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Roles of Na^{+}–Ca^{2+} exchange and of mitochondria in the regulation of presynaptic Ca^{2+} and spontaneous glutamate release

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THE ROYAL SOCIETY

Roles of Na^+–Ca^{2+} exchange and of mitochondria in the regulation of presynaptic Ca^{2+} and spontaneous glutamate release

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The release of neurotransmitter from presynaptic terminals depends on an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In addition to the opening of presynaptic Ca^{2+} channels during excitation, other Ca^{2+} transport systems may be involved in changes in $[Ca^{2+}]_i$. We have studied the regulation of $[Ca^{2+}]_i$ in nerve terminals of hippocampal cells in culture by the Na^+–Ca^{2+} exchanger and by mitochondria. In addition, we have measured changes in the frequency of spontaneous excitatory postsynaptic currents (sEPSC) before and after the inhibition of the exchanger and of mitochondrial metabolism. We found rather heterogeneous $[Ca^{2+}]_i$ responses of individual presynaptic terminals after inhibition of Na^+–Ca^{2+} exchange. The increase in $[Ca^{2+}]_i$ became more uniform and much larger after additional treatment of the cells with mitochondrial inhibitors. Correspondingly, sEPSC frequencies changed very little when only Na^+–Ca^{2+} exchange was inhibited, but increased dramatically after additional inhibition of mitochondria. Our results provide evidence for prominent roles of Na^+–Ca^{2+} exchange and mitochondria in presynaptic Ca^{2+} regulation and spontaneous glutamate release.

Keywords: Na^+–Ca^{2+} exchange; mitochondria; hippocampal cells; spontaneous excitatory postsynaptic currents (sEPSC); glutamate release; presynaptic Ca^{2+} concentration

1. INTRODUCTION

A brief rise of the Ca^{2+} concentration ($[Ca^{2+}]_i$) in synaptic terminals is required to initiate fast exocytosis of synaptic vesicles (for a review see Zucker 1993). The high $[Ca^{2+}]_i$ achieved during excitation must be quickly reduced after cessation of action potential activity.

In previous studies, we have shown (Reuter & Porzig 1995; Bouron & Reuter 1996) that the plasmalemmal Na^+–Ca^{2+} exchanger plays a major role in the regulation of $[Ca^{2+}]_i$ in synaptic boutons of hippocampal neurons and that it influences synaptic vesicle recycling. Na^+–Ca^{2+} exchange, first discovered in heart muscle (Reuter & Seitz 1968) and squid axon (Blaustein & Hodgkin 1969), is a powerful high-capacity, low-affinity Ca^{2+} transport system driven by the electrochemical Na^+ gradient across the membrane (for reviews see Carafoli 1987; Hilgemann *et al*. 1996). The Na^+–Ca^{2+} exchanger is expressed in hippocampal cells, and immunocytochemistry revealed a predominant colocalization with functional synapses (Juhaszova *et al*. 1996; Reuter & Porzig 1995). Replacement of extracellular Na^+ by Li^+ increased $[Ca^{2+}]_i$ during stimulation in nerve terminals, resulting in faster initial rates of exocytosis of synaptic vesicles (Reuter & Porzig 1995). However, individual terminals reacted differently to the inhibition of the exchanger. On average, the fall in $[Ca^{2+}]_i$ was greatly slowed down, but in some terminals the slow fall was preceded by a fast initial decrease. This inhomogeneity of individual nerve terminals in their response to blockade of the exchanger could possibly be linked to variable occurrences of mitochondria in the terminals (Shepherd & Harris 1998; for a review see Edwards 1995). If mitochondria are present, they may act as additional buffers for cytosolic Ca^{2+}.

Mitochondria can sequester and release large amounts of calcium (Blaustein 1988; Carafoli 1979; Babcock & Hille 1998; Miller 1998). Calcium uptake is mediated by a Ca^{2+} selective channel—the mitochondrial Ca^{2+} uniporter—driven by the large inside-negative membrane potential. The high Ca^{2+} flux rate through the uniporter can modulate amplitudes and spatio-temporal organization of the cytosolic calcium signals (Thayer & Miller 1990; Budd & Nicholls 1996; Simpson & Russel 1996). Ca^{2+} ions accumulated in the mitochondrial matrix can be exported into the cytoplasm by a mitochondrial Na^+–Ca^{2+} exchanger (Carafoli 1979; Babcock & Hille 1998; Miller 1998). After loading the mitochondrial matrix with Ca^{2+}, the subsequent export can greatly prolong cytoplasmic Ca^{2+} signals, even after the cessation of the original stimulus (Tang & Zucker 1997). Mitochondrial Ca^{2+} can also be rapidly released by the transient dissipation of the transmembrane proton gradient through opening of the permeability transition pore of the inner membrane. The existence of such mitochondrial depolarization spikes has recently been demonstrated in isolated mitochondria (Hüser *et al*. 1998; Ichas *et al*. 1997; Miller 1997). Drugs that rapidly dissipate the mitochondrial proton gradient can mimic the opening of this pore

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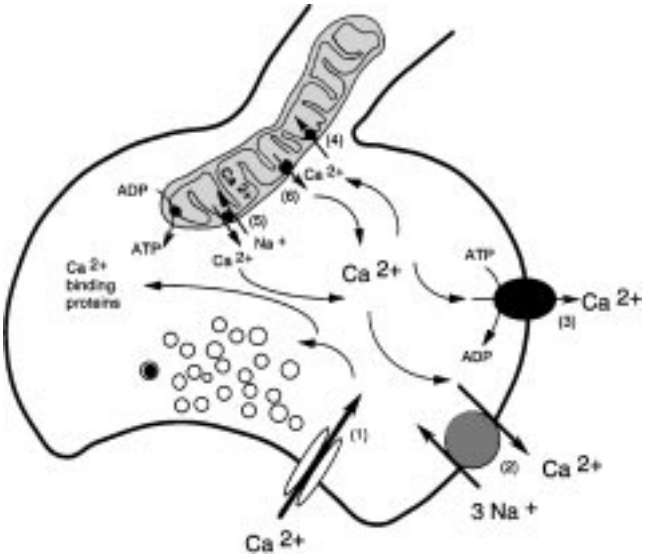

Figure 1. Pathways of Ca^{2+} handling in a nerve terminal. The scheme shows the membrane transport systems, the transport systems of mitochondria and the soluble Ca^{2+} binding proteins (modified from Blaustein 1988). Ca^{2+} enters the bouton through channels in the plasma membrane (1) and is extruded by Na^+–Ca^{2+} exchanger (2) and the Ca^{2+} ATPase (3). Mitochondria can sequester and release large amounts of calcium. Calcium uptake is mediated by the mitochondrial Ca^{2+} uniporter (4). Ca^{2+} ions accumulated in the mitochondrial matrix can be exported into the cytoplasm by a mitochondrial Na^+–Ca^{2+} exchanger (5) or it can also be rapidly released through opening of the permeability transition pore of the inner membrane (6).

and also release Ca^{2+} into the cytoplasm (Rizzuto *et al.* 1992; Budd & Nicholls 1996). Their long-lasting action, however, disrupts mitochondrial Ca^{2+} uptake mechanisms as well (Wang & Thayer 1996). A general scheme of Ca^{2+} handling in an individual nerve terminal is illustrated in figure 1.

Here, we provide evidence that mitochondria in nerve terminals, together with the plasmalemmal Na^+–Ca^{2+} exchange are heavily involved in the control of $[Ca^{2+}]_i$ and, thus, in the regulation of transmitter release.

2. MATERIALS AND METHODS

(a) *Dissociated hippocampal cell culture*

Both hippocampi were dissected from the brain of one neonatal Sprague–Dawley rat (P1-3) and processed according to a protocol modified from Malgaroli & Tsien (1992) and Ryan *et al.* (1993). Briefly, the tissue was incubated in 3.4 mg ml^{-1} trypsin type IX and 0.9 mg ml^{-1} DNAse type IV, then mechanically dissociated in Ca^{2+}-free Hanks' solution supplemented with 12 mM $MgSO_4$, 0.4 mg ml^{-1} DNAse and 3 mg ml^{-1} BSA. The cell suspension obtained was centrifuged twice (80g) and resuspended to estimate cell density on a Coulter counter. Hippocampal cells were then plated at a density of 715 mm^{-2} on poly-L-ornithine-coated coverslips within a cloning cylinder (28 mm^2) attached to the coverslip with high vacuum silicon grease. The cells were incubated in minimal essential medium, supplemented with 292 mg l^{-1} glutamax I, 5000 mg l^{-1} glucose, 25 mg l^{-1} insulin, 100 mg l1 transferrin, 5 mg l^{-1} gentamycin and 10% foetal calf serum. Cultures were maintained in a CO_2 enriched atmosphere (6.5%) at 37 °C. On the second day *in vitro*, the serum concentration in the medium was reduced to 5% and cytosine b-D-arabinofuranoside and B27 supplement were added to a final concentration of 1 μM and 2%, respectively. The medium was changed every 2–3 days.

For fluorescence measurements and electrophysiological experiments cell cultures (day 7–14 *in vitro*) were transferred to a saline solution containing 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM$CaCl_2$, 30 mM glucose and 10 mM HEPES buffered to pH 7.4. To depolarize mitochondria the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazone (FCCP; 1 μM) was applied to the bath solution for 2–5 min. Alternatively, the inhibitor of cytochrome bc1 in the mitochondrial respiratory chain, antimycin A (1 μM), was used to block electron transport (Tokutake *et al.* 1994). To counteract consumption of cytoplasmic ATP, the ATPsynthase (F_0F_1-ATPase) blocker oligomycin (1 μM) was applied together with FCCP or antimycin A. In some control experiments oligomycin was left out or applied alone. NaCl was replaced for about 2 min by equimolar LiCl to block the Na^+–Ca^{2+} exchanger before and during mitochondrial depolarization. To manipulate intracellular pH the NH_4Cl (20 mM) prepulse technique was applied (Roos & Boron 1981; Chatton *et al.* 1997). Briefly, addition of NH_4Cl at a constant bath pH of 7.4 results in cellular alcalinization because of diffusion of NH_3 across the membrane and its protonation to NH_4^+ in the cytosol. Replacement of the NH_4Cl-containing solution with control saline causes acidification because of outward diffusion of NH_3 after deprotonation. All experiments were performed at room temperature (22 °C).

(b) *Fluorescence microscopy*

To monitor relative changes in the intraterminal calcium concentration, $[Ca^{2+}]_i$, hippocampal cells were loaded for 30 min with 10 μM of the cell permeant fluo-3 acetoxy methyl ester (fluo-3 AM). The cultures were placed into a chamber that permitted constant saline superfusion (1–3 ml min^{-1}) and was equipped with platinum electrodes for electrical field stimulation (20 Hz, 1 ms current pulses). The chamber was put on the stage of an inverted microscope which is part of a confocal laser scan microscope (Zeiss LSM 410), and cells were viewed with a 40 × oil immersion objective (Plan Neofluar, 1.3 na). A region of the peripheral neurite meshwork was selected with a thin, non-confluent astrocytic layer underneath. Calcium influx through postsynaptic glutamatergic N-methyl-D-aspartate (NMDA) or a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors was blocked by R(-) 2-amino-5-phoshonopentanoic acid (D-AP5, 25 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), respectively. Fluo-3 was excited by the 488 nm line of an Argon laser and the fluorescence emitted (peak 530 nm) was filtered through a 515–560 nm filter with the pinhole about 10% open. Fluo-3 fluorescence changes were sampled at scanning rates of 2 s per image. To identify fluo-3-labelled spots as synaptic boutons, the fluorescent styryl membrane probe FM4-64 (15 μM; excitation 543 nm, emission 590 nm) was added to the solution at the end of the experiment. Cells were then stimulated for 10–20 s (20 Hz) and left in FM4-64 containing solution for another 60 s to ensure complete endocytosis of synaptic vescicles and maximal dye loading (Ryan *et al.* 1993). Areas (<1 μm^2) of intense FM4-64 uptake which were able to release the dye in response to a second period of stimulation (90 s, 20 Hz) were identified as functional synapses. Changes in fluorescence were normalized to that at the beginning of the scanning period, and averages of 10–15 boutons were plotted.

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For measurements of intracellular pH, neuronal cells were loaded for 20–30 min with 0.2–0.3 μM 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM). The cell cultures were mounted on the stage of a Nikon inverted epifluorescence microscope equipped with a video camera that was attached to an image intensifier (Chatton *et al*. 1997). Nerve cell somata were viewed through an oil immersion 100 × 1.3 na objective. The video image was digitized using an image processor that allowed the selection of regions of interest and intensity measurements inside single cells. A filter wheel was used for rapid automatic exchange of the excitation filters to sequentially illuminate the specimen with 440 and 480 nm light. The filter cube contained a 510 nm dichroic mirror and a 520 nm emission filter. Excitation ratios of 480:440 nm were computed. Calibration of intracellular pH was performed at the end of each experiment using the nigericin technique (Thomas *et al*. 1979). Data are presented as means ± s.e.m. of values collected in several pyramidal-like perikarya from individual preparations.

(c) *Electrophysiology*

Spontaneous postsynaptic excitatory currents (sEPSCs) were recorded by the patch-clamp method in whole cell configuration. The membrane potential was clamped at −50 mV. To block Ca^{2+} currents, $GABA_A$ receptor-mediated inhibitory currents and action potentials, the saline contained 100 μM $CdCl_2$, 10 μM bicuculline and 0.1 μM tetrodotoxin (TTX). Rapid, randomly occurring excitatory currents through glutamatergic AMPA-type channels could be measured under these conditions. They could be inhibited with 10 μM CNQX (Bouron & Reuter 1996). In the presence of TTX, these currents correspond to miniature EPSCs elicited by the release of glutamate quanta.

Patch pipettes (3–4 MΩ) contained 135 mM CsF, 2 mM $MgCl_2$, 10 mM EGTA, 5 mM Mg_2ATP, 0.2 mM Tris-GTP and 10 mM HEPES (pH 7.1). Filtered (1 kHz) currents were amplified, continuously monitored on the oscilloscope and digitized at 10 kHz every minute for 9 s. These data were stored on the hard disk of a computer. For the analysis of the sEPSCs a program provided by Dr A. Dityatev (Hamburg University) was used. The software is based on the algorithm of Ankri *et al*. (1994) and reliably detects randomly occurring sEPSCs, measuring their amplitude, kinetics and interevent interval (Bouron & Reuter 1996). Rise time and decay time are expressed as mean ± s.e.m. of normalized values. Frequency and amplitude of sEPSCs under different conditions are presented as means ± s.e.m. of absolute values recorded from 6 to 19 pyramidal-like neurons from 2 to 9 different cultures. The paired non-parametric Wilcoxon sign rank test was used for statistics. Significance was set at $p < 0.05$.

In some experiments, hippocampal cells were loaded with the membrane permeable acetoxy methyl esther of BAPTA (BAPTA-AM, 10 μM) to buffer the intracellular calcium concentration during blockade of mitochondrial function. BAPTA-AM incubation was done at room temperature for 10 min during recordings, and baseline sEPSCs were sampled before and following the buffer-loading procedure to keep track of any changes. Optimal incubation time was assessed by fluorescence microscopy. After loading with fura-2 (5 μM for 20–30 min at 37 °C) the decline in $[Ca^{2+}]_i$ during BAPTA-AM incubation was monitored using 340/380 nm excitation ratio.

3. RESULTS

During a brief (*ca*. 2 s) period of electrical stimulation of the cells in NaCl-containing solution, fluo-3 fluorescence, as an indicator of $[Ca^{2+}]_i$, increased two- to sixfold in synaptic boutons and, after cessation of stimulation, it rapidly returned to baseline within about 5 s (figure 2). The filled squares in figure 2*a* represent the average responses of 12 boutons. When NaCl was replaced by LiCl, to block plasmalemmmal Na^+–Ca^{2+} exchange, and the cells were stimulated again, the decrease of fluo-3 fluorescence after stimulation was delayed. In agreement with previous results (Reuter & Porzig 1995), we found heterogeneous decay kinetics between individual boutons in LiCl solution (not shown). In some terminals fluo-3 fluorescence fell rapidly, though incompletely, while in others the $[Ca^{2+}]_i$ remained elevated and decreased only on readdition of NaCl solution. This heterogeneity in $[Ca^{2+}]_i$ changes between individual boutons could be due to a lack of mitochondria in about 50% of the boutons (Shepherd & Harris 1998). When mitochondrial calcium uptake was blocked with FCCP in the presence of oligomycin, the relative changes of the synaptic $[Ca^{2+}]_i$ were more homogenous in Na^+-free medium. Fluo-3 fluorescence decreased very slowly after stimulation and returned to baseline only after readdition of the NaCl solution (figure 2*a*, open squares). FCCP was always coapplied with oligomycin, to minimize ATP depletion. Mitochondrial ATP synthesis is linked to the outward proton gradient across the inner mitochondrial membrane. Dissipation of this gradient with FCCP will cause the enzyme to function as an ATPase, eventually leading to the consumption of cytoplasmic ATP. This can be prevented with oligomycin, a specific inhibitor of mitochondrial ATPsynthase (Budd & Nicholls, 1996).

The large increase in synaptic $[Ca^{2+}]_i$ during excitation was rapidly reduced in control conditions when Ca^{2+} extrusion and uptake systems were both functional. When FCCP was applied after a brief period of stimulation, the mitochondrial potential was presumably dissipated, thus releasing the Ca^{2+} previously accumulated in these organelles. During prolonged exposure to FCCP and oligomycin, the increase in fluo-3 fluorescence stabilized at about twofold baseline levels. Subsequent inhibition of the plasmalemmal Na^+–Ca^{2+} exchanger by replacement of Na^+ by Li^+ caused a sixfold increase in fluo-3 fluorescence even without stimulation (figure 2*b*). Replacement of Na^+ by Li^+ alone always produced only a small increase in resting $[Ca^{2+}]_i$ (figure 2*a*).

An increase of $[Ca^{2+}]_i$ is known to trigger exocytosis of neurotransmitter-containing vesicles. The rise in $[Ca^{2+}]_i$ observed in synaptic terminals during the blockade of mitochondrial metabolism could either enhance neurotransmitter release by itself, or additional inhibition of calcium extrusion systems may be required to trigger exocytosis. To answer this question, we studied the effects of FCCP and oligomycin on the frequency of sEPSCs as a measure of neurotransmitter release. Figure 3*a* shows representative traces recorded at different times during exposure to LiCl or FCCP. Baseline sEPSC frequency was very little affected in the presence of LiCl. Two minutes exposure to FCCP and oligomycin increased the

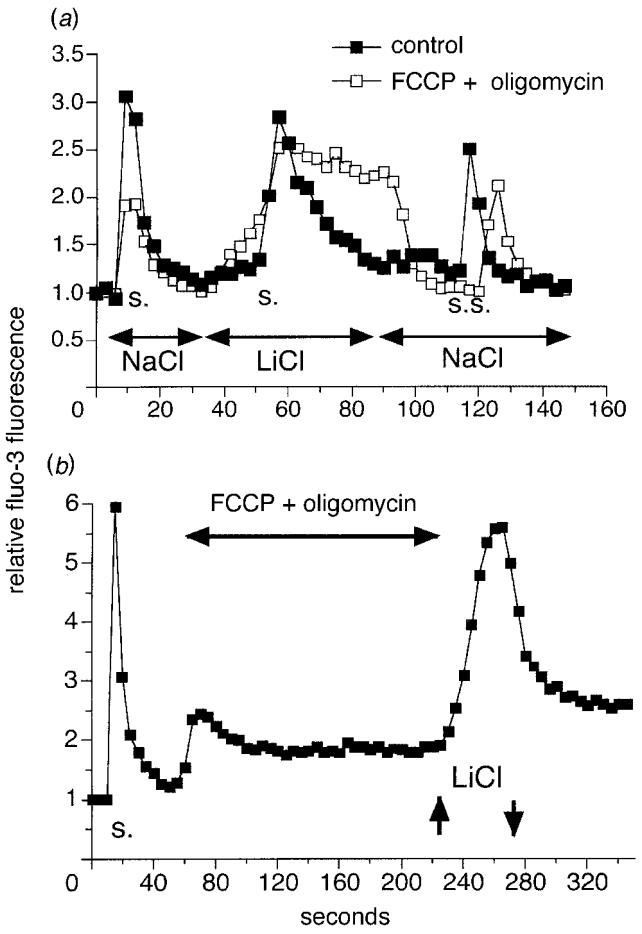

Figure 2. Inhibition of Na^+–Ca^{2+} exchange and mitochondrial depolarization by the protonophore FCCP elevate $[Ca^{2+}]_i$. (*a*) In NaCl-containing solution, fluo-3 fluorescence in presynaptic terminals increased two- to threefold during brief electrical stimulation (s.) of the cells, and rapidly returned to baseline when stimulation was stopped. When NaCl was replaced by LiCl to block Na^+–Ca^{2+} exchange and the cells were stimulated again, the decrease of fluo-3 fluorescence after stimulation was delayed (filled squares). When mitochondrial calcium uptake was blocked with FCCP in the presence of oligomycin, fluo-3 fluorescence decreased very slowly after stimulation in Li^+ -containing solution and returned to the baseline only after readdition of NaCl (open squares). (*b*) Mitochondrial depolarization with FCCP, applied after a brief period of stimulation (s.), released the Ca^{2+} previously accumulated in these organelles thus increasing $[Ca^{2+}]_i$ to twofold above control. Subsequent replacement of Na^+ with Li^+ caused a sixfold increase in fluo-3 fluorescence even without stimulation.

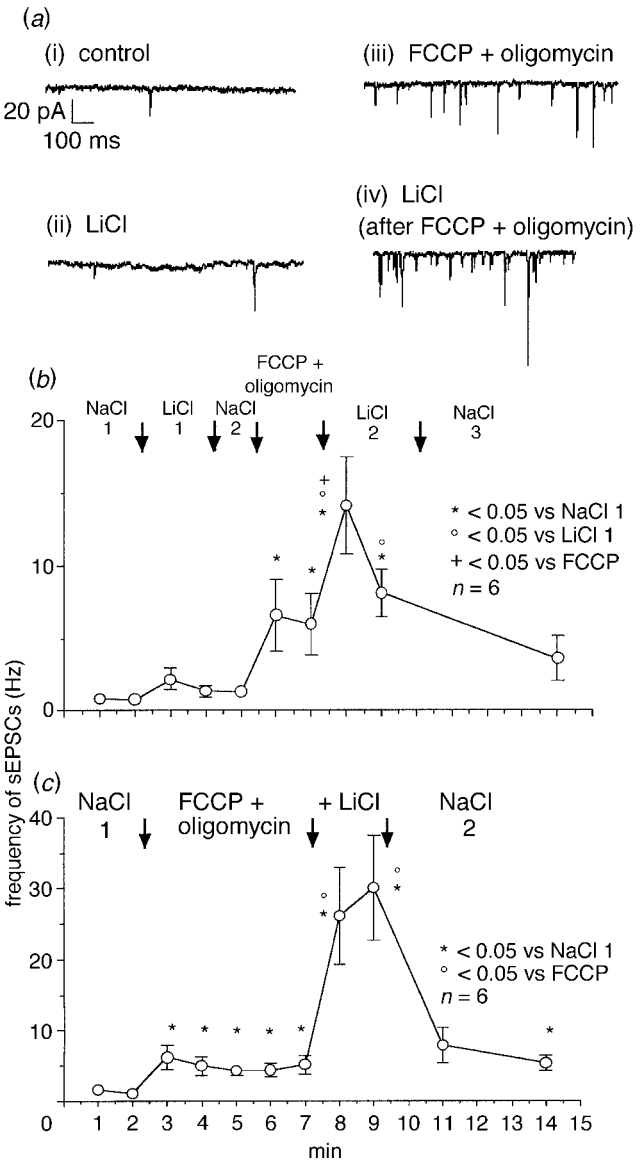

Figure 3. FCCP induced mitochondrial depolarization can increase glutamate release. This effect is enhanced when Ca^{2+} extrusion via the Na^+–Ca^{2+} exchanger is additionally impaired. (*a*) Representative traces recorded at different times during exposure to LiCl or FCCP and oligomycin. (*b*) Control sEPSC frequency was very little affected by LiCl. Two minutes exposure to FCCP and oligomycin led to a rapid fivefold increase of sEPSC frequency. If LiCl was applied a second time shortly after the exposure to FCCP a 15-fold increase in sEPSC frequency occurred. (*c*) A 5-min application of FCCP and oligomycin resulted again in a rapid and stable increase of the sEPSC frequency. When LiCl solution was applied in the presence of FCCP, a 25-fold increase of the sEPSC frequency was observed that remained stable until NaCl solution was reapplied. The numbers underneath NaCl and LiCl indicate the sequences of repeated solution changes.

sEPSC frequency. The effect of FCCP was rapid, leading on average to a significant fivefold increase of sEPSC frequency. In the experiments shown in figure 3*a* and 3*b*, LiCl was applied a second time shortly after the exposure to FCCP. This condition is similar to the calcium measurements shown figure 2*b*. A 15-fold increase in sEPSC frequency paralleled the large rise in $[Ca^{2+}]_i$. Mean sEPSC frequency fell already during the second minute of exposure to the LiCl-containing solution when FCCP was absent and decreased further after the readdition of NaCl. The initial decrease may depend on the rate of recovery of mitochondrial function. We tested this possibility in a second group of cells. In this experiment a 5-min application of FCCP again resulted in a rapid and stable, about fivefold, increase of the sEPSC frequency. When LiCl solution was applied in the presence of FCCP, a 25-fold increase of the sEPSC frequency above control levels was observed that remained stable until NaCl solution was reapplied (figure 3*c*). This suggests that blockade of mitochondrial function by FCCP alone is sufficient to cause an increase of glutamate release and that this effect is dramatically enhanced when Ca^{2+} extrusion via the

Table 1. *FCCP and oligomycin had no significant effect on the amplitudes or the kinetics of sEPSC*

drug	amplitude (pAmp)	rise time (s)	decay time (ms)	*n*	*p*
FCCP + oligomycin	1.19 ± 0.35	1.00 ± 0.05	1.24 ± 0.46	6	n.s.
FCCP + oligomycin + LiCl	1.33 ± 0.47	1.14 ± 0.08	0.86 ± 0.22	6	n.s.
FCCP + oligomycin	1.30 ± 0.19	0.97 ± 0.04	1.16 ± 0.31	14	n.s.
BAPTA-AM + FCCP + oligomycin	1.01 ± 0.10	1.07 ± 0.09	0.89 ± 0.08	6	n.s.

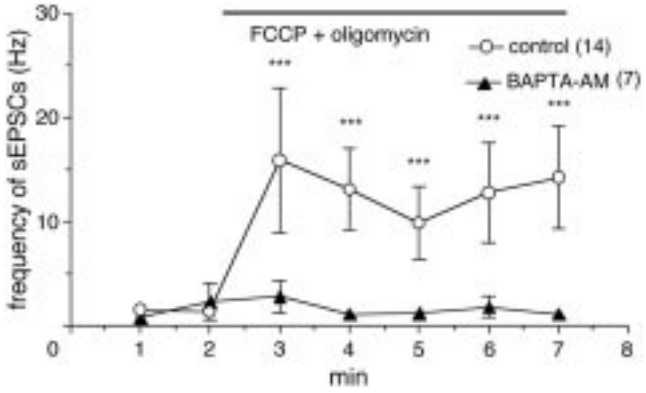

Figure 4. Effects of protonophore induced Ca^{2+} release on sEPSC frequency are inhibited by BAPTA-AM. Loading the cells with the calcium chelator BAPTA-AM (10 μM) did not affect control sEPSC frequency but completely prevented its increase in the presence of FCCP and oligomycin.
*** $p < 0.001$.

plasmalemmal Na^+–Ca^{2+} exchanger is additionally impaired.

FCCP and oligomycin had no significant effect on the amplitude or the kinetics of sEPSC (table 1) indicating a lack of effect on AMPA-type postsynaptic receptors.

Figure 4 provides further evidence for a link between mitochondrial Ca^{2+} handling and sEPSC frequency. Loading the cells with the calcium chelator BAPTA-AM (10 μM) did not affect control sEPSC frequency but completely prevented its increase in the presence of FCCP and oligomycin.

Oligomycin alone did not increase significantly sEPSC frequency (1.24 ± 0.21 above control, $n = 6$). However FCCP alone led to a marked, but variable, increase (10.74 ± 4.02 above control, $n = 6$, $p < 0.05$) of the sEPSC frequency.

Instead of the protonophore FCCP, we have also used antimycin A, an inhibitor of the cytochrome bc1 complex in the mitochondrial respiratory chain, in combination with oligomycin. The effects of antimycin A on the frequency of the sEPSCs were tested on 16 cells. sEPSC frequency increased slowly during a 5-min exposure to the drug together with oligomycin. Significant two- to three-fold changes in the sEPSC frequency were obtained after 4-min exposure to the drugs (figure 5*a*iii). In eight of these cells we also replaced NaCl with LiCl. After 5 min of antimycin A application, a further significant increase of the sEPSC frequency was observed when LiCl was added (figure 5*b*). The changes in sEPSC frequency were more delayed and less pronounced than with FCCP. Antimycin A had no effects on the amplitude (1.37 ± 0.11 increase above control), or kinetics of the sEPSC. Antimycin A was alone responsible for the significant increase of the sEPSC frequency (2.67 ± 1.28, $n = 8$, $p < 0.05$ above control). Elevation of sEPSC frequency in the presence of antimycin A was completely prevented only in seven out of ten BAPTA loaded cells. Changes in $[Ca^{2+}]_i$ caused by antimycin A were measured with fluo-3 in separate experiments. Figure 5*d* shows the average of $[Ca^{2+}]_i$ changes detected in 13 individual boutons. Elevation of $[Ca^{2+}]_i$ was slow over the 5-min exposure to antimycin A. On addition of LiCl, a sudden elevation of $[Ca^{2+}]_i$ occurred. $[Ca^{2+}]_i$ increased further when the cells were stimulated.

As mitochondria represent the primary site of oxygen consumption, their blockade may result in an increase of anaerobic glucose utilization and thereby in intracellular acidification. Intracellular pH measurements in the cell soma were performed by means of BCECF-AM (0.2–0.3 μM) (table 2). Exposure to FCCP in the presence of oligomycin for an average of 4 min caused a small but significant acidification. Since rebound acidification after an ammonium pulse was found to increase sEPSC frequency, we tested antimycin A as an alternative to FCCP. Antimycin A did not affect intracellular pH, even when coapplied with LiCl (table 2).

4. DISCUSSION

Neurons possess several mechanisms to rapidly and efficiently buffer changes in $[Ca^{2+}]_i$ that occur with evoked Ca^{2+} influx or intracellular Ca^{2+} mobilization. The homeostatic mechanisms generally consist of: (i) plasma membrane-bound Ca^{2+} extrusion systems, such as the Na^+–Ca^{2+} exchanger and the ATP-dependent Ca^{2+} pump; (ii) intracellular organelles capable of Ca^{2+} uptake and release like mitochondria and endoplasmic reticulum; and (iii) cytosolic Ca^{2+} binding proteins (Blaustein 1988). The same mechanisms, with the exception of the endoplasmic reticulum (Edwards 1995; Reuter & Porzig 1995), are active in synaptic boutons, but their mutual interactions in handling local $[Ca^{2+}]_i$ changes and in modulating neurotransmitter release are incompletely understood. This study provides evidence for prominent roles of Na^+–Ca^{2+} exchange and mitochondrial Ca^{2+} uptake in the clearance of Ca^{2+} loads and maintainance of the resting $[Ca^{2+}]_i$ in synapses of hippocampal cells in culture. Inhibition of Na^+–Ca^{2+} exchange during stimulation increases $[Ca^{2+}]_i$ in intact synaptic terminals (Reuter & Porzig 1995). We confirm that the recovery of $[Ca^{2+}]_i$ after stimulation is slowed down during blockade of the Na^+–Ca^{2+} exchanger, and we show that it can be further impaired when Ca^{2+} uptake into mitochondria is additionally inhibited.

The ability of mitochondria to reversibly sequester cytoplasmic Ca^{2+} in neurons has been shown during physiological and pathological Ca^{2+} loading. It is

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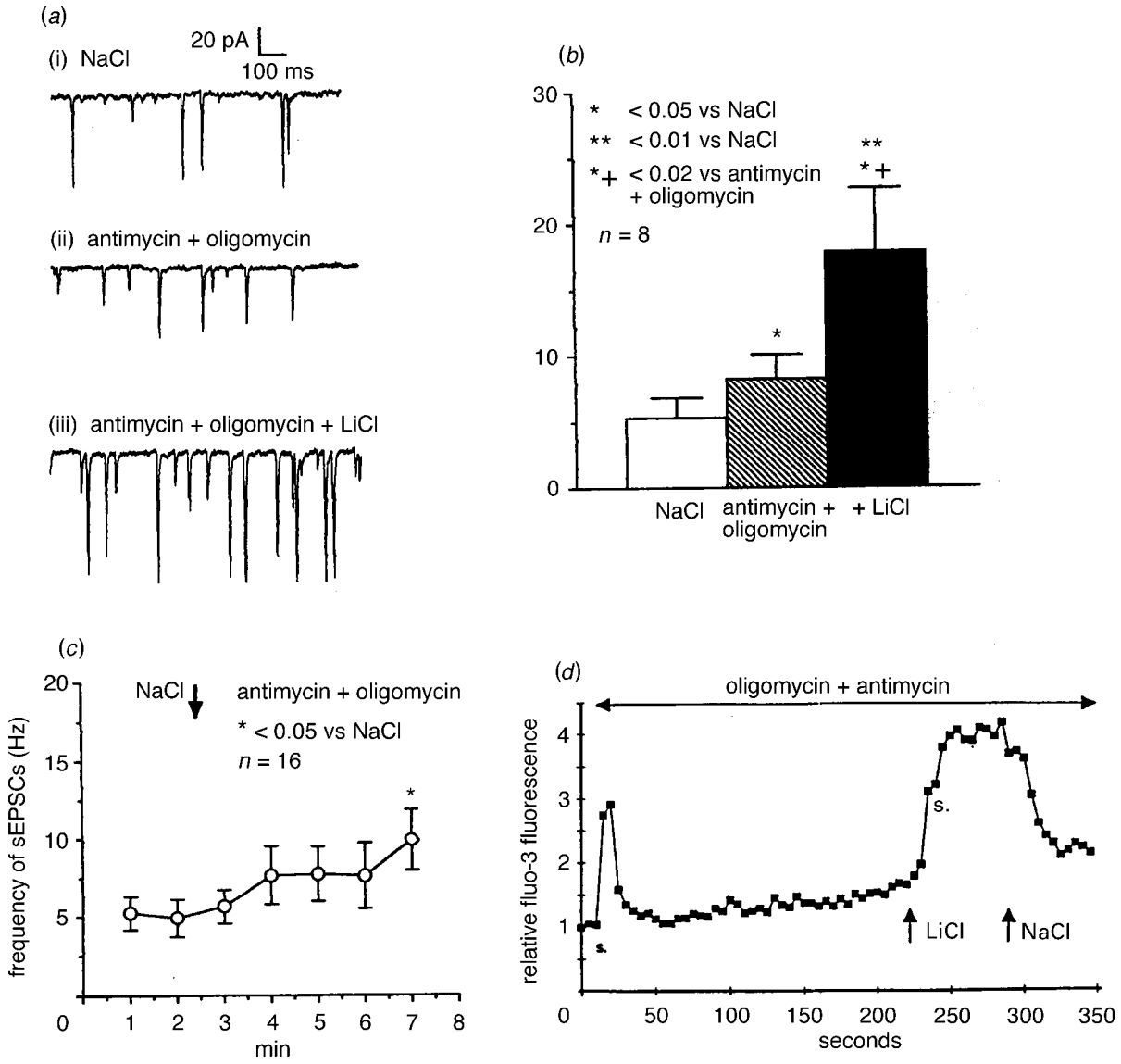

Figure 5. Blockade of the mitochondrial electron transport with antimycin A increases sEPSC frequency and synaptic $[Ca^{2+}]_i$. Effects are enhanced by additional inhibition of Ca^{2+} extrusion via the Na^+–Ca^{2+} exchanger. Representative traces (*a*) and mean sEPSC frequency (*b*) recorded at 5-min exposure to antimycin A and oligomycin, and after two additional minutes in the presence of LiCl. Significant two- to threefold changes in the sEPSCs frequency were obtained with antimycin A. Subsequent replacement of NaCl by LiCl caused a further significant increase of the sEPSC frequency. (*c*) Changes in sEPSC frequency caused by antimycin A were more delayed and less pronounced than those achieved with the protonophore FCCP. This was also true for changes in fluo-3 fluorescence measured in separate experiments (*d*). Brief (*ca.* 2 s) periods of electrical stimulation are indicated by (s.)

generally accepted that mitochondria can limit the increase of $[Ca^{2+}]_i$ above critical levels (Werth & Thayer 1994; White & Reynolds 1995; Wang & Thayer 1996). However, Budd & Nicholls (1996) have recently shown that the KCl-evoked Ca^{2+} elevation is decreased when mitochondrial Ca^{2+} uptake is abolished. The role of mitochondria in the regulation of $[Ca^{2+}]_i$ within nerve terminals is also controversial. Nachshen (1985) reported that Na^+–Ca^{2+} exchange but not mitochondrial Ca^{2+} uptake regulates $[Ca^{2+}]_i$ in synaptosomes, while other authors (Akerman & Nicholls 1981) showed that during KCl depolarization Ca^{2+} accumulates in mitochondria of such isolated nerve terminals. However, the total synaptosomal Ca^{2+} load that follows KCl depolarization is reduced when mitochondrial function is abolished (Akerman & Nicholls 1981). In isolated neurohypophysial nerve endings, mitochondrial Ca^{2+} uptake is essential to limit changes in $[Ca^{2+}]_i$ during phasic bursting activity needed for the release of neuropeptides (Stuenkel 1994). Post-tetanic potentiation has been shown to depend on mitochondrial Ca^{2+} sequestration during extensive presynaptic activity and on Ca^{2+} efflux into the cytosol after tetanus (Tang & Zucker 1997). In agreement with these recent studies (Stuenkel 1994; Tang & Zucker 1997), we suggest that presynaptic boutons that contain mitochondria have a faster Ca^{2+} clearance after depolarization than synapses where mitochondria are absent or specifically blocked.

We also provide evidence that mitochondrial Ca^{2+} uptake or release can regulate exocytosis of synaptic

Table 2. *Exposure to FCCP in the presence of oligomycin for an average of four min caused a small but significant intracellular acidification*

(Since acidification following an ammonium pulse also increased sEPSC frequency, the inhibitor of cytochrome bc1 of the mitochondrial respiratory chain antimycin A was tested as an alternative. Antimycin A did not affect intracellular pH, even when coapplied with LiCl.)

	intracellular pH (pH_i)				
drug	pH_i Ctrl	pH_i drug	ΔpH_i	*n*	*p*
acid load (after NH_4Cl)	6.70 ± 0.04	6.42 ± 0.03	-0.28 ± 0.04	17	<0.001
FCCP + oligomycin	6.67 ± 0.04	6.52 ± 0.02	-0.16 ± 0.05	7	0.028
antim + oligomycin	6.82 ± 0.19	6.72 ± 0.14	-0.03 ± 0.02	6	n.s.
antim + oligomycin + LiCl	6.67 ± 0.04	6.65 ± 0.03	-0.02 ± 0.02	7	n.s.
	sEPSC frequency (Hz)				
drug	Hz ctrl	Hz drug	ratio	*n*	*p*
acid load (after NH_4Cl)	4.82 ± 1.79	40.63 ± 14.14	9.97 ± 3.14	6	0.031

vesicles. We observed a significant increase of spontaneous quantal glutamate release during mitochondrial inhibition and showed that it is mediated by a rise in $[Ca^{2+}]_i$. A blockade of the Na^+–Ca^{2+} exchanger alone, however, was not sufficient to cause a Ca^{2+} accumulation high enough to trigger exocytosis. Increased Ca^{2+} influx through voltage-gated Ca^{2+} channels has been observed in neurons during exposure to mitochondrial uncouplers (Nowicky & Duchen 1990). In our experiments Ca^{2+} channels were blocked with Cd^{2+}. We argue, therefore, that the rise in synaptic $[Ca^{2+}]_i$ observed during mitochondrial inhibition, and the subsequent increase in sEPSC frequency, depend on the outflow of Ca^{2+} ions through the depolarized inner mitochondrial membrane and on the prolonged impairment of Ca^{2+} uptake into these organelles. Simultaneous monitoring of $[Ca^{2+}]_i$ and of mitochondrial Ca^{2+} levels revealed that the rise in $[Ca^{2+}]_i$, usually observed on exposure to the protonophore FCCP, is paralleled by a reciprocal fall in the mitochondrial Ca^{2+} concentration (Rizzuto *et al*. 1992). However, the rise in $[Ca^{2+}]_i$ accompanying a blockade of mitochondrial function could be secondary to ATP depletion (Budd & Nicholls 1996), thus affecting the plasma membrane Ca^{2+} pump activity (Carafoli 1987). To reduce the risk of ATP depletion, oligomycin was always coapplied with the protonophore FCCP or the electron transport inhibitor antimycin A. This treatment successfully prevented consumption of cytoplasmic ATP in other studies (Nachshen 1984; Budd & Nicholls 1996). Moreover, a 5-min exposure to oligomycin alone to selectively block mitochondrial ATP production without reversal of the mitochondrial membrane potential did not affect sEPSC frequency. Loading the cells with the calcium chelator BAPTA-AM (10 µM) prevented the increase of sEPSC frequency induced by FCCP plus oligomycin. Therefore, release of Ca^{2+} from mitochondria and a blockade of Ca^{2+} uptake during mitochondrial inhibition are the most likely determinants of the rise in $[Ca^{2+}]_i$ and for the subsequent increase of neurotransmitter release in our experiments. These effects were greatly enhanced by additional inhibition of the plasma membrane Na^+–Ca^{2+} exchange, indicating that both, mitochondria and exchanger are very important for the regulation of $[Ca^{2+}]_i$ and neurotransmitter release.

Intracellular pH (pHi) levels decreased slightly after exposure to FCCP and oligomycin, while antimycin A together with oligomycin did not lead to cellular acidification. We found that a fall in pHi after an ammonium pulse can significantly increase the sEPSC frequency. This result is in accordance with other studies showing that spontaneous neurotransmitter release is enhanced in synaptosomes during acidification (Drapeau & Nachshen 1988). Since the increase in sEPSC frequency that we have observed with FCCP and oligomycin was completely prevented when $[Ca^{2+}]_i$ changes were buffered with BAPTA, we conclude that the small acidification was not important for the neurotransmitter release.

In conclusion, we have shown that the plasma membrane Na^+–Ca^{2+} exchanger and mitochondria can cooperate in the regulation of $[Ca^{2+}]_i$ in individual presynaptic boutons of hippocampal cells. We have also demonstrated that the Ca^{2+} handling by mitochondria and the exchanger is important for spontaneous neurotransmitter release.

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