation between synaptic or intrinsic membrane currents and ΔΨ and thus metabolic changes in single hippocampal neurons within the network of the slice.

In the present study, we have used photomultiplier-based optical techniques in hippocampal slices to investigate whether long-term recording of ΔΨ is feasible in individual CA1 pyramidal neurons that are dialyzed with Rh-123 via the patch electrode. It is known from measurements on isolated neurons or mitochondria that a rise of [Ca\textsuperscript{2+}]_i produces a robust depolarization of ΔΨ (Duchen 1992; Duchen and Biscoe 1992; Gunter et al. 1994; Loew et al. 1994; McCormack et al. 1990). Accordingly, we have studied the extent to which a rise of [Ca\textsuperscript{2+}]_i, evoked by depolarization of the plasma membrane, affects ΔΨ in whole cell recorded hippocampal neurons in situ. For comparison of the effects of plasma membrane depolarization with ΔΨ responses due to direct modulation of energy metabolism, we have analyzed the effects on [Ca\textsuperscript{2+}]_i, and ΔΨ of block of aerobic metabolism by CN\textsuperscript{−} (Ballanyi and Kulik 1998; Biscoe and Duchen 1990; Duchen 1992). We further-
more have used digital imaging techniques to study putative spatial differences of activity- and metabolism-related changes of $\Delta \Psi$ and $[\text{Ca}^{2+}]$. The results show that this novel technique is an appropriate tool for monitoring the temporal and causal relation between membrane excitability and metabolism of individual neurons in a functional network.

**METHODS**

**Preparation and solutions**

The experiments were performed on hippocampal slices from 9- to 14-day-old Wistar rats of either sex. The animals were anesthetized with ether and decapitated. The forebrain with the hippocampus was isolated and kept for 5 min in ice-cold artificial cerebrospinal fluid (standard solution; composition see following text; $\text{Ca}^{2+}$ concentration reduced to 0.5 mM). Eight to 10 longitudinal slices (200 $\mu$m) were cut from the ventral side in ice-cold low $\text{Ca}^{2+}$ solution. Before transfer to the recording chamber, the slices were stored at 30°C in standard solution. The recording chamber (volume, 3 ml) was superfused with oxygenated standard solution (flow rate, 5 ml/min, 30°C) of the following composition (in mM): 118 NaCl, 3 KCl, 1 MgCl$_2$, 1.5 CaCl$_2$, 25 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, and 10 D-glucose. The pH was adjusted to 7.4 by gassing with 95% O$_2$–5% CO$_2$. Chemical anoxia was induced by addition of 1 mM NaCN to the standard solution (Ballanyi and Kulik 1998). In the $\text{Ca}^{2+}$-free solution, which also contained 1 mM EGTA as a $\text{Ca}^{2+}$ buffer, the $\text{Mg}^{2+}$ concentration was elevated to 5 mM. Drugs were purchased from Sigma (München, Germany), Biomol (Köln, Germany), or Tocris Cookson (Bristol, UK).

**Intracellular recording**

Patch pipettes were produced from borosilicate glass capillaries (GC 150TF, Clark Electromedical Instruments, Pangbourne, UK) using a horizontal electrode puller (Zeitz, München, Germany). The standard patch pipette solution (osmolarity, 270–285 mosmol) contained (in mM) 140 potassium gluconate, 1 MgCl$_2$, 0.5 CaCl$_2$, 10 HEPES, 1 K$_2$-BAPTA, and 1 Na$_2$-ATP; pH 7.3–7.4. For measurements of $[\text{Ca}^{2+}]$, the patch solution contained neither $\text{Ca}^{2+}$ nor BAPTA because of the $\text{Ca}^{2+}$-buffering properties of fura-2 (Neher 1989; Tsien 1990). The DC resistance of the electrodes ranged from 4 to 6 MΩ. Fura-2 (100 $\mu$M; Molecular Probes, Eugene, OR) or 1–10 $\mu$g/ml Rh-123 (Sigma) was added to the pipette solution before the experiment. In one series of experiments, 100 $\mu$M esiun-B also was added to the patch electrode solution. Whole cell recordings were performed on superficial CA1 pyramidal neurons under visual control using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany), driven by Pulse/Pulsefit software (HEKA) on a PowerPC (Apple Computer, Cupertino, CA). Seal resistance ranged from 1 to 3 GΩ, and series resistance was between 10 and 25 MΩ. Membrane conductance ($g_{m}$) was measured by application of hyperpolarizing voltage pulses (−20 mV) with a duration of 500 ms. Holding potential in voltage clamp was −60 mV.

**Fluorescence measurements**

Fluorescence measurements were done with either a photomultiplier (Luois and Neumann, Ratingen, Germany) or an imaging system using a 12-bit CCD camera (T.I.L.L. Photonics, Planegg, Germany) that was fixed to an upright microscope (Standard-16 or Axioskop, Zeiss, Oberkochen, Germany). The microscope was equipped with epifluorescence optics and a monochromator (Polychrome II, T.I.L.L. Photonics) to allow for fluorescence excitation at both 360 and 390 nm ($[\text{Ca}^{2+}]$ measurements) or 485 nm ($\Delta \Psi$ measurements). Emission was measured at 510 nm ($[\text{Ca}^{2+}]$ measurements) or 530 nm ($\Delta \Psi$ measurements). In one set of experiments, both fura-2 and Rh-123 were added to the patch electrode solution for simultaneous measurements of $[\text{Ca}^{2+}]$, and $\Delta \Psi$. In this case, alternating excitation was done at 390 and 485 nm, and emission was measured at 530 nm. For the photomultiplier system, a pinhole diaphragm was used to avoid disturbance from background illumination. The diaphragm limited the region from which light was collected from the cell soma and proximal dendrites to a circular spot of 20 μm diam.

Fluorescence ratios were converted into $[\text{Ca}^{2+}]$, by using Eq. 1:

$$[\text{Ca}^{2+}] = K R - R_{\min }/ (R_{\max } - R)$$

(Gryniewicz et al. 1985), in which $R$ is the fluorescence ratio (360 nm/390 nm) and $K$ is the effective dissociation constant of fura-2. In vivo calibration to determine $R_{\min }$, $R_{\max }$, and $K$ was performed according to the method described by Neher (1989). Briefly, measurements were performed with three different pipette solutions (pH 7.1) that contained (in mM) 130 KCl, 1 MgCl$_2$, 10 BAPTA, 10 HEPES, and 1 Na$_2$-ATP (low calcium; $R_{\min }$); 130 KCl, 1 MgCl$_2$, 3 CaCl$_2$, 4 BAPTA, 10 HEPES, and 1 Na$_2$-ATP (intermediate $\text{Ca}^{2+}$; 300 nM, according to a $K_{0}$ of 107 nM for BAPTA (Tsien 1980)); or 130 KCl, 1 MgCl$_2$, 10 CaCl$_2$, 10 HEPES, and 1 Na$_2$-ATP (high $\text{Ca}^{2+}$; $R_{\max }$). To each solution, 100 μM fura-2 was added. The resulting intracellular fluorescence ratios were calculated according to Eq. 1. $K$ was calculated as $K = 300$ nM ($R_{\max } - R$)/($R - R_{\min }$).

**Data analysis**

Fluorescence and electrophysiological signals were sampled at 3 Hz by the PowerPC (Apple) via the ITC-16 interface of the EPC-9 amplifier using the X-Chart extension of the Pulse/Pulsefit software (HEKA). Analysis of the data was done with IGOR software (WaveMetrics, Lake Oswego, OR). Images were sampled at a rate of 1–10 Hz on an IBM-compatible computer using T.I.L.L. vision software. Further image processing was done using Adobe Photoshop software (Adobe Systems, Mountain View, CA) and CANDAS (Deneba software, Miami, FL). Values are means ± SE.

**RESULTS**

**Dialysis of CA1 pyramidal neurons with Rh-123**

The permeant dye Rh-123 accumulates in polarized intracellular compartments such as mitochondria (Chen 1988; Duchen 1992, 1999; Johnson et al. 1980; Scaduto and Grottohann 1999). At appropriate concentrations, the accumulated dye will self-quench, thereby reducing its quantum yield. Mitochondrial depolarization induces release of Rh-123 into the cytoplasm where, because of its dilution, it will produce a larger fluorescence signal. So far the dye has been used almost exclusively in cells that were loaded with the dye on bath application. In the present study, we have elaborated whether Rh-123 can be applied via the patch electrode to neurons within brain slices to continuously measure relative changes of mitochondrial membrane potential ($\Delta \Psi$).

In previous reports on acutely dissociated or cultured neurons, 10 μg/ml was typically used for bulk-loading with Rh-123 (Nowicky and Duchen 1998; Schuchmann et al. 1998; see also Bindokas et al. 1998; Chen 1988). With this concentration of Rh-123 in the patch electrode, a stable rhodamine fluorescence signal (RFS), which indicated a rather constant concentration of the dye in the cytoplasm, was observed within 166 ± 72 s after establishing the whole cell configuration. In contrast, stabilization of the fluorescence signal was delayed three- to fourfold while dialyzing the cells with 5 μg/ml of the dye. In particular, when series resistance was relatively high (>15 MΩ), the RFS continued to drift throughout the whole cell recording period with 5 μg/ml Rh-123 in the electrode. Be-
cause of the even greater time delay to establish a stable intracellular fluorescence signal, it was not appropriate to dialyze the cells with Rh-123 at a concentration of \(<5 \mu g/ml\). Accordingly, because of the more rapid equilibration time and the better signal-to-noise ratio, a concentration of 10 \(\mu g/ml\) of the dye was used routinely for this study. Under these conditions, \(I_m\) and \(g_{in}(\sim21 \pm 12 \text{ pA}; 2.9 \pm 1.9 \text{ nS}; n = 39)\) as well as the RFS were stable for time periods of \(\approx2 \text{ h}\), indicating lack of cytotoxic effects of the drug as well as lack of photodamage (see also Chen 1988; Johnson et al. 1980). Similar stable whole cell recordings were obtained on dialysis of the cells with either fura-2 alone or on simultaneous administration of both dyes. In some slices, leakage of Rh-123 from the patch electrode before establishing a GΩ seal produced dye labeling of tissue in the vicinity of the recorded neuron. Such unspecific labeling was avoided by prefilling of the electrodes with 2 \(\mu l\) of intracellular solution that did not contain the dye.

**Calibration of Rh-123 fluorescence signal**

A depolarizing change of \(\Delta \Psi\) is indicated by an increase in RFS in response to dequenching of the dye after release from mitochondria. In isolated mitochondria, the magnitude of the Rh-123 signal had been shown to vary linearly with \(\Delta \Psi\) (Duchen 1992; Emaus et al. 1986). Additional factors, including total cell volume and mitochondrial volume fraction, influence the intensity of the Rh-123 fluorescence monitored in intact cells. Even among neurons of the same population, these factors may in principal vary considerably. Most of this variability is removed when rhodamine fluorescence intensity, monitored as the output voltage of the photomultiplier tube (Fig. 1), is normalized to the baseline observed in the resting cell that is examined after equilibration of dye from the patch pipette but before stimulus.

The *inset axis* in Fig. 1A (left) illustrates application of this procedure to produce the RFS (%), the normalized rhodamine fluorescence signal in percent of control, that we routinely analyzed. Stimulus by depolarization of the plasma membrane from −60 to 0 mV for 15 s evoked a \(>1\text{-nA}\) outward current and a transient increase in the rhodamine intensity by \(\approx50\%\) indicating a relative depolarization (see also Fig. 1. B and C). Figure 1A, right, illustrates a more elaborate procedure, not used routinely, for calibration (or rather normalization) of Rh-123 fluorescence intensity to the maximal responses observed for fully energized and fully depolarized mitochondria. Oligomycin, by inhibiting the mitochondrial ATP synthase, allows \(\Delta \Psi\) to become maximally hyperpolarized. The protophore p-trifluoromethoxy-phenylhydrazone (FCCP), by directly dissipating the mitochondrial transmembrane \(H^+\) gradient, depolarizes \(\Delta \Psi\). The *right-hand inset axis* is scaled to assumed values of −200 and −60 mV for \(\Delta \Psi\) of fully hyperpolarized and -depolarized mitochondria (Scaduto and Grotyohann 1999). On average oligomycin (10 \(\mu M\)) led to a decrease of RFS by 52 ± 7\%, whereas subsequent addition of FCCP (1 \(\mu M\)) produced an RFS increase of 295 ± 17\% \((n = 7; \text{Fig. 1, A and C})\). The latter type of normalization gives a rough estimate for the activity of the ATP synthase and therefore of basic metabolic activity. As evident by the small standard error bars in Fig. 1, B and C, baseline levels as well as experimentally evoked changes of \(\Delta \Psi\) were rather uniform. These results show on the one hand that the variability in steady-state levels of dye distribution is very low, suggesting that resting \(\Delta \Psi\) is rather similar between individual cells. On the other hand, it is evident that dialysis with “fresh” dye via the patch electrode does not buffer or impede dynamic changes of \(\Delta \Psi\) (compare Neher 1989; Trapp et al. 1996a).

**\(\Delta \Psi\) signals due to plasmalemmal depolarization**

The origin of the RFS increase due to plasmalemmal depolarization that indicates depolarization of \(\Delta \Psi\) was analyzed further. Depolarization to 0 mV for 15 s led to a slowly inactivating outward current with a magnitude that varied in individual cells between 0.3 and 3 nA \((n = 48)\). With a delay of 3.3 ± 0.8 s \((n = 11)\) after onset of the outward current, a mean RFS increase of 36 ± 14\% was observed \((n = 11; \text{Fig. 2, A and B})\). After termination of the plasmalemmal depolarization, the RFS increase and thus \(\Delta \Psi\) recovered to baseline with a mono-exponential time course \((\tau = 19.2 \pm 8.9 \text{ s}; n = 11)\). In four cells analyzed, hyperpolarization from −60 to −120 mV for 15 s evoked a sustained inward current by between −300 and −900 pA without

![FIG. 1. Calibration of Rh-123 fluorescence signal (RFS) in voltage-clamped CA1 pyramidal neurons of hippocampal slices. A: after stabilization of the RFS −12 min subsequent to establishing the whole cell recording, plasmalemmal depolarization for 15 s from −60 to 0 mV led to an increase in rhodamine fluorescence intensity that is proportional to the output voltage of the photomultiplier tube (numbers represent mV). Increased rhodamine fluorescence intensity corresponds to a relative depolarization of mitochondrial membrane potential (\(\Delta \Psi\)). RFS routinely is calibrated (or rather normalized) to percent of control, where 100% corresponds to the signal prior to stimulation. As further possibility for normalization, maximal mitochondrial hyperpolarization (to about −200 mV) can be evoked by the antibiotics oligomycin (10 \(\mu g/ml\)), whereas maximal depolarization (to around −60 mV) is induced by 1 \(\mu M\) of the mitochondrial uncoupler p-trifluoromethoxy-phenylhydrazone (FCCP). [Values are taken from the literature (for references, see Emaus et al. 1986; Scaduto and Grotyohann 1999).] B: mean values for RFS intensity baseline, for the peak response to plasmalemmal depolarization, and for oligomycin with or without FCCP \((n = 5)\). C: statistical representation of the evoked changes of RFS in B normalized as percent of control.](http://jn.physiology.org/Downloadedfrom)
an effect on $\Delta \Psi$ (Fig. 2B). The voltage threshold for the depolarization-evoked RFS differed in individual cells between $-45$ and $-60$ mV and a pulse duration of $>1$ s was necessary to evoke an RFS signal in response to depolarization to $0$ mV. The rise of RFS saturated at depolarizations to between 0 and $+30$ mV, and the mean RFS increase for depolarization to $+30$ mV was $38 \pm 6.9\%$ ($n = 4$). A maximum RFS increase was seen in response to a 10-s depolarization (Fig. 3A). For longer plasmalemmal depolarizations, $\leq 30$ s in length, the RFS transient stabilized at a maximal value for the duration of the stimulus (Fig. 3A). The depolarization-evoked RFS responses were accompanied by changes of $[\text{Ca}^{2+}]_\text{i}$ baseline ($101 \pm 5$ nM; $n = 9$). On 15-s depolarization to $-40$, $-20$, 0, and $+20$ mV, $[\text{Ca}^{2+}]_\text{i}$ rose by $23 \pm 9$, $120 \pm 28$, $281 \pm 168.1$, and $358 \pm 12$ nM, respectively, in these cells after a delay of $<1$ s (Fig. 2C). Little or no recovery occurred until the stimulus terminated, even when the pulses had a duration of 30 s (Fig. 3B). Hyperpolarization of three cells to $-120$ mV did not affect $[\text{Ca}^{2+}]_\text{i}$ baseline (Fig. 2D).

**Role of $\text{Ca}^{2+}$ influx in voltage-dependent $\Delta \Psi$ depolarization**

The preceding results suggest that the $\Delta \Psi$ depolarization is related causally to the depolarization-induced $[\text{Ca}^{2+}]_\text{i}$ transient that results from $\text{Ca}^{2+}$ entry through voltage-gated $\text{Ca}^{2+}$ channels (for references, see Trapp et al. 1996b). To support this assumption, the slices were superfused with $\text{Ca}^{2+}$-free saline that also contained $5 \text{ mM Mg}^{2+}$ and $1 \text{ mM}$ of the $\text{Ca}^{2+}$ chelator EGTA. This solution not only blocked the stimulus-induced $[\text{Ca}^{2+}]_\text{i}$ rises ($n = 4$) but also abolished the concomitant $\Delta \Psi$ depolarization ($n = 5$; Fig. 4). Furthermore the $\text{Ca}^{2+}$-free superfusate led to a reversible decrease in the RFS by $\leq 25\%$, representing $\Delta \Psi$ hyperpolarization (Fig. 4A) and a decrease in $[\text{Ca}^{2+}]_\text{i}$ baseline by $42.1 \pm 1.5$ nM (Fig. 4B). Besides these effects, the $\text{Ca}^{2+}$-free solution attenuated the slowly inactivating plateau value of the depolarization-induced outward current by $\leq 20\%$ (Fig. 4). Very similar effects on $\Delta \Psi$ ($n = 5$), $[\text{Ca}^{2+}]_\text{i}$ ($n = 4$), and $I_\text{m}$ were observed on bath application of a mixture of $200 \text{ nM}$ of the blockers of voltage-activated $\text{Ca}^{2+}$ channels $\text{Ni}^{2+}$ and $\text{Cd}^{2+}$ (not shown) (compare Trapp et al. 1996b). For an estimation of the temporal correlation of the depolarization-evoked $\Delta \Psi$ and $[\text{Ca}^{2+}]_\text{i}$ transient, the cells were dialyzed with both fura-2 and Rh-123 in one series of experiments. Also under these nonparametric conditions, a decrease in the fura-2 fluorescence (390-nm excitation) corresponds to a rise of $[\text{Ca}^{2+}]_\text{i}$ (see METHODS) (Nowicky and Duchen 1998). The evoked $[\text{Ca}^{2+}]_\text{i}$ increase was found to precede the depolarization-evoked RFS rise by $1.6 \pm 0.2$ s ($n = 4$). After termination of the depolarizing stimulus, RFS had recovered by between 45 and 60% at the time when $[\text{Ca}^{2+}]_\text{i}$ had almost returned to baseline (Fig. 5).

**Eosin-induced potentiation of voltage-dependent $\Delta \Psi$ depolarization**

Previous studies established that a plasmalemmal $\text{Ca}^{2+}/\text{H}^+$ pump (Carafoli 1991) has a major contribution to the recovery from a cytosolic $\text{Ca}^{2+}$ load due to voltage-gated $\text{Ca}^{2+}$ channels (Benham et al. 1992; Werth et al. 1996). Accordingly, it recently was demonstrated that block of this $\text{Ca}^{2+}$ extrusion...
mechanism with eosin (Choi and Eisner 1999; Gatto et al. 1995) produces a considerable delay of recovery of [Ca\(^{2+}\)], rises evoked by membrane depolarization such as that used in the present study (Trapp et al. 1996b). In accordance with the latter study, we have added 100 \( \mu \)M eosin-B to the Rh-123-containing intracellular solution to block the Ca\(^{2+}\)/H\(^{+}\) pump.

In six neurons tested, depolarization of the plasma membrane to 0 mV for 15 s resulted in a RFS increase that was up to fourfold larger than that of control measurements. Furthermore, as exemplified in Fig. 6A, repetitive administration of depolarizing pulses resulted in both, a consecutive increase in the peak of the \( \Delta \Psi \) signal and delayed recovery to baseline level. Addition of the drug to the fura-2-containing patch electrode revealed that recovery from depolarization-evoked [Ca\(^{2+}\)], rises also was altered considerably (Fig. 6, B and C). In the eosin-dialyzed cells, recovery of Ca\(^{2+}\) from the plasmalemmal depolarization reached 90% after 83.8 \( \pm \) 10.3 s (\( n = 5 \)) versus 8.6 \( \pm \) 0.8 s in four control cells. In four of six CA1 neurons, this impairment of [Ca\(^{2+}\)] recovery led to a consecutive rise in [Ca\(^{2+}\)], baseline by between 30 and 120 nM. However, this effect was not as pronounced as the stepwise elevation of RFS evoked by eosin-B (compare Fig. 6, A and B).

**Comparison of voltage-dependent and metabolism-related RFS changes**

In contrast to the potentiating effect of eosin-B on RFS increases in response to consecutive application of depolarizing pulses, the voltage-dependent RFS rise tended to decrease in magnitude under control conditions. This became evident when the cells were depolarized repeatedly from −60 to 0 mV at a stimulus interval of 1–6 min. The mitochondrial depolarization during the second stimulus was potentiated by ≤60% (Figs. 2 and 4), whereas further periods of depolarization attenuated the \( \Delta \Psi \) response in ≤60% of observations (\( n = 17 \); Fig. 7, A and B). In individual cells, no \( \Delta \Psi \) depolarization could be elicited by the fourth or fifth depolarization although the membrane current response was not altered (not shown). This attenuating effect was not due to washout of Ca\(^{2+}\) currents and subsequent decrease in the magnitude of depolarization-induced [Ca\(^{2+}\)], transients as these were stable or even increased over time periods of >1 h in 13 cells tested (Fig. 7, C and D). To investigate, whether the decrease in the magnitude of the depolarization-induced \( \Delta \Psi \) depolarization was due to a methodological artifact resulting in incapability of Rh-123 to respond to \( \Delta \Psi \) changes, the effects of bath application of CN\(^−\) (1 mM) were compared with those of depolarization of the plasma membrane. CN\(^−\) is established to cause a major \( \Delta \Psi \) depolarization by blocking aerobic metabolism (Duchen and Bisconoe 1992; McCormack et al. 1990; Miller 1991). In 13 of 35 CA1 pyramidal cells, application of CN\(^−\) for 30 s evoked an outward current of 50 \( \pm \) 11 pA and a concomitant \( I_{\text{m}} \) increase by between 20 and 90% (Figs. 7 and 8). In the remaining 22 neurons, application of CN\(^−\) for 30 s either did not change (60% of cases) resting \( I_{\text{m}} \) or \( g_{\text{m}} \) (Fig. 9) or evoked an inward current (<50 pA; Fig. 10) and \( g_{\text{m}} \) rise (≤30% of cells).

Despite the moderate effects of short application of CN\(^−\) on
basic membrane properties, the drug produced a prominent increase in RFS indicating depolarization of \( \Delta \Psi \). In the experiment of Fig. 7A, the cell was depolarized repeatedly for 15 s and then exposed to \( \text{CN}^- \). This revealed that the \( \Delta \Psi \) response to depolarization progressively decreased in magnitude. In contrast, the response to \( \text{CN}^- \), which was initially more than twofold larger than the response to depolarization, was more and more potentiated (Fig. 7A). As exemplified with the neuron of Fig. 7C, both these experimental procedures had opposite effects on \([\text{Ca}^{2+}]_i\). \( \text{CN}^- \) elevated \([\text{Ca}^{2+}]_i\) by 50 nM, whereas depolarization to 0 mV caused a steadily increasing rise of intracellular \( \text{Ca}^{2+} \). The mean \( \text{CN}^- \)-induced RFS increase for the third and fourth application stabilized at values of between 200 and 270% of control (Fig. 7B), and the concomitant \([\text{Ca}^{2+}]_i\) rise ranged between 10 and 45 nM (Fig. 7D). To exclude that the RFS responses to \( \text{CN}^- \) are due to a nonspecific action of the agent, the effects of block of aerobic metabolism by rotenone, an inhibitor of complex-I NADH dehydrogenase, were tested. Bath application of 1 \( \mu \text{M} \) rotenone (1 min) produced a RFS increase that was very similar with that evoked by \( \text{CN}^- \) (178 ± 87%, \( n = 15 \); not shown).

### Relation between metabolism-induced RFS changes and \([\text{Ca}^{2+}]_i\)

The preceding experiments showed that the prominent RFS increase in response to block of aerobic metabolism with \( \text{CN}^- \) occurs without a major change of \([\text{Ca}^{2+}]_i\). For further characterization of the relation of \( \Delta \Psi \) and intracellular \( \text{Ca}^{2+} \) during metabolic manipulation, the effects of oligomycin and FCCP were analyzed. The cell illustrated in Fig. 8A responded to repetitive application of \( \text{CN}^- \) with an almost identical increase in RFS. Subsequent exposure of oligomycin resulted in the typical decrease of RFS (compare Fig. 1). In the presence of

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**FIG. 6.** Inhibitory effects of intracellular eosin on recovery from voltage-dependent \( \Delta \Psi \) depolarization and \([\text{Ca}^{2+}]_i\) rise. A: continuous recording started \( \sim 5 \) min after establishing the whole cell configuration with a patch electrode that contained 100 \( \mu \text{M} \) of the \( \text{Ca}^{2+}/\text{H}^+ \) pump blocker eosin-B in addition to Rh-123. Plasmaemmal depolarization from \( -60 \) to 0 mV led to a progressive increase in the RFS, indicating consecutive diminution of mitochondrial potential. B: in a different neuron that was dialysed with eosin-B and fura-2, recovery from plasmalemmal depolarization as in A was delayed considerably with regard to control measurements without intracellular eosin as exemplified for 1 cell (gray traces) in C.

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**FIG. 7.** Effects of consecutive periods of plasma membrane depolarization and metabolic inhibition on \( \Delta \Psi \) and \([\text{Ca}^{2+}]_i\). A: cell was subjected to regular application of depolarizing pulses (from \(-60 \) to 0 mV) and bath application of \( \text{CN}^- \) (1 mM). \( \Delta \Psi \) depolarization (evident as an increase in RFS) as evoked by voltage pulses was attenuated consecutively in this cell. In contrast, the depolarizing \( \Delta \Psi \) response to block of aerobic metabolism by \( \text{CN}^- \) progressively increased. B: statistical analysis revealed attenuation of the voltage-dependent (●) \( \Delta \Psi \) depolarization (after a slight potentiation during the 2nd stimulation) whereas the mitochondrial response to \( \text{CN}^- \) (□) increased in particular during the 2nd administration (\( n = 5 \)). C: in this pyramidal neuron, consecutive depolarization led to potentiation of the rise of intracellular \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+}]_i\)], whereas the moderate [\( \text{Ca}^{2+}]_i\)], increase due to \( \text{CN}^- \) slightly decreased in magnitude. D: statistical analysis shows that the rises of [\( \text{Ca}^{2+}]_i\)], during depolarization and \( \text{CN}^- \) were rather stable (\( n = 5 \)).
oligomycin, the recovery of the RFS rise due to a further application of CN\(^{-}\) was faster than under control although the peak response was potentiated. Addition of FCCP to the oligomycin-containing superfusate after recovery from CN\(^{-}\) induced a RFS increase to the same level as seen during CN\(^{-}\) in the presence of oligomycin. On average, the CN\(^{-}\)-evoked RFS rise in this series of four experiments was 202 ± 20%, whereas the potentiated response in the presence of oligomycin was 285 ± 25%. Oligomycin produced a hyperpolarization by 46 ± 10%, whereas FCCP evoked a RFS increase by 300 ± 20% in the presence of oligomycin. In five different neurons, CN\(^{-}\) elevated [Ca\(^{2+}\)], by <30 nM. Exposure of these cells to oligomycin neither had an effect on [Ca\(^{2+}\)], baseline nor were the very moderate CN\(^{-}\)-evoked intracellular Ca\(^{2+}\) rises potentiated. Also FCCP only produced a [Ca\(^{2+}\)], rise of <30 nM in the presence of oligomycin (Fig. 8B). Oligomycin did not affect \(I_m\) or \(g_m\), whereas FCCP induced an inward current in two of the five CA1 neurons.

**Imaging of voltage-dependent and CN\(^{-}\)-induced \(\Delta \Psi\) depolarization**

The experiments described so far showed that reproducible \(\Delta \Psi\) changes can be recorded for extended time periods with a photomultiplier-based optical system in voltage-clamped CA1 neurons in situ. In a final approach, imaging was used in 12 (Rh-123 measurements) and 11 ([Ca\(^{2+}\)], measurements) neurons to determine the extent to which spatial resolution of the intracellular fluorescent signals could be resolved. In the example of Fig. 9, the Rh-123 fluorescence under resting conditions was more prominent in somatic regions close to the nucleus. On plasmalemmal depolarization, the Rh-123 fluorescence increased primarily in this area and invaded neither the region of the nucleus nor sites close to the plasma membrane. On exposure of the cell to CN\(^{-}\), the Rh-123 fluorescence increase, which was considerably larger than that on depolarization of the plasma membrane, again appeared rather localized in somatic regions that were not occupied by the nucleus. CN\(^{-}\) did not produce a major signal in the vicinity of the plasma membrane (Fig. 9B). In contrast, the depolarization-evoked rise of [Ca\(^{2+}\)], distributed rapidly and uniformly over the entire soma (Fig. 10A), whereas the CN\(^{-}\)-induced minor rise of [Ca\(^{2+}\)], was diffusely distributed over the somatic region (Fig. 10B).

**DISCUSSION**

In the first part of RESULTS, which contains a summary of the principal of \(\Delta \Psi\) measurements with Rh-123, it was demonstrated that mammalian neurons of brain slices can be dialyzed via the whole cell recording patch electrode with Rh-123 for long-term recording of mitochondrial membrane potential and therefore metabolic activity. It also was shown that different ways of normalization of the Rh-123 fluorescence signal are possible. Furthermore it was found that FCCP induces a prominent increase in RFS indicating mitochondrial depolarization whereas oligomycin evokes a noticeable hyperpolarization similar to previous studies on isolated cells (Duchen 1992, 1999; Duchen and Bisoe 1992). This indicates a wide dynamic range of activity-related mitochondrial membrane potential changes in neurons in situ. Accordingly we demonstrated that depolarization of the plasma membrane elicits a reversible \(\Delta \Psi\) depolarization that is likely to be due to stimulation of Ca\(^{2+}\) uptake into mitochondria. In contrast, a prominent CN\(^{-}\)-induced mitochondrial depolarization developed in the absence of a major cytosolic Ca\(^{2+}\) increase as a consequence of block of the electron transport chain. On the basis of methodological considerations, future applications of the technique for simultaneous measurement of cell metabolism and excitability are considered.

**Ca\(^{2+}\)-dependent \(\Delta \Psi\) depolarization**

Previous studies using extracted mitochondria (Gunter et al. 1994; McCormack et al. 1990) or isolated cells (Duchen 1992, 1999; Duchen and Bisoe 1992; Loew et al. 1994; Nowicky and Duchen 1998) have established that a rise of cytosolic [Ca\(^{2+}\)], depolarizes mitochondria. As shown in detail in the latter studies, this mitochondrial depolarization is secondary to influx of Ca\(^{2+}\) into these organelles via an electrogenic Ca\(^{2+}\) uniporter conductive pathway (see also Babcock et al. 1997; David et al. 1998). At present, it is thought that the increase of intramitochondrial Ca\(^{2+}\) is not only important for buffering of cytosolic Ca\(^{2+}\) loads (Babcock and Hille 1998; Herrington et al. 1996; Werth et al. 1996) but also serves to stimulate aerobic energy production (Gunter et al. 1994; McCormack et al. 1990). This view gains support from the finding of an initial oxidation of NAD(P)H and FADH, which turns into a secondary increase in the reduced state of both enzymes (Duchen 1992, 1999; Schuchmann et al. 1998). In the
present study, a robust rise of \([\text{Ca}^{2+}]_i\) by 200–500 nM was evoked in the CA1 pyramidal cells by depolarization of the plasma membrane. The magnitudes of the depolarization-induced \([\text{Ca}^{2+}]_i\) rise and \(\Delta \Psi\) depolarization were parallel with the voltage dependence of voltage-gated \(\text{Ca}^{2+}\) channels in CA1 cells (for references, see Trapp et al. 1996b; see also Duchen 1992). That the \(\Delta \Psi\) depolarization was indeed caused by \(\text{Ca}^{2+}\) entry through voltage-gated \(\text{Ca}^{2+}\) channels and was not due to a direct effect of the plasmalemmal depolarization is indicated by the blocking effect of \(\text{Ni}^{2+}/\text{Cd}^{2+}\) and \(\text{Ca}^{2+}\)-free superfusate on both the \([\text{Ca}^{2+}]_i\) and \(\Delta \Psi\) response (Duchen 1992).

The fall of Rh-123 fluorescence on omission of extracellular \(\text{Ca}^{2+}\) could be due to a decrease in the demand for mitochondrial ATP production (see preceding text). As an alternative,
mitochondria might mediate buffering of cytosolic Ca\textsuperscript{2+} even under resting conditions. Involvement of mitochondria in Ca\textsuperscript{2+} regulation in the hippocampal neurons is suggested by the finding that inhibition of the plasmalemmal Ca\textsuperscript{2+}/H\textsuperscript{+} pump by eosin-B prolonged recovery of both the $\Delta \Psi$ and the [Ca\textsuperscript{2+}]	extsubscript{i} transient on plasmalemmal depolarization. The potentiation of the peak $\Delta \Psi$ response on repetitive periods of plasmalemmal depolarization in the presence of eosin-B can be explained by the observation that recovery of RFS from a single stimulus was much slower than that of the [Ca\textsuperscript{2+}]	extsubscript{i} transient. These results support previous assumptions that a plasmalemmal Ca\textsuperscript{2+}/H\textsuperscript{+} pump plays a major role in cellular Ca\textsuperscript{2+} homeostasis (Benham et al. 1992; Choi and Eisner 1999; Trapp et al. 1996b; Werth et al. 1996). They also suggest that impairment of Ca\textsuperscript{2+} homeostasis results in a pronounced Ca\textsuperscript{2+} load of mitochondria in the CA1 pyramidal neurons. Future monitoring of mitochondrial Ca\textsuperscript{2+} with dyes such as rhod-2 will elucidate the role of mitochondria in Ca\textsuperscript{2+} homeostasis in these central neurons as previously demonstrated for other excitable cells (Babcock and Hille 1998; Babcock et al. 1997; David et al. 1998; Pivovarova et al. 1999; Werth and Thayer 1994).

That influx of Ca\textsuperscript{2+} initiates the mitochondrial depolarization and not vice versa, that mitochondrial depolarization promotes the cytosolic Ca\textsuperscript{2+} signal, is indicated by the results from the simultaneous fura-2 and Rh-123 measurements. These recordings showed that the [Ca\textsuperscript{2+}]	extsubscript{i} rise precedes the $\Delta \Psi$ response on average by 1.6 s. As suspected by Nowicky and Duchen (1998) on the basis of similar simultaneous measurements in fura-2 and Rh-123 bulk-loaded dissociated hippocampal cells, the time lag might be smaller because the Rh-123 signal reflects unquenched dye that left the mitochondrial compartment on depolarization of the organelles (see also Chen 1998). As the fura-2 signal during the simultaneous recordings was not ratiometrically measured in this series of experiments of the present study, the Ca\textsuperscript{2+} transient could not be quantified. Ratiometric [Ca\textsuperscript{2+}]	extsubscript{i} measurements are, in principal, possible during simultaneous monitoring of $\Delta \Psi$ and [Ca\textsuperscript{2+}]	extsubscript{i}, provided that the optical set up allows for excitation with three alternating wavelengths (Nowicky and Duchen 1998). The fura-2 signal did not appear to interfere with the RFS, but the extent to which a major change in Rh-123 fluorescence might influence the fura-2 signal (Duchen 1992) needs to be analyzed in future studies.

It was found with long-term recording that the average magnitude of the $\Delta \Psi$ depolarization decreased in $\sim$50% of cells and even was abolished in individual neurons on consecutive administration of depolarizing voltage steps. Nevertheless, the persistence and even potentiation of the $\Delta \Psi$ response in response to CN\textsuperscript{-} (see following text) excludes that wash-out or bleaching of Rh-123 is responsible for this effect. It is probable, but deserves further experimental analysis, that the attenuation of the depolarization-evoked $\Delta \Psi$ response represents saturation of mitochondrial Ca\textsuperscript{2+} stores, which inhibits further Ca\textsuperscript{2+} entry (Duchen 1992; Pivovarova et al. 1999; Werth and Thayer 1994).

Depolarization voltage steps with a duration of $>1$ s were necessary to reveal a mitochondrial depolarization. We tested here the suitability of the method of dialysis of neurons with Rh-123 with a simple experimental procedure that is not hampered by signals originating from synaptic and metabolic interactions within the slice. We chose depolarization of the plasma membrane as a test protocol because the effects of [Ca\textsuperscript{2+}]	extsubscript{i} rise due to voltage-activated Ca\textsuperscript{2+} channels on mitochondrial energetics have been elucidated in detail in isolated neurons and glia (Duchen 1992, 1999; Duchen and Biscoe 1992; Nowicky and Duchen 1998). More prominent $\Delta \Psi$ depolarizations are likely to be revealed in future studies on synaptic activation for several reasons. On tetanic stimulation, that is necessary to evoke synaptic plasticity (Andersen et al. 1977; Bliss and Collingridge 1993; Edwards 1995; Tang and Zucker 1997) not only intracellular Ca\textsuperscript{2+} is elevated but also intracellular Na\textsuperscript{+} increases by several tens of millimolar (Ballanyi et al. 1984; Yu and Salter 1998). As indicated by a concomitant prominent fall of tissue oxygen, that correlates with the kinetics of Na\textsuperscript{+}/K\textsuperscript{+} pump activation (for references, see Brockhaus et al. 1993), the activity-related Na\textsuperscript{+} rise also should stimulate aerobic metabolism and thus dissipate mitochondrial potential.

Furthermore the [Ca\textsuperscript{2+}]	extsubscript{i} rise associated with synaptic activity of hippocampal neurons typically exceeds that evoked by exclusive activation of voltage-gated Ca\textsuperscript{2+} channels due to activation of both ionotropic and metabotropic glutamate receptors (Alford et al. 1993; Magee et al. 1995; Murphy and Miller 1988; Regehr and Tank 1994; Regehr et al. 1989). Accordingly, it was shown that activation of ionotropic glutamate receptors evokes a profound $\Delta \Psi$ depolarization in ester-loaded neurons (Bindokas et al. 1998; Budd and Nicholls 1996; Hoyt et al. 1998; Schinder et al. 1996; Schuchmann et al. 1998; White and Reynolds 1996). The recovery from glutamate receptor-dependent mitochondrial depolarization was sometimes considerably slower than that of the concomitant [Ca\textsuperscript{2+}]	extsubscript{i} rise (Schuchmann et al. 1998) and even could be incomplete, in particular if the N-methyl-d-aspartate type of glutamate receptors was involved (Khodorov et al. 1996; Schinder et al. 1996; White and Reynolds 1996). This led to the assumption by the latter authors that Ca\textsuperscript{2+}-dependent mitochondrial disfunction is a primary event in glutamate-induced neurotoxicity due to anoxic/ischemic insults (Budd and Nicholls 1996; Choi 1988; Kristian and Siesjö 1996; Schanne et al. 1979; Stout et al. 1998).

CN\textsuperscript{-}-induced $\Delta \Psi$ depolarization

In contrast to a maximal $\Delta \Psi$ response of 50% RFS increase on depolarization of the plasma membrane, block of aerobic metabolism produced a mitochondrial depolarization that could amount to 300% RFS of control. One explanation of these differences might be that only those mitochondria close to the cell membrane contribute to the changes of RFS due to plasmalemmal depolarization. [Ca\textsuperscript{2+}]	extsubscript{i} rises in more central microdomains of the cytosol might be too small to induce mitochondrial depolarization (for references, see Duchen 1999; Pivovarova et al. 1999). Nevertheless, the CN\textsuperscript{-} effects substantiate the preceding assumption that the depolarization-induced Rh-123 signals are not at saturating levels of the dynamic range of cellular $\Delta \Psi$ changes. This suggests that the expected changes in mitochondrial potential during intense neuronal activity could in principal be well resolved. Because of the strong temperature dependence of $\Delta \Psi$ depolarizations associated with metabolic blockade (Duchen and Biscoe 1992) even larger responses to CN\textsuperscript{-} are probably inducible at 36–37°C instead of 30°C used in the present study. The reduced in
vitro temperature also might explain the moderate effects of CN" on membrane current and [Ca\(^{2+}\)] (for references, see Morris et al. 1991). Also the short application time of 30 s might contribute to the small effects on current and intracellular Ca\(^{2+}\). Accordingly, exposure of isolated CA1 neurons to CN" for 2 min was found to promote a [Ca\(^{2+}\)] rise of 100 nM, leading to a pronounced hyperpolarization by opening of Ca\(^{2+}\)-activated K\(^+\) channels (Nowicky and Duchen 1998). It is also possible that the moderate CN"-induced [Ca\(^{2+}\)] rise is partly due to dialysis of the cells (compare Bickler and Hansen 1998; Yamagushi et al. 1998). However, no difference in the CN"-related [Ca\(^{2+}\)] increase was found between whole cell recorded and intact dorsal vagal medullary neurons (Ballanyi and Kulik 1998). In the latter study, it was suggested that the small initial [Ca\(^{2+}\)] rise in the vagal neurons is mediated by Ca\(^{2+}\) release from mitochondria as also was suggested for Purkinje cells of cerebellar slices (Ballanyi et al. 1999). Although the mechanism of the initial moderate rise of intracellular Ca\(^{2+}\) in the CA1 neurons during CN" remains to be elucidated, our measurements clearly show that changes in mitochondrial function, and therefore of metabolism, can occur in the absence of a major cytosolic Ca\(^{2+}\) signal.

The CN"-induced ΔΨ signal appears to represent mitochondrial depolarization and not an artificial response to the drug as block of aerobic metabolism by rotenone produced a very similar effect. The prominent ΔΨ responses on blockade of aerobic metabolism occurred despite dialysis of the neurons with ATP via the patch electrode. This supports previous assumptions that the mitochondrial depolarization is not primarily related to a decrease in the ATP/ADP\,P\, ratio (Duchen 1992; Duchen and Biscoe 1992). In agreement with conclusions from the latter studies, the present results suggest that the ΔΨ depolarization induced by the metabolic inhibitors is caused by block of electron transport through the respiratory chain. Nevertheless, dialysis of the cells with ATP-containing patch pipette solution might partly counteract the depolarizing effect of CN" on mitochondrial potential by providing the fuel for reverse-mode operation of the ATP synthase, which then would hyperpolarize ΔΨ (Babcock et al. 1997). However, this mechanism does not appear to have a major contribution as block of the ATP synthase with oligomycin only led to a modest potentiation of the CN"-induced mitochondrial depolarization. Interestingly, the oligomycin-potentiated CN"-evoked depolarization was almost identical with the expected maximal depolarization induced by FCCP (Duchen 1992).

**Future applications**

High-resolution imaging techniques using fluorophores that are only sensitive to very high Ca\(^{2+}\) levels demonstrated that [Ca\(^{2+}\)] rises can amount to several μM in dendrites during tetanic stimulation that is necessary to induce long-term changes of synaptic plasticity (Regehr and Tank 1992). Because these activity-related Ca\(^{2+}\) transients last for up to several seconds, it can be assumed on the basis of the present results that they induce depolarization of mitochondria. It is established that mitochondria are present not only in the soma but also along the entire dendritic tree of CA1 neurons (Nafstad and Blackstad 1966; Siklos and Kuhn 1994). Thus it should be possible to monitor localized metabolic activity by means of dynamic changes of ΔΨ in different compartments of mammalian neurons in situ. In the present study, spatial resolution was hampered by the fact that ΔΨ imaging was not done with confocal or two-photon optical techniques. Accordingly, resolution of mitochondrial signals or of [Ca\(^{2+}\)]\(^+\) rises in small dendrites or even spines was not possible. Nevertheless it was revealed that Rh-123 fluorescence under resting conditions was distributed nonuniformly in spots and that the region of the nucleus did not show fluorescence. This is consistent with findings in cultured hippocampal neurons in which mitochondria were found to be clustered in particular in the perinuclear somatic region (Bindokas et al. 1998; Schinder et al. 1996) as also shown for other cell types (Chen 1988; Duchen 1999; White and Reynolds 1996). Ester loading of hippocampal slices with ΔΨ-sensitive dyes recently was demonstrated as a powerful tool to study the relation of metabolism and synchronized electrical activity. In extension of this work, we have presented here a method for selective labeling of individual neurons within functionally intact neuronal networks. This allows for simultaneous recording of membrane excitability, energy metabolism and [Ca\(^{2+}\)]. The method also should be applicable to presynaptic boutons (David et al. 1998; Tang and Zucker 1997) as well as to single glial cells in brain slices (Kulik et al. 1999). Therefore it should be possible to elaborate in future studies how stimulated neuronal and/or glial metabolism (Tsacopoulos and Magistretti 1996) contributes to synaptic plasticity (Edwards 1995; Tang and Zucker 1997) or pathological processes such as epilepsy (Duchen 1992; Lee et al. 1984; Schuchmann et al. 1999) or ischemia-related neurotoxicity (Budd and Nicholls 1996; Choi 1988; Kristian and Siesjö 1996; Schanne et al. 1978).

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