Metabotropic Receptor-Mediated Ca\(^{2+}\) Signaling Elevates Mitochondrial Ca\(^{2+}\) and Stimulates Oxidative Metabolism in Hippocampal Slice Cultures

Oliver Kann, Richard Kovács, and Uwe Heinemann

Department of Neurophysiology, Charité, Humboldt University, D-10117 Berlin, Germany; and Department of Neurochemistry, Hungarian Academy of Sciences, Budapest 1025, Hungary

Submitted 15 January 2003; accepted in final form 17 April 2003

Kann, Oliver, Richard Kovács, and Uwe Heinemann. Metabotropic receptor-mediated Ca\(^{2+}\) signaling elevates mitochondrial Ca\(^{2+}\) and stimulates oxidative metabolism in hippocampal slice cultures. J Neurophysiol 90: 613–621, 2003. First published April 30, 2003; 10.1152/jn.00042.2003. Metabotropic receptors modulate numerous cellular processes by intracellular Ca\(^{2+}\) signaling, but less is known about their role in regulating mitochondrial metabolic function within the CNS. In this study, we demonstrate in area CA3 of rat organotypic hippocampal slice cultures that glutamatergic, serotonergic, and muscarinic metabotropic receptor ligands, namely trans-azetidine-2,4-dicarboxylic acid, α-methyl-5-hydroxytryptamine, and carbachol, transiently increase mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{mt}\)) as recorded by changes in Rhod-2 fluorescence, stimulate mitochondrial oxidative metabolism as revealed by elevations in NAD(P)H fluorescence, and induce K\(^{+}\) outward currents as monitored by rapid increases in extracellular K\(^{+}\) concentration ([K\(^{+}\)]\(_{e}\)). Carbachol (1–1,000 μM) elevated NAD(P)H fluorescence by ≤14% ΔF/ΔF\(_{0}\) and increased [K\(^{+}\)]\(_{e}\) by ≤4.3 mM in a dose-dependent manner. Carbachol-induced responses persisted in [Ca\(^{2+}\)]\(_{mt}\)-free solution and blockade of ionotropic glutamatergic and nicotinic receptors. Under similar conditions caffeine, known to cause Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CIR), also evoked elevations in [Ca\(^{2+}\)]\(_{mt}\), NAD(P)H fluorescence and [K\(^{+}\)]\(_{e}\) that, in contrast to carbachol-induced responses, displayed oscillations. After depletion of intracellular Ca\(^{2+}\) stores by carbachol in Ca\(^{2+}\)-free solution, re-application of 1.6 mM Ca\(^{2+}\)-containing solution triggered marked elevations in [Ca\(^{2+}\)]\(_{mt}\), NAD(P)H fluorescence and [K\(^{+}\)]\(_{e}\). These data indicate that metabotropic transmission effectively regulates mitochondrial oxidative metabolism via diverse receptor types in hippocampal cells and that inonitol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release (ICR) or CIR or capacitative Ca\(^{2+}\) entry might suffice in stimulating oxidative metabolism by elevating [Ca\(^{2+}\)]\(_{mt}\). Thus activation of metabotropic receptors might significantly contribute to generation of ATP within neurons and glial cells.

INTRODUCTION

Metabotropic signal transmission coupled to guanine nucleotide-binding proteins (G proteins) is induced by a variety of small-molecule ligands including acetylcholine, biogenic amines like histamine and serotonin as well as amino acids like γ-aminobutyric acid (GABA) and glutamate. One of the intracellular signaling cascades activated by metabotropic receptors is generation of second-messenger inositol 1,4,5-trisphosphate (InsP\(_{3}\)), which acts on InsP\(_{3}\) receptors to release Ca\(^{2+}\) from endoplasmatic reticulum (ER; InsP\(_{3}\)-induced Ca\(^{2+}\) release, IICR). In concert with ryanodine receptors, which are also located at the ER and potentially mediate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), complex spatiotemporal Ca\(^{2+}\) signals can be generated within excitable and nonexcitable cells (Clapham 1995; Meldolesi 2001; Verkhratsky et al. 1998).

It is assumed that 60% of ATP consumption within the CNS is used for ion transport across plasma membranes to maintain or restore neuronal excitability and another 10–20% for the process of neurotransmission (Ames 2000). Most of ATP is generated within mitochondria by oxidative metabolism that primarily uses pyruvate to generate NADH and FADH\(_{2}\) in the Krebs’ cycle (Ames 2000; Duchen 1999). These reduced co-factors are essential to establish a potential across the inner mitochondrial membrane that drives F\(_{1}\)F\(_{0}\)-ATP synthase to phosphorylate ADP, thereby generating ATP (Ames 2000; Mitchell 1966). The current concept concerning the regulation of oxidative metabolism primarily emerges from studies of isolated mitochondria and cultures of nonexcitable cells (Hansford and Zorov 1998; McCormack et al. 1990; Pralong et al. 1994; Robb-Gaspers et al. 1998; Veronina et al. 2002). This concept involves mitochondrial Ca\(^{2+}\), which regulates the activity of different dehydrogenases within the Krebs’ cycle in the nanomolar to micromolar range (Denton et al. 1978; McCormack 1985; Rutter 1990), as well as the ratios of substrates like ADP/ATP, NAD\(^{+}\)/NADH or CoA/acetyl CoA (Hansford 1980; Reed and Yeaman 1987).

The capability of diverse metabotropic transmitters in generating complex spatiotemporal Ca\(^{2+}\) signals within nerve and glial cells has been well documented (Berridge 1998; Blaustein and Golovina 2001; Verkhovsky et al. 1998). Moreover, via intracellular Ca\(^{2+}\)-signaling metabotropic transmitters trigger several cellular processes and influence membrane excitability, dendritic integration, and synaptic plasticity (Nakamura et al. 1999; Tsubokawa and Ross 1997; Vanderklish and Edelman 2002). Surprisingly, less is known about the role of metabotropic transmitter signaling in regulation of energy homeostasis, namely generation of ATP within cells of the CNS. However, it is essential for our understanding of neurophysiology as well as of pathophysiology of various neurological diseases (Kovács et al. 2001; Mattson 2000; Schapira 1999; Schuchmann et al. 1998) whether metabotropic receptor-evoked in-
creases in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{c}}\)]\(_{\text{m}}\)) are sufficient to elevate mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{m}}\)]\(_{\text{m}}\)) and to have, thereby, functional consequences on mitochondrial oxidative metabolism.

We therefore investigated the effects of different Ca\(^{2+}\)-mobilizing metabotropic receptor ligands and, in detail, cellular Ca\(^{2+}\) release and Ca\(^{2+}\) entry pathways by combining microfluorimetric and electrophysiological techniques. Rhod-2-based fluorimetry was used to monitor changes in [Ca\(^{2+}\)]\(_{\text{m}}\) (Babcok et al. 1997; Billups and Forsythe 2002; Rutter et al. 1996). We recently verified this method in organotypic hippocampal slice cultures by demonstrating that mitochondrial uncoupler, carbonyl cyanide \(m\)-chlorophenyl hydrazone (CCCP), strongly reduced stimulus-induced elevations in Rhod-2 fluorescence (Kann et al. 2003). NAD(P)H fluorescence signals provide an intrinsic parameter of mitochondrial metabolic function within living cells (Hajnóczky et al. 1995; Schuchmann et al. 1998). When excited by UV light, NAD(P)H fluorescence originates from fluororescing reduced forms of nicotinamide adenine dinucleotides, NADH, and NADPH, whereas the oxidized forms are nonfluorescent (Aubin 1979). Simultaneously to Rhod-2 or NAD(P)H fluorescence recordings, we monitored changes in extracellular K\(^{+}\) concentration ([K\(^{+}\)]\(_{\text{o}}\)) using ion-sensitive microelectrodes (Cordingley and Somjen 1978; Heinemann and Lux 1975) to get insight into K\(^{+}\) fluxes occurring at the plasma membranes on application of the ligands.

Our data provide evidence that ligands of metabotropic glutamatergic, serotonergic, and muscarinic receptors and more in detail IICR, CICR, as well as capacitative Ca\(^{2+}\) entry, increase [Ca\(^{2+}\)]\(_{\text{m}}\) effectively, stimulate mitochondrial oxidative metabolism, and induce K\(^{+}\) outward currents.

**METHODS**

**Tissue culture**

Organotypic hippocampal slice cultures were prepared as described previously (Stoppini et al. 1991). In brief, hippocampal slices (400 \(\mu\)m) were cut from 7- to 9-day-old Wistar rats under sterile conditions in gassed (95\% O\(_{2}\)-5\% CO\(_{2}\)) ice-cold minimal essential medium (MEM, Gibco, Invitrogen, Karlsruhe, Germany). Slices were maintained on a biomembrane surface (0.4 \(\mu\)m, Millicell-CM, Millipore, Eschborn, Germany) between culture medium (50\% MEM, 25\% Hank’s balanced salt solution (Sigma, Taufkirchen, Germany), 25\% horse serum (Gibco), and 2 \(m\) M l-glutamine at pH 7.3) and humidified atmosphere (5\% CO\(_{2}\), 36.5\(^\circ\)C) in an incubator (Unitherm 150, UniEquip, Martinsried, Germany). Culture medium was completely replaced twice a week. Slice cultures were used for experiments after 7–10 days in vitro. All animals were housed, cared and killed in accordance with the recommendations of the European Commission and the Berlin Animal Ethics Committee.

**Solutions and recordings**

Slice cultures on excised membranes were superfused in the recording chamber with gassed (20\% O\(_{2}\)-5\% CO\(_{2}\)) artificial cerebrospinal fluid (ACSF) at 34 \(\pm\) 1\(^\circ\)C that contained (in mM) 129 NaCl, 3 KCl, 1.25 NaN\(_{3}\)PO\(_{4}\), 1.8 MgSO\(_{4}\), 1.6 CaCl\(_{2}\), 21 NaHCO\(_{3}\), and 10 glucose (Sigma); pH 7.35. Ca\(^{2+}\)-free ACSF was similar but with 1.6 \(m\) M MgCl\(_{2}\) in place of CaCl\(_{2}\) and 0.5 \(m\) M EGTA. Stock solutions of (AFA), 2-amino-5-phosphonopentanoic acid (o-AP5), 6-cyano-7-nitroquinolxaline-2,3-dione (CNQX), \(\alpha\)-methyl-5-hydroxytryptamine maleate (\(\alpha\)-Me-5-HT), (S)-\(\alpha\)-methyl-4-carboxyphenylglycine (MCPG), mianserin (Biotrend/Tocris, Köln, Germany), atropine, mecamylamine, carbachol, and caffeine (Sigma) were freshly dissolved in ACSF and applied via the superfusion system (rate at 4.5 \(m\)l/min). To avoid the induction of artifacts in NAD(P)H fluorescence signals by applying hyperosmolar, 20 \(m\) M caffeine containing ACSF, slice cultures were allowed to adapt to hyperosmolar (Ca\(^{2+}\)-free) ACSF (addition of 20 \(m\) M saccharose, Sigma) before and after caffeine application. The recording chamber was mounted on an epifluorescence microscope (Axioplan, Zeiss, Jena, Germany) equipped with a \(\times\)20 water-immersion objective (0.5 numerical aperture). For recordings, a double-barrelled K\(^{+}\)-sensitive/fieldd potential recording microelectrode was placed in stratum pyramidale of area CA3, and a monopolar stimulation electrode (glass pipette filled with ACSF, tip diameter: 5–10 \(\mu\)m) was positioned >350 \(\mu\)m away in st. radiatum. Slice cultures were accepted for experiments when evoked postsynaptic field potentials (single pulse of 0.1 ms) displayed amplitudes of >0.5 mV.

**Ion-sensitive microelectrodes**

DC-coupled recordings of field potentials and changes in [K\(^{+}\)]\(_{\text{o}}\), were performed with double-barrelled K\(^{+}\)-sensitive and reference microelectrodes manufactured and calibrated as described previously (Heinemann and Arens 1992). In brief, electrodes were pulled from double-barrelled theta glass (Science Products, Hoøheim, Germany). The reference barrel was filled with 154 \(m\) M NaCl solution, the ion-sensitive barrel with potassium ionophore I cocktail A (6031; Fluka Chemie, Buchs, Switzerland) and 100 \(m\) M KCl. Ion-sensitive microelectrodes with a sensitivity of 59 \(\pm\) 2 mV to a 10-fold increase in [K\(^{+}\)]\(_{\text{o}}\) were used for experiments. The amplifier was equipped with negative capacitance feedback control, which permitted recordings of changes in [K\(^{+}\)]\(_{\text{o}}\), with time constants of 50–200 ms. Signals from the electrode were digitised at 10 Hz using a standard PC and FeliX software (Photon Technology Instruments, Wedel, Germany).

**Microfluorimetric measurements of [Ca\(^{2+}\)]\(_{\text{m}}\) and NAD(P)H**

Slice cultures were stained with cell-permeable Rhod-2 AM (5 \(\mu\)M, Molecular Probes, Leiden, The Netherlands) in ACSF for 60 min at 36.5\(^\circ\)C; this facilitates accumulation of this positively charged Ca\(^{2+}\) indicator into mitochondria (Minta et al. 1989). To allow for hydrolysis of the dye-esters and to promote mitochondrial compartmentalization of Rhod-2 AM, slice cultures were then maintained in ACSF for 60–90 min at 36.5\(^\circ\)C. Excitation wavelengths (NAD(P)H, 360 nm; Rhod-2, 530 nm) were set by a monochromator system (Photon Technology Instruments). Emission light from st. pyramidal and st. radiatum of area CA3 was detected by a photomultiplier (SMT, Seefeld, Germany) at >590 nm (Rhod-2) or 460 nm (NAD(P)H) (Aubin 1979). Because the emission spectra of NADH and NADPH overlap, NAD(P)H indicates that the recorded fluorescence might have originated from either one or both. Photomultiplier data were recorded on computer disk at 10 Hz simultaneously with signals of the K\(^{+}\)-sensitive electrode. Fluorescence signals of Rhod-2 and NAD(P)H are presented as changes in \(\%\Delta F/F_{0}\) (\(\Delta F/F_{0}\) \(\times\) 100) where \(F_{0}\) is the averaged fluorescence of a 30-s period before bath application of an agonist. Illustrated traces of Rhod-2 fluorescence were corrected for bleaching and washout of the dye.

**Calculations and statistics**

To translate the recorded potential values (mV) in [K\(^{+}\)]\(_{\text{o}}\), a modified Nernst equation was used

\[
\log [\text{ion}]_{\text{o}} = E_{m}(s_{v})^{-1} + \log [\text{ion}]_{\text{rest}},
\]

with \(E_{m}\), recorded potential; \(s_{v}\), electrode slope obtained at calibration; \(v\), valence of the specific ion; [ion]\(_{\text{rest}}\), ion concentration at rest; and [ion]\(_{\text{o}}\), ion concentration during activation.
Experimental data from slice cultures (n) were obtained from at least three different preparations. All results of a particular experiment were pooled and are given as means ± SE. Groups were compared by ANOVA.

RESULTS

Application of metabotropic receptor ligands induces elevations in [Ca\(^{2+}\)]\(_{\text{m}}\), NAD(P)H fluorescence and [K\(^+\)]\(_{\text{o}}\)

Three different metabotropic receptor agonists, namely, the glutamatergic ligand, ADA (Kapur et al. 2001; Manahan-Vaughan et al. 1996), the serotonergic ligand, α-Me-5-HT (Baxter et al. 1995; Rainnie 1999), and the muscarinic ligand, carbachol (Irving and Collingridge 1998; Müller and Connor 1991), were tested for their capacity to evoke changes in [Ca\(^{2+}\)]\(_{\text{m}}\), NAD(P)H fluorescence, and [K\(^+\)]\(_{\text{o}}\) in area CA3 of organotypic hippocampal slice cultures. In these experiments, each ligand was applied via the bath solution for 60 s, while Rhod-2 or NAD(P)H fluorescence signals from st. pyramidale and st. radiatum were recorded simultaneously to electrophysiological monitoring of [K\(^+\)]\(_{\text{o}}\) from st. pyramidal.

All three ligands (ADA, 1 mM; α-Me-5-HT, 40 μM; carbachol, 10 μM) evoked reversible monotonic elevations in [Ca\(^{2+}\)]\(_{\text{m}}\) [each: 3 of 3 slice cultures tested (n = 3/3)] and in NAD(P)H fluorescence (n = 4/4, each) (Figs. 1–3). Elevations in both fluorescence signals were tightly associated with transient increases in [K\(^+\)]\(_{\text{o}}\), ranging from ~0.1 mM (α-Me-5-HT, 40 μM) to 1.8 mM (ADA, 1 mM) from the baseline of 3 mM. Interestingly, on any application of metabotropic receptor ligands, [Ca\(^{2+}\)]\(_{\text{m}}\) and NAD(P)H fluorescence were still elevated at times when [K\(^+\)]\(_{\text{o}}\) had decreased to preapplication levels.

FIG. 1. Application of a metabotropic glutamatergic receptor ligand induces elevations in [Ca\(^{2+}\)]\(_{\text{m}}\), NAD(P)H fluorescence and [K\(^+\)]\(_{\text{o}}\). Bath application of metabotropic glutamatergic ligand trans-azetidine-2,4-dicarboxylic acid (ADA) evoked elevations in [Ca\(^{2+}\)]\(_{\text{m}}\) (Rhod-2 fluorescence), NAD(P)H fluorescence, and [K\(^+\)]\(_{\text{o}}\) in area CA3 of organotypic hippocampal slice cultures. ADA (1 mM) was bath applied for 60 s (●), while Rhod-2 or NAD(P)H fluorescence signals from st. pyramidale and st. radiatum were recorded simultaneously to electrophysiological monitoring of [K\(^+\)]\(_{\text{o}}\) from st. pyramidal. Changes in fluorescence signals were expressed as %ΔF/ΔF\(_{\text{o}}\).

FIG. 2. Application of a metabotropic serotonergic receptor ligand induces elevations in [Ca\(^{2+}\)]\(_{\text{m}}\), NAD(P)H fluorescence and [K\(^+\)]\(_{\text{o}}\). Bath application of metabotropic serotonergic ligand α-methyl-5-hydroxytryptamine (α-Me-5-HT) evoked elevations in [Ca\(^{2+}\)]\(_{\text{m}}\) (Rhod-2 fluorescence), NAD(P)H fluorescence, and [K\(^+\)]\(_{\text{o}}\) in area CA3 of organotypic hippocampal slice cultures. α-Me-5-HT (40 μM) was bath applied for 60 s (●), while Rhod-2 or NAD(P)H fluorescence signals from st. pyramidale and st. radiatum were recorded simultaneously to electrophysiological monitoring of [K\(^+\)]\(_{\text{o}}\) from st. pyramidale. Changes in fluorescence signals were expressed as %ΔF/ΔF\(_{\text{o}}\).

FIG. 3. Application of a muscarinic receptor ligand induces elevations in [Ca\(^{2+}\)]\(_{\text{m}}\), NAD(P)H fluorescence and [K\(^+\)]\(_{\text{o}}\). Bath application of muscarinic ligand carbachol (CCh, 10 μM, 60 s, ■) evoked elevations in [Ca\(^{2+}\)]\(_{\text{m}}\) (Rhod-2 fluorescence), NAD(P)H fluorescence, and [K\(^+\)]\(_{\text{o}}\) in area CA3 of organotypic hippocampal slice cultures. Rhod-2 or NAD(P)H fluorescence signals from st. pyramidale and st. radiatum were recorded simultaneously to electrophysiological monitoring of [K\(^+\)]\(_{\text{o}}\) from st. pyramidale. Changes in fluorescence signals were expressed as %ΔF/ΔF\(_{\text{o}}\).
mM), α-Me-5-HT (40 μM), or carbachol (100 μM) were completely blocked in the presence of MCPG (500 μM, not shown), mianserin (10 μM, not shown), or atropine (1 μM, Fig. 4C), respectively (n = 3; each). The ADA-evoked elevations in [Ca^{2+}]_m, NAD(P)H fluorescence and in [K^+]_o persisted during blockade of ionotropic glutamate receptors by CNQX (60 μM) and t-AP5 (60 μM) (not shown).

To characterize the relationship between metabotropic ligand-induced elevations in NAD(P)H fluorescence and increases in [K^+]_o, we focused on carbachol. Carbachol was applied at different concentrations over the range of 1–1,000 μM in 14 slice cultures. Maximal responses in NAD(P)H fluorescence and [K^+]_o were evoked by 1,000 μM carbachol whereas a concentration of 1 μM carbachol was effective only in one of three slice cultures. The dose-response curve had an EC_{50} value of 34.9 μM (Fig. 4A). When elevations in NAD(P)H fluorescence evoked by application of carbachol at different concentrations were plotted as a function of increases in [K^+]_o, linear regression analysis revealed a positive correlation between both parameters (r = 0.62; P < 0.001; n = 39; Fig. 4B). Because we recently observed strong positive correlations between stimulus-induced increases in [K^+]_o as a measure of neuronal activation, NAD(P)H signals, and rises in [Ca^{2+}]_m (Kann et al. 2003), the positive correlation between carbachol-induced elevations in [K^+]_o and NAD(P)H signals might also provide indirect evidence for a fine-tuned coupling of elevations in [Ca^{2+}]_m/[Ca^{2+}]_l and stimulation of mitochondrial oxidative metabolism.

The data from these experiments indicate that different InsP_3-coupled, metabotropic receptor ligands increase [Ca^{2+}]_l effectively to stimulate mitochondrial oxidative metabolism, and induce K^+ outward currents.

**IICR and CICR elevate [Ca^{2+}]_m, NAD(P)H fluorescence and [K^+]_o.**

Because metabotropic receptor-evoked elevations in [Ca^{2+}]_c might be due to activation of Ca^{2+} entry and Ca^{2+} release pathways, we investigated next whether release of Ca^{2+} from ER was sufficient to mimic the effects of metabotropic receptor ligands on [Ca^{2+}]_m, NAD(P)H fluorescence and [K^+]_o.

To isolate IICR from capacitative Ca^{2+} entry, a mechanism that is considered to refill depleted Ca^{2+} stores (Nilius and Droogmans 2001; Parekh and Penner 1997), carbachol was applied in Ca^{2+}-free ACSF. Before application of carbachol, slice cultures were superfused with Ca^{2+}-free ACSF for 6 min. This superfusion period sufficed to completely block evoked postsynaptic field potentials and stimulus-induced elevations in [Ca^{2+}]_l/[Ca^{2+}]_m in hippocampal slice cultures (Kann et al. 2003) as well as depolarization-induced intracellular Ca^{2+} increases in pyramidal cells of acute hippocampal slices (Schuchmann et al. 2000). Because Ca^{2+} release from intracellular stores might trigger release of neurotransmitters (Cochilla and Alford 1998; Emptage et al. 2001), Ca^{2+}-free ACSF additionally contained antagonists, CNQX and t-AP5 (60 μM, each) to block any putative activation of ionotropic glutamate receptors. Under these conditions, application of carbachol (500 μM, 60 s) induced a biphasic NAD(P)H fluorescence signal in which an initial drop became more apparent as compared with lower carbachol concentrations (Fig. 5A, see also Fig. 3). The initial drop was followed by a long-lasting, monotonic elevation of the signal (7.9 ± 0.7%/ΔF/F_0) for ≤15 min (n = 3/3). Initially, these changes in NAD(P)H fluorescence were associated with increases in [K^+]_o by 1.6 ± 0.1 mM from basal levels (n = 3/3; Fig. 5A). These carbachol-induced biphasic changes in NAD(P)H fluorescence and the increases in [K^+]_o were also evoked by prolonged carbachol application (500 μM) for 180 s, and they were resistant to additional application of the nonspecific nicotinic acetylcholine receptor antagonist, mecamylamine (20 μM), still indicating the muscarinic nature of the responses at higher concentrations of carbachol (not shown).

Caffeine, in millimolar concentrations, is known to cause CICR by activation of ryanodine receptors located on the membrane of ER. Thus caffeine provides an alternative tool in initiating Ca^{2+} release from intracellular stores (McPherson et al. 1991; Shmigol et al. 1996). To avoid artificial changes in NAD(P)H fluorescence due to ATP consumption associated with cellular responses to changes of osmolarity under 20 mM caffeine, slice cultures were superfused with hyperosmolar Ca^{2+}-free ACSF (substitution of 20 mM saccharose instead of caffeine) before and after application of the ligand. Caffeine (20 mM, 180 s) evoked long-lasting elevations in NAD(P)H.
Ca\(^{2+}\) entry after store depletion increases \([Ca^{2+}]_m\), NAD(P)H fluorescence and \([K^+]_o\).

Ca\(^{2+}\) entry after depletion of intracellular Ca\(^{2+}\) stores provides a further mechanism that elevates \([Ca^{2+}]_c\) and that is thought to replenish the Ca\(^{2+}\) stores (Emptage et al. 2001; Parekh and Penner 1997). Thus we next focused on the effects of Ca\(^{2+}\) entry after Ca\(^{2+}\) store depletion on \([Ca^{2+}]_m\), NAD(P)H fluorescence and \([K^+]_o\). Store depletion was achieved by application of carbachol in Ca\(^{2+}\)-free ACSF. In some slice cultures, application of the ligand was performed twice, while first Rhod-2 fluorescence and \([K^+]_o\) and subsequently NAD(P)H fluorescence and \([K^+]_o\) were recorded to monitor fluorescence parameters under identical conditions. Recordings of stimulus-induced postsynaptic field potentials (single pulse of 0.1 ms) before and after such experiments documented that the viability of the slice cultures was only slightly affected by the experimental procedure (Fig. 6B).

Interestingly, application of Ca\(^{2+}\)-free ACSF did not only

\[ \text{fluorescence (11.7 \pm 0.9\%/F_0), and transient increases in [K\(^+]_o \text{ by 1.9 \pm 0.1 mM (n = 3/3). In contrast to application of carbachol, caffeine-induced elevations in NAD(P)H fluorescence displayed marked oscillations in NAD(P)H fluorescence that displayed slow and fast components in the range of seconds (B, top).} \]

These data indicate that Ca\(^{2+}\) mobilization from intracellular Ca\(^{2+}\) stores due to either CICR or IICR is sufficient to induce oscillatory as well as nonoscillatory elevations in \([Ca^{2+}]_m\), NAD(P)H fluorescence and \([K^+]_o\).
decrease [Ca\(^{2+}\)]_o (e.g., Schuchmann et al. 2000) but also [Ca\(^{2+}\)]_m (Fig. 6A, top). Application of carbachol in Ca\(^{2+}\)-free ACSF induced elevations in [Ca\(^{2+}\)]_m (2.4 ± 0.2\%ΔF/F_0; n = 4/4), NAD(P)H fluorescence (11.2 ± 1.3\%ΔF/F_0; n = 5/5), and [K\(^+\)]_o (1.9 ± 0.3 mM; n = 7/7) that displayed different time courses of recovery (Fig. 6A). Re-application of Ca\(^{2+}\)-containing ACSF after 5 min triggered rapid elevations in [Ca\(^{2+}\)]_m (6.5 ± 0.9\%ΔF/F_0; n = 4/4) that, in comparison to the onset of the experiments, exceeded their basal levels (Fig. 6A, top). These rapid elevations in [Ca\(^{2+}\)]_m were associated with additional, monotonic elevations in NAD(P)H fluorescence (3.9 ± 0.7\%ΔF/F_0; n = 5/5) that arose from carbachol-induced elevated levels (Fig. 6A, middle). Elevations in both signals were accompanied by rises in [K\(^+\)]_o, whose amplitudes were only 37% (0.7 ± 0.2 mM; n = 7/7) of those evoked during application of carbachol in Ca\(^{2+}\)-free ACSF (1.9 ± 0.3 mM; P < 0.05; Fig. 6A, bottom). Notably, the increase in [K\(^+\)]_o was transient and displayed a faster recovery than elevations in [Ca\(^{2+}\)]_m and NAD(P)H fluorescence. The effects of carbachol application in Ca\(^{2+}\)-free solution as well as of re-application of Ca\(^{2+}\)-containing ACSF on [Ca\(^{2+}\)]_m, NAD(P)H fluorescence, and [K\(^+\)]_o were also present during combined blockade of ionotropic glutamate receptors by CNQX/DO-AP5 (60 \muM, each) and nicotinic acetylcholine receptors by mecamylamine (20 \muM) (not shown).

These data indicate that even capacitative Ca\(^{2+}\) entry after depletion of intracellular Ca\(^{2+}\) stores by ICR triggers not only elevations in [Ca\(^{2+}\)]_m but also in NAD(P)H fluorescence and [K\(^+\)]_o.

**DISCUSSION**

The main conclusion from the present study is that by activation of metabotropic receptors, transmitter release might induce transient Ca\(^{2+}\) elevations not only in the cytosol of hippocampal cells (Carmant et al. 1997; Irving and Collingridge 1998; Jaffe and Brown 1994) but also in their mitochondria, which results in stimulation of mitochondrial oxidative metabolism. Moreover, different pathways that contribute to metabotropic receptor-mediated Ca\(^{2+}\) signaling, namely IICR, CICR, and capacitative Ca\(^{2+}\) entry, suffice in elevating [Ca\(^{2+}\)]_m and in stimulating a metabolic response. Thus activation of metabotropic receptors does not only affect modulation of membrane excitability, dendritic integration, and synaptic plasticity (Nakamura et al. 1999; Tsubokawa and Ross 1997; Vanderklish and Edelman 2002) but also does effectively regulate oxidative metabolism providing the generation of ATP within the cells.

**Activation of metabotropic receptors in hippocampal neurons**

In extensive and profound studies, it has been described that activation of metabotropic glutamatergic (Jaffe and Brown 1994; Kapur et al. 2001; Rae et al. 2000; Shirasaki et al. 1994) and muscarinic receptors (Beier and Barish 2000; Egorov et al. 1999; Irving and Collingridge 1998; Power and Sah 2002) generates cytoplasmic Ca\(^{2+}\) signals in hippocampal neurons. Here, we demonstrate that metabotropic glutamatergic and muscarinic receptor ligands, ADA and carbachol, also induce elevations in [Ca\(^{2+}\)]_m that are tightly associated with elevations in NAD(P)H fluorescence and transient increases in [K\(^+\)]_o (Figs. 1 and 3). Using the selective agonist α-Me-5-HT (Fig. 2), we extend these observations to a third metabotropic receptor family, namely serotonergic 5-HT\(_2\) receptors that, so far, have been described to evoke intracellular Ca\(^{2+}\) signals in ovary cells (Porter et al. 1999), astrocytes (Sanden et al. 2000), and a neuronal-like cell line (Jerman et al. 2001).

In our pharmacological approach, concentrations of the agonists applied via the bath solution were used in the micromolar to millimolar range, and it has been reported that, e.g., the concentration of glutamate peaked at 1.1 mM at cultured hippocampal synapses (Clements et al. 1992). However, further studies in neuronal tissue employing metabotropic receptor antagonists and stimulation in the physiological range might strengthen our observations with respect to functional meaning.

**Rhod-2 fluorescence and [Ca\(^{2+}\)]_m**

We used the fluorescence indicator Rhod-2 AM, the positive charge of which facilitates its accumulation within mitochondria and that has been used in a variety of preparations to monitor mitochondrial Ca\(^{2+}\) signaling (Billups and Forsythe 2002; Hajnóczky et al. 1995; Hoth et al. 1997; Rutter et al. 1996). It should be noted that the positive charge does not necessarily imply the exclusive presence of Rhod-2 within mitochondria after the loading procedure (Bindokas et al. 1998; Kaftan et al. 2000). However, we recently verified in organotypic hippocampal slice cultures that, in comparison to Fluo-3, Rhod-2 primarily reflects changes in [Ca\(^{2+}\)]_m by demonstrating that Rhod-2 fluorescence signals evolved by repetitive stimulation displayed different kinetics and that the mitochondrial uncoupler, CCCP strongly reduced stimulus-induced elevations in Rhod-2 fluorescence (Kann et al. 2003; see also Kovács et al. 2001).

The three metabotropic receptor ligands, ADA, carbachol, and α-Me-5-HT induced monotonic elevations in Rhod-2 fluorescence signals that declined over minutes reflecting transient elevations in [Ca\(^{2+}\)]_m (Figs. 1–3). These data demonstrate that InsP\(_3\)-coupled activation of metabotropic receptors in a neuronal preparation is sufficient to induce Ca\(^{2+}\) uptake by mitochondria, which extends observations in nonneuronal cells (Hajnóczky et al. 1995; Robb-Gaspers et al. 1998; Voronina et al. 2002). Similarly, IICR and CICR, evoked under Ca\(^{2+}\)-free conditions and blockade of ionotropic receptors, as well as capacitative Ca\(^{2+}\) entry after store depletion (Figs. 5 and 6), that have been reported to increase [Ca\(^{2+}\)]_m in many cell types including neurons (Clapham 1995; Parekh and Penner 1997; Pozzan et al. 1994; Verkhratsky and Shmigol 1996), caused elevations in Rhod-2 fluorescence, indicating substantial mitochondrial Ca\(^{2+}\) uptake (see also Collins et al. 2001; Hoth et al. 1997). Thus activation of metabotropic, InsP\(_3\)-coupled receptors in hippocampal cells might recruit different Ca\(^{2+}\) sources in generating spatiotemporal Ca\(^{2+}\) signals that will still affect [Ca\(^{2+}\)]_m and, thereby, will have functional implications on shaping cytoplasmic Ca\(^{2+}\) dynamics as well as on stimulating oxidative metabolism.
NAD(P)H fluorescence and mitochondrial oxidative metabolism

The metabotropic receptor ligands also induced monotonic elevations in NAD(P)H fluorescence signals that were tightly coupled to the elevations in $[\text{Ca}^{2+}]_{\text{i}}$ and $[\text{K}^+]_{\text{o}}$ (Figs. 1–3 and 6). These data indicate the efficacy of metabolotropic receptor-induced elevations in $[\text{Ca}^{2+}]_{\text{i}}$ to stimulate mitochondrial dehydrogenases, a process that results in an enhanced generation of NADH and NADPH (Hansford and Zorov 1998; McCormack et al. 1990). Thus our observations confirm the current concept on $\text{Ca}^{2+}$ regulation in mitochondrial oxidative metabolism that, on the cellular level, is based on studies with InsP$_3$-coupled hormones in nonneuronal cells (Hajnóczky et al. 1995; Robb-Gaspers et al. 1998; Voronina et al. 2002). For neuronal cells, the $\text{Ca}^{2+}$ dependence of stimulus-induced NAD(P)H fluorescence signals has been exclusively established by applying electrical depolarizing stimuli to acutely isolated sensory neurons and hippocampal slice cultures (Duchen 1992; Kann et al. 2003). However, the capacity of metabotropic receptor-mediated $\text{Ca}^{2+}$ signaling in affecting $[\text{Ca}^{2+}]_{\text{i}}$ and stimulating a metabolic response has not been tested. Thus our data extend the concept of $\text{Ca}^{2+}$ regulation in mitochondrial oxidative metabolism for neuronal preparations to InsP$_3$-coupled, metabotropic transmitter signaling.

Interestingly, application of carbachol and caffeine under $\text{Ca}^{2+}$-free conditions resulted in differing shapes of elevations in NAD(P)H fluorescence signals (Fig. 5). Higher concentrations of carbachol induced biphasic NAD(P)H signals that were composed of an initial decline and a prolonged monotonic elevation similar to those obtained by depolarisation of brain slices (Lipton 1973), organotypic slice cultures (Kann et al. 2003; Kovács et al. 2001), or dissociated sensory neurons (Duchen 1992). In contrast, application of caffeine induced elevations in NAD(P)H signals displaying slow and fast decreasing as well as increasing components. Due to technical limitation, that is, the lack of simultaneous temporal recordings of NAD(P)H and Rhod-2 fluorescence as well as the lack of simultaneous spatial recordings of fluorescence signals and $[\text{K}^+]_{\text{o}}$ (see following text), we were not able to fully correlate decreasing and increasing components of caffeine-induced changes in NAD(P)H fluorescence with those in Rhod-2 and $[\text{K}^+]_{\text{o}}$. However, it is likely that the oscillatory nature of caffeine-induced changes in NAD(P)H signals reflects an overlay of several biphasic signals that were evoked, e.g., by enhancement of synchronized $\text{Ca}^{2+}$ release and/or $\text{Ca}^{2+}$ waves due to activation/sensitization of ryanodine receptors by caffeine.

Surprisingly, not only $\text{Ca}^{2+}$ release from ER but also $\text{Ca}^{2+}$ entry after depletion of intracellular $\text{Ca}^{2+}$ stores (Fig. 6) evoked elevations in Rhod-2 and additional elevations in NAD(P)H fluorescence. This suggests that capacitative $\text{Ca}^{2+}$ entry results in mitochondrial $\text{Ca}^{2+}$ uptake that is functionally integrated into a metabolic response (Rohács et al. 1997, adrenal glomerulosa cells). This observation might extend the knowledge on functional implications of capacitative $\text{Ca}^{2+}$ entry (Nilius and Droogmans 2001; Parekh and Penner 1997).

By inducing metabotropic receptor-mediated $\text{Ca}^{2+}$ signaling, we recorded long-lasting elevations in NAD(P)H fluorescence that, by applying diverse stimuli, have been also observed in neuronal and nonneuronal cells in vitro (Duchen 1992; Hajnóczky et al. 1995; Kovács et al. 2001; Robb-Gaspers et al. 1998; Rohács et al. 1997; Voronina et al. 2002). However, epileptiform neuronal activity or cortical spreading depression has been reported to predominantly correlate with decreases in NAD(P)H fluorescence in vivo that were sparsely followed by elevations (Jöbsis et al. 1971; Mayevsky and Chance 1975). These deviant findings in the in vitro and in vivo situation might relate to cellular oxygen supply, changes in the vascular compartment with alterations of cerebral blood flow and effects of anesthetics on mitochondria (Anderson et al. 2002; Hertsens et al. 1984).

$[\text{K}^+]_{\text{o}}$ and ionic changes at the plasma membranes

In general, $\text{K}^+$ release from neurons results from membrane depolarizations that increase the driving force for $\text{K}^+$ currents through different types of $K^+$ channels, like voltage-dependent $K^+$ channels and $\text{Ca}^{2+}$-activated $K^+$ channels. Thus during neuronal activity $[\text{K}^+]_{\text{o}}$ does increase by $<3 \text{mM}$ from basal levels under physiological conditions (Amzica and Steriade 2000; Heinemann and Lux 1975, 1977; Lothman and Somjen 1975) or might exceed tens of millimolar under pathophysiological conditions (Lux et al. 1986; Nicholson et al. 1978). The fast recovery of increases in $[\text{K}^+]_{\text{o}}$ reflects clearance of $\text{K}^+$ from the extracellular space that is determined by active as well as passive uptake mechanisms of neurons and glial cells (D’Ambrosio et al. 2002; Newman 1995; Somjen 1995).

In the present study, metabotropic receptor ligands evoked transient increases in $[\text{K}^+]_{\text{o}}$, displaying a faster recovery than elevations in NAD(P)H fluorescence (Figs. 1–3). Moreover, application of carbachol or caffeine, even in the presence of $\text{Ca}^{2+}$-free solution and ionotropic receptor antagonists, as well as re-application of $\text{Ca}^{2+}$-containing solution after store depletion by carbachol, resulted in transient increases in $[\text{K}^+]_{\text{o}}$ of $<2 \text{mM}$ from basal levels (Figs. 5 and 6). These changes in $[\text{K}^+]_{\text{o}}$ were monitored by $\text{K}^+$-sensitive microelectrodes that measure accumulation of $\text{K}^+$ in a restricted extracellular space, irrespective of whether $\text{K}^+$ is released from dendrites, somata, axons, or presynaptic terminals.

Different types of $K^+$ channels and mechanisms might have contributed to these increases in $[\text{K}^+]_{\text{o}}$: 1) $\text{Ca}^{2+}$-activated $K^+$ channels (Knaus et al. 1996; Poole and Johnston 1999) that were activated by IICR and CICR (Sah and Faber 2002; Vergara et al. 1998), 2) voltage-dependent $K^+$ channels (Klee et al. 1995; Mitterdorfer and Bean 2002), and 3) modulation of two-pore domain $K^+$ channels determining resting (background) conductances (Goldstein et al. 2001; Talley et al. 2001), carbachol-mediated suppression of M-currents (Marrion 1997), activation of $\text{Ca}^{2+}$-release activated channels (Hoth and Penner 1992; Nilius and Droogmans 2001; Parekh and Penner 1997), and/or activation of $\text{Ca}^{2+}$-activated nonselective cation channels (Partridge and Valenzuela 2000; Petersen 2002) that all favor neuronal depolarization. However, the precise nature of the ion channels and mechanisms involved in the increases in $[\text{K}^+]_{\text{o}}$ that we report on has to be left to further studies.

Because the amplitude of transient increases in $[\text{K}^+]_{\text{o}}$ provides an indirect measure of the degree of depolarisation and/or elevations in $[\text{Ca}^{2+}]_c$, the significant positive correlation between increases in $[\text{K}^+]_{\text{o}}$ and elevations in NAD(P)H fluorescence (Fig. 4) might indicate a tight coupling between metabotropic receptor-mediated neuronal activity and mitochondrial
metabolic function. This coupling seems to be fine-tuned and not characterized by an all-or-nothing response.

The authors thank Drs. H. Siegmund and H.-J. Gabriel for excellent technical assistance and Drs. A. Friedman, S. Kirischuk, and S. Schuchmann for helpful discussions.

DISCLOSURES

This work was supported by the German Research Foundation (DFG, SFB 507, He 1128/13-1) and a fellowship of the European Union to R. Kovács (ICA1 CT 2002 70007).

REFERENCES


Pozzan T, Rizzuto R, Volpe P, and Meldolesi J.

Power JM and Sah P.


