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In Situ Fluorescence Imaging of Glutamate-Evoked Mitochondrial Na^+ Responses in Astrocytes

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ABSTRACT
Astrocytes can experience large intracellular Na^+ changes following the activation of the Na^+-coupled glutamate transport. The present study investigated whether cytosolic Na^+ changes are transmitted to mitochondria, which could therefore influence their function and contribute to the overall intracellular Na^+ regulation. Mitochondrial Na^+ (Na^+_{mit}) changes were monitored using the Na^+-sensitive fluorescent probe CoroNa Red (CR) in intact primary cortical astrocytes, as opposed to the classical isolated mitochondria preparation. The mitochondrial localization and Na^+ sensitivity of the dye were first verified and indicated that it can be safely used as a selective Na^+_{mit} indicator. We found by simultaneously monitoring cytosolic and mitochondrial Na^+ using sodium-binding benzofuran isophthalate and CR, respectively, that glutamate-evoked cytosolic Na^+ elevations are transmitted to mitochondria. The resting Na^+_{mit} concentration was estimated at 19.0 ± 0.8 mM, reaching 30.1 ± 1.2 mM during 200 μM glutamate application. Blockers of conductances potentially mediating Na^+ entry (calcium uniporter, monovalent cation conductances, K^+_{ATP} channels) were not able to prevent the Na^+_{mit} response to glutamate. However, Ca^{2+} and its exchange with Na^+ appear to play an important role in mediating mitochondrial Na^+ entry as chelating intracellular Ca^{2+} with BAPTA or inhibiting Na^+/Ca^{2+} exchanger with CGP-37157 diminished the Na^+_{mit} response. Moreover, intracellular Ca^{2+} increase achieved by photoactivation of caged Ca^{2+} also induced a Na^+_{mit} elevation. Inhibition of mitochondrial Na/H antiporter using ethylisopropyl-amiloride caused a steady increase in Na^+_{mit} without increasing cytosolic Na^+, indicating that Na^+ extrusion from mitochondria is mediated by these exchangers. Thus, mitochondria in intact astrocytes are equipped to efficiently sense cellular Na^+ signals and to dynamically regulate their Na^+ content.

INTRODUCTION

After its synaptic release, glutamate is rapidly taken up by surrounding astrocytes, thus preventing the excitotoxic buildup of extracellular glutamate. This extremely efficient transport system of astrocytes utilizes the steep electrochemical gradient of Na^+ across the cell membrane as the driving force (Danbolt, 2001). As a consequence of this transport activity, the astrocytes are among the few cell types that can experience large variations in their cytosolic Na^+ concentration (Na^+_{cyt}), which can increase by 20–30 mM in a few seconds (Bernardinelli et al., 2004; Chatton et al., 2000; Rose and Ransom, 1996).

The Na^+_{cyt} increases lead to a >2-fold enhancement of Na^+/K^+-ATPase activity for extracellular glutamate concentrations expected to be seen by astrocytes during neuronal activity (Chatton et al., 2000, 2003), thus causing an energy burden sufficient to lead to a drop in cellular ATP levels (Chatton and Magistretti, 2005). This increased energy need is believed to stimulate the astrocyte energy metabolism and glucose uptake. These processes appear to be spatially and temporally coordinated by an elaborate dynamic intercellular signaling (Bernardinelli et al., 2004).

Mitochondria play a central role in cellular ATP production as they host the citric acid cycle and oxidative phosphorylation. Despite the highly efficient mitochondrial energy production, it appears that the glutamate-evoked metabolic response of astrocytes is primarily glycolytic (Pellerin and Magistretti, 1994).

If glutamate-evoked variations of Na^+_{cyt} were transmitted to mitochondria, they could participate to the modulation of the overall Na^+ and metabolic responses of astrocytes. Mitochondria are thought to express a complex repertoire of cation conductances and transporters (Bernardi, 1999; Brierley et al., 1994) that could mediate a significant Na^+ flux across mitochondrial membranes. Because electrophysiological approaches are extremely difficult to apply on mitochondria due to the small size of these organelles, these conductances have been unveiled mostly using pharmacological approaches on isolated mitochondria. Studying mitochondrial function in situ in intact living cells is complicated by the presence of the cell membrane. Nevertheless, these types of studies become accessible owing to the development of fluorescent indicators of mitochondrial functions and imaging tools. In particular, Ca^{2+} is known to be

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exchanged across the inner mitochondrial membrane in a dynamic way (Malli et al., 2003) in register with cytosolic Ca^{2+} changes. As other cations, Na^+ is expected to be strongly attracted into mitochondria because of the highly negative electrical potential inside the mitochondrial matrix. Recently, a Na^+ fluorescent indicator CoroNa Red (CR) was described (Jayaraman et al., 2001a,b), and shown to localize in mitochondria of different cell types (Baron et al., 2005; Yang et al., 2004), opening to new perspectives for the study of mitochondrial Na^+ homeostasis and its potential link to energy metabolism in astrocytes. Here, we investigated the Na^+ dynamics in mitochondria in their native cellular environment and, more specifically, assessed whether Na^+_{cyt} changes observed in response to glutamate transport activation are transmitted to mitochondria. We show that mitochondria in intact astrocytes possess mechanisms for efficiently sensing cytosolic Na^+ changes and dynamically regulating their Na^+ content.

MATERIALS AND METHODS

Cell Culture

Cortical astrocytes in primary culture were obtained from 1- to 3-day-old OF1 and C57BL/6 mice as described previously (Sorg and Magistretti, 1992). Cells were grown at confluency for 3 weeks on glass coverslips in DME medium supplemented with 10% FCS.

Fluorimetric Characterization

The Na^+ sensitivity and selectivity of CoroNa Red (CR; Molecular Probes, Eugene, OR) was tested in vitro on a spectrofluorimeter (Perkin–Elmer, Wellesley, MA) in the absence of cells or mitochondria. Fluorescence was excited at 530 nm and measured at 580 nm. CR was dissolved at 0.1 μM in intracellular-like saline containing (mM) the following: K^+-gluconate, 138, NaCl 8; $MgCl_2$, 5; EGTA, 1; $CaCl_2$, 0.5; HEPES, 10, pH 7.2. For Na^+ titration, NaCl concentration was varied from 0 to 400 mM. Solutions with various pH values were titrated using KOH. Sensitivity of the CR to K^+ was tested by adding known amounts of K^+-gluconate. CR sensitivity to Ca^{2+} was tested using solutions of defined free Ca^{2+} concentration in the presence of 1 mM EGTA as calculated using the Maxchelator software (Chris Patton, Stanford University, http://www.stanford.edu/~cpatton/maxc.html).

Fluorescence Imaging

Cells were loaded at 37°C for 18 min with 1 μM CR in a HEPES-buffered balanced solution (see below) and then superfused at 37°C in a thermostated chamber (Chatton et al., 2000). MitoTracker Green FM (5 μM, Molecular Probes) was loaded using the same procedure. For simultaneous cytosolic and mitochondrial Na^+ imaging, cells were loaded for 75 min with 15 μM of the acetoxymethyl ester derivative of sodium-binding benzofuran isophthalate (SBFI-AM, Teflabs, Austin, TX) at 37°C, and with CR (1 μM) for the last 18 min of incubation.

Dynamic intracellular ion imaging was performed on an inverted epifluorescence microscope (Axiovert 100M, Carl Zeiss, Germany) using a 40 × 1.3 N.A. oil-immersion objective lens. Fluorescence excitation wavelengths were selected using a monochromator (Till Photonics, Planegg, Germany) and fluorescence was detected using a 12-bit cooled CCD camera (Princeton Instruments, Trenton, NJ). CR fluorescence was excited at 560 nm and detected at >580 nm. For dual CR and SBFI imaging, a double band dichroic mirror (~420 and ~575 nm) and emission filter (~510 and ~600 nm) were used (Chroma Technology Rockingham, VT), and fluorescence was sequentially excited at 340, 380, and 550 nm. Image acquisition was computer-controlled using the software Metafluor (Universal Imaging, Reading, PA) running on a Pentium computer. Regions of interest with high density of mitochondria and excluding nuclei were selected in individual cells, and the average fluorescence signal inside these regions was analyzed over time.

Flash Photolysis

Ca^{2+} photorelease was performed using a high power UV LED (365 nm/100 mW) system as previously described (Bernardinelli et al., 2005). The UV light was delivered to the specimen by means of a multimode fused silica 50-μm core optical fiber positioned 20 μm above the cell surface to illuminate a spot of about 30 × 60 μm^2 corresponding to 3–4 cells of the confluent monolayer. For these experiments, we used the caged Ca^{2+} compound *o*-nitrophenyl EGTA (NP-EGTA) that gets fragmented by UV light in two parts having negligible Ca^{2+} affinities, resulting in fast Ca^{2+} release (Ellis-Davies et al., 1996). NP-EGTA was loaded into astrocytes using its membrane-permeant derivative NP-EGTA-AM (30 min, 8 μM; Molecular Probes). Cells were then placed back in the incubator for 3–4 h to allow the compound to become gradually saturated with Ca^{2+} before loading the Ca^{2+}-probe Fluo-4 AM (6 μM, 30 min; Teflabs) or CR as described earlier. For these experiments, we used an inverted epifluorescence microscope (Diaphot 300, Nikon, Tokyo, Japan) equipped with a 40 × 1.3 N.A. oil-immersion objective lens (Nikon), a fast filter wheel for selection of excitation wavelengths (Sutter Instruments, Novato, CA), and a Gen III^+ intensified CCD Camera (VideoScope International, Washington DC).

Confocal Imaging

Confocal imaging of CR and MitoTracker Green FM was performed on living cells on a LSM 510 Meta confocal microscope with a 63 × 1.4 N.A. oil immersion objective (Carl Zeiss), with sequential excitation at 543 and 488 nm, respectively. For dual staining experiments, a double band primary dichroic mirror was used (488/543 nm), and the fluorescence emission was detected on two channels (496–529 nm and 561–636 nm, respectively)

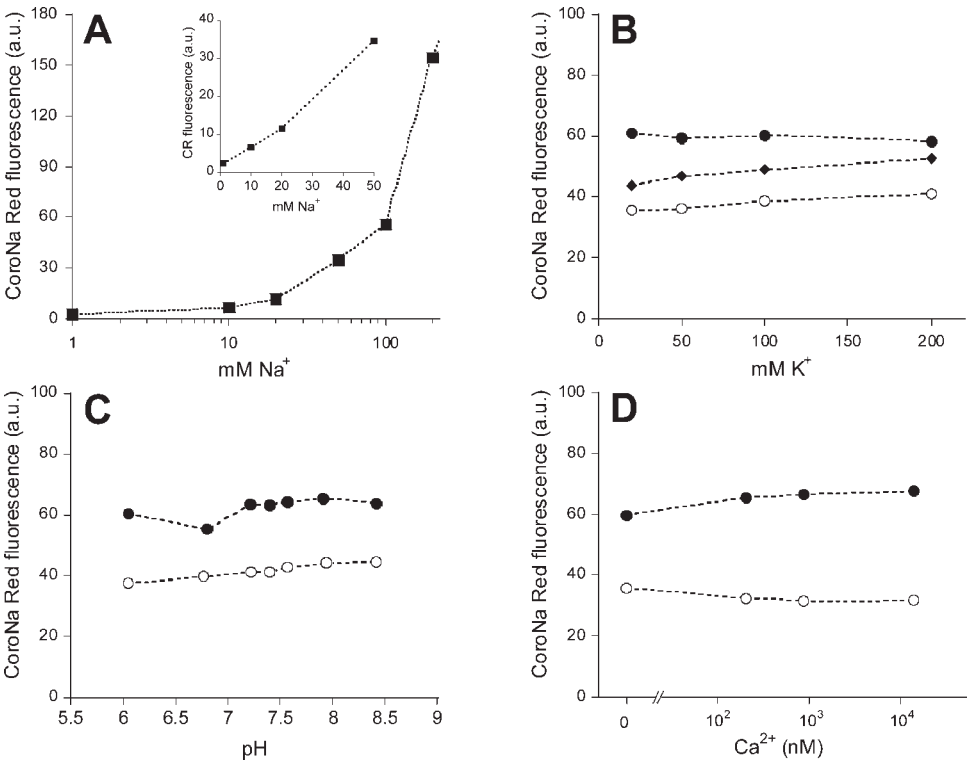

Fig. 1. Fluorometric characterization of CR cation sensitivity. CR dissolved at 0.1 μM in intracellular-like solution (see Methods) was excited at 530 nm and emission was recorded at 580 nm in the absence of cells or mitochondria. (**A**) Sensitivity to increasing medium Na^+ concentration in the range 0–400 mM. For graphical clarity only values up to 200 mM are shown, although higher concentrations display monotonic fluorescence increase. The inset shows the fluorescence change in the range 0–50 mM Na^+. Sensitivity to ambient K^+ (**B**), pH (**C**), and Ca^{2+} (**D**) measured at constant Na^+ concentration of 8 mM (○), 20 mM (◆), or 50 mM (●).

selected on the photomultiplier array spectral detector (Meta detector). The spectral detector was also used to record dye emission spectra from single mitochondria.

Experimental Solutions

Solutions contained (mM) the following: NaCl, 135; KCl, 5.4; $NaHCO_3$, 25; $CaCl_2$, 1.3; $MgSO_4$, 0.8; NaH_2PO_4, 0.78; glucose, 5, bubbled with 5% CO_2/95% air. The solution used to deliver BAPTA-AM (50 μM) to cells contained in addition 1 g% bovine serum albumin and 10 mM HEPES (pH 7.4). The solution for cellular dye loading contained (mM) the following: NaCl, 135; KCl, 5.4; HEPES, 20; $CaCl_2$, 1.3; $MgSO_4$, 0.8; NaH_2PO_4, 0.78; glucose, 20 (pH 7.4), and was supplemented with 0.1% Pluronic F-127 (Molecular Probes). For experiments involving La^{3+}, because of the poor solubility of this ion in salines containing bicarbonate and phosphate, we used the following solutions (mM): NaCl, 160.8; KCl, 5.4; $CaCl_2$, 1.3; $MgSO_4$, 0.8; HEPES, 20; glucose, 5, bubbled with air and adjusted to pH 7.4.

In situ calibration of CR signal was attempted using a protocol previously used for mitochondrial (Yang et al., 2004) and cytosolic Na^+ calibration (Chatton et al., 2000, 2003). Cells were permeabilized for monovalent cations using 6 μg/ml gramicidin and 10 μM monensin with simultaneous inhibition the Na^+/K^+-ATPase using 1 mM ouabain. Cells were then sequentially perfused with solutions buffered at pH 7.2 with 20 mM HEPES and containing 0, 10, 20, and 50 mM Na^+, respectively, and 30 mM Cl^- and 136 mM gluconate with a constant total concentration of Na^+ and K^+ of 165 mM. Individual four-point calibration curves were computed for cells in the field of view and used to convert CR fluorescence ratio values into Na^+ concentrations. During the entire procedure, the mitochondrial staining pattern was not altered.

Materials

Ouabain was from Fluka (Buchs, Switzerland). CGP-37157, U37883A, and glibenclamide were from Biomol-Anawa Trading (Zurich, Switzerland). RU-360 was from Calbiochem. Ethyl-isopropyl amiloride (EIPA) was gift from Dr. H. Lang (Aventis Pharma, Frankfurt, Germany). BAPTA-AM was from Molecular Probes. All other compounds were from Sigma.

RESULTS

Corona Red Characterization

The ability of CR to monitor Na^+ was first assessed in vitro by spectrofluorimetry using experimental solutions mimicking the cellular ionic environment. CR fluorescence increased monotonically as a function of Na^+ concentration in the range 0–400 mM, consistent with the K_D of ~200 mM reported by the manufacturer. Figure 1A depicts the fluorescence response of CR in the range 0–200 mM Na^+ that has physiological relevance, and indicates that the probe displays significant fluorescence change in this range. We then tested the ability of the probe to discriminate against other cations. Figure 1B shows that K^+ in the range 20–200 mM has only minor influence on CR fluorescence. Neither pH in the range 6–

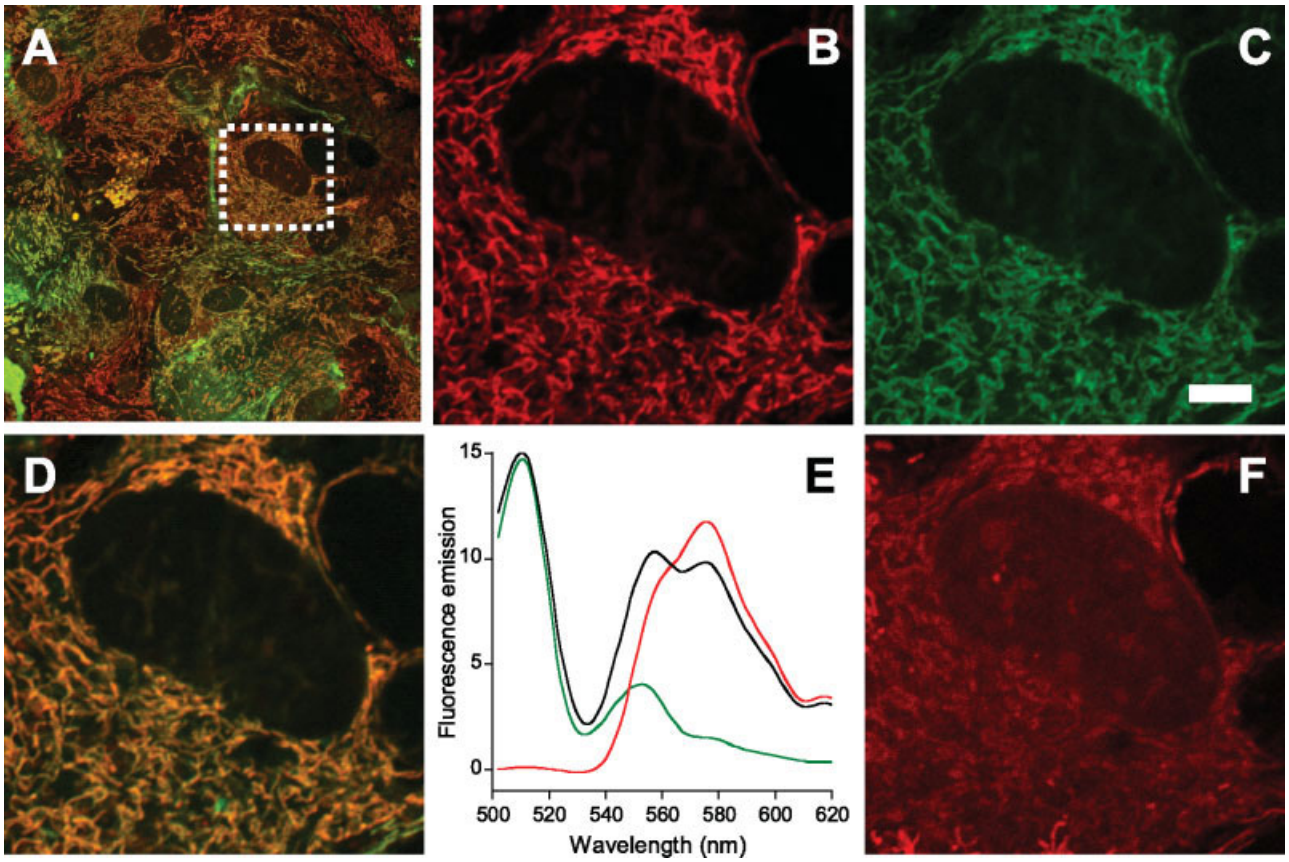

Fig. 2. Mitochondrial localization of CR signal. Confocal images of living astrocytes loaded with MitoTracker Green (MTG) and CR (**A**). Images of CR (**B**), MTG (**C**), and the overlay of both (**D**) at higher magnification for the region depicted in A (dotted box). Scale bar, 5 μM. (**E**) Emission spectra recorded in individual mitochondria from cells loaded with MTG (green), with CR (red), and with both (black) show that mitochondria contain the spectral signature of both dyes. (**F**) Longer incubations or higher concentrations of CR results in a diffuse staining, where cytosolic and nuclear distribution becomes more prominent.

8.5 (Fig. 1C) nor Ca^{2+} in the range 0–10 μM (Fig. 1D) markedly influenced CR fluorescence in the presence of 8 or 50 mM Na^+. One can conclude from this in vitro characterization that CR can be safely used as a Na^+-sensitive probe, which is consistent with previous reports (Jayaraman et al., 2001a,b).

We then tested whether CR staining of living astrocytes was specific to mitochondria. After a 18 min loading time, 0.7-μm thick confocal optical sections of CR-loaded cells displayed the typical pattern of mitochondrial loading, with dark nuclei and punctated staining of rod-like structures (Figs. 2A–D). Close-up view shows that CR stain matched almost perfectly the mitochondrial stain Mitotracker Green FM loaded in the same cells. To ascertain that MitoTracker dye and CR localized within the same organelles, fluorescence emission spectra were recorded using the confocal spectral detector in structures of living cells with typical mitochondrial appearance and showed the spectral signature of both dyes (Fig. 2E), with an emission peak at ~600 nm for CR and at ~520 nm for MitoTracker Green FM. These results indicated that CR indeed localizes into mitochondria of living astrocytes. However, as observed by others (Yang et al., 2004), when longer loading time or higher CR concentrations are used, CR stained not only mitochondria but also the rest of the cell, as shown by the diffuse staining in Fig. 2F. For the entire study, we optimized the loading conditions to maximize mitochondrial loading and systematically discarded any experiment where cytosolic fluorescent staining was observed at the beginning or during the course of an experiment.

Cytosolic Na^+ Changes are Transmitted to Mitochondria

Glutamate is known to induce robust Na^+_{cyt} increases in astrocytes mediated almost entirely by an avid Na^+-coupled uptake mechanism (Chatton et al., 2000). To test whether these Na^+ increases are transmitted to mitochondria, astrocytes were loaded with CR and fluorescence changes were monitored by dynamic fluorescence microscopy. Glutamate superfusion induced a clear-cut Na^+_{mit} signal increase that returned to baseline after glutamate washout (Fig. 3A). Because glutamate interacts with several classes of receptors and is a metabolic substrate of mitochondria, we assessed whether the ob-

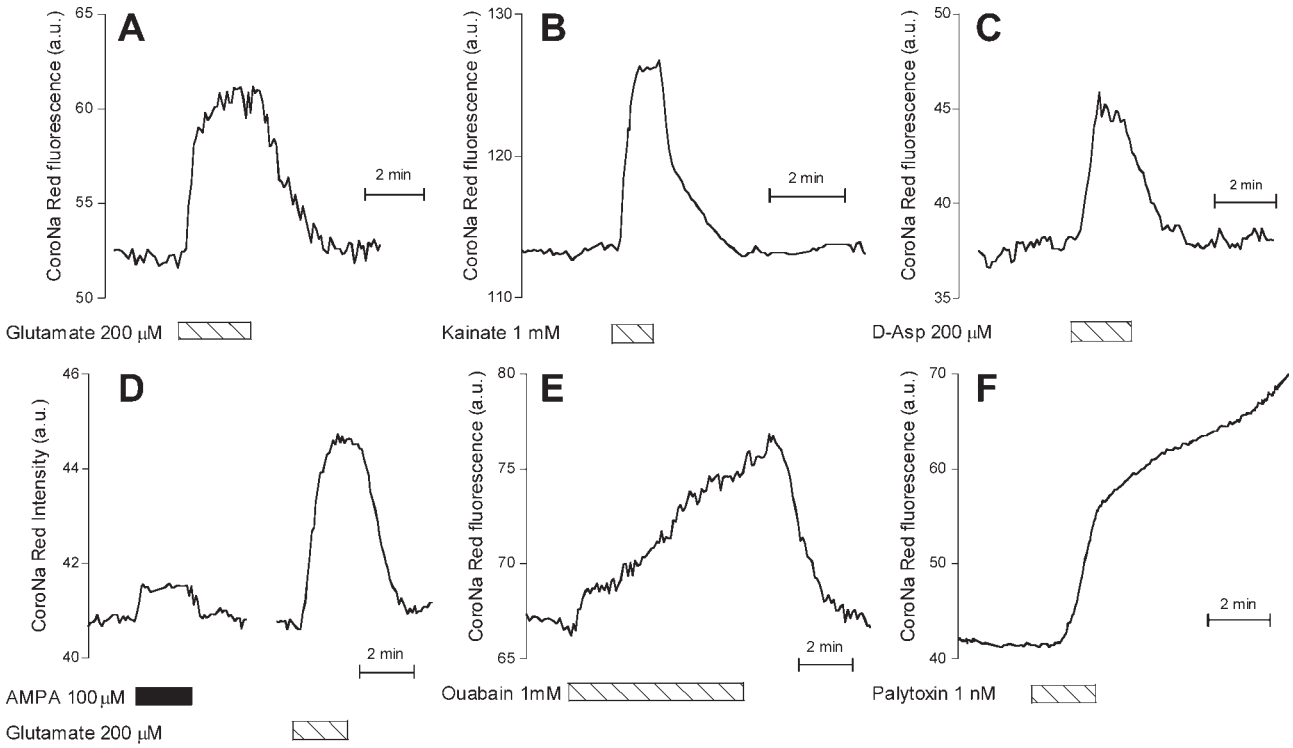

Fig. 3. Agents known to increase cytosolic Na^+ in astrocytes lead to Na^+_{mit} increase. Representative traces of Na^+_{mit} changes in single cells are shown for bath application of (**A**) glutamate (200 μM; n = 48 cells from 7 experiments), (**B**) kainate (1 mM, n = 68 cells from 9 experiments), (**C**) D-aspartate (200 μM; n = 72 cells from 8 experiments), (**D**) AMPA (100 μM) compared to glutamate (200 μM) on the same cell (n = 38 cells from 5 experiments), (**E**) ouabain (1 mM; n = 12 cells from 2 experiments), and (**F**) palytoxin (1 nM; n = 24 cells from 3 experiments). The paradigm of drug applications is indicated in the bottom of each graph by dashed rectangles.

served Na^+_{mit} increase could be induced by other means. Activation of non-NMDA receptors using kainate that is known to induce Na^+_{cyt} responses (Chatton et al., 2000) also produced robust Na^+_{mit} responses (Fig. 3B), as well a the glutamate transporter substrate D-aspartate (Fig. 3C). Maximal activation of AMPA receptors using AMPA was found to cause a weak cytosolic Na^+ response with an amplitude of 23% of the response to glutamate (Chatton et al., 2000). This was found to be also the case on the Na^+_{mit} response (Fig. 3D). Application of ouabain, which increases Na^+_{cyt} by specifically inhibiting the cellular Na^+ extrusion by the Na^+/K^+-ATPase, led to a steady increase in Na^+_{mit} (Fig. 3E). Finally, application of palytoxin, a 2,700 kDa polypeptide toxin, which binds to the Na^+/K^+-ATPase on its extracellular side and transforms it into a nonselective cation channel (Horisberger et al., 2004), induced a massive and irreversible increase in Na^+_{mit} (Fig. 3F).

Estimation of Na^+_{mit} Concentration in Intact Astrocytes

To obtain estimates of the resting Na^+_{mit} concentration as well as the amplitude of the observed Na^+_{mit} response, attempts were made to calibrate CR fluorescence in situ. The approaches described by others in Madin Darby canine kidney (MDCK) cells (Baron et al., 2005) used permabilization of plasma membrane with ionophore in addition to mitochondrial depolarization using p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP). In our hands, FCCP could not be used as it resulted in a rapid loss of mitochondrial staining and widespread cellular dye redistribution. We used instead the approach proposed in another study in cardiomyocytes (Yang et al., 2004), in which the plasma membrane Na^+/K^+-ATPase was inhibited using ouabain, and the ionophores monensin and gramicidin were added to permeabilize both plasma and intracellular membranes for monovalent cations. Four solutions with Na^+ concentrations ranging from 0 to 50 mM were then applied to cells and the CR fluorescence signal measured. Under these conditions, CR remained associated to mitochondria throughout the entire procedure. Figure 4A shows an experiment during which a first 200 μM glutamate application was performed and evoked the CR response described above, after which a zero Na^+ bath application rapidly decreased the signal. Subsequent addition of permeabilization cocktail further reduced the signal. Bath Na^+ concentration was then stepwise increased and resulted in proportionate CR fluorescence increase. Plateau values of CR fluorescence for each applied Na^+ concentration and each cell under study were measured and show a monotonic increase with respect to Na^+

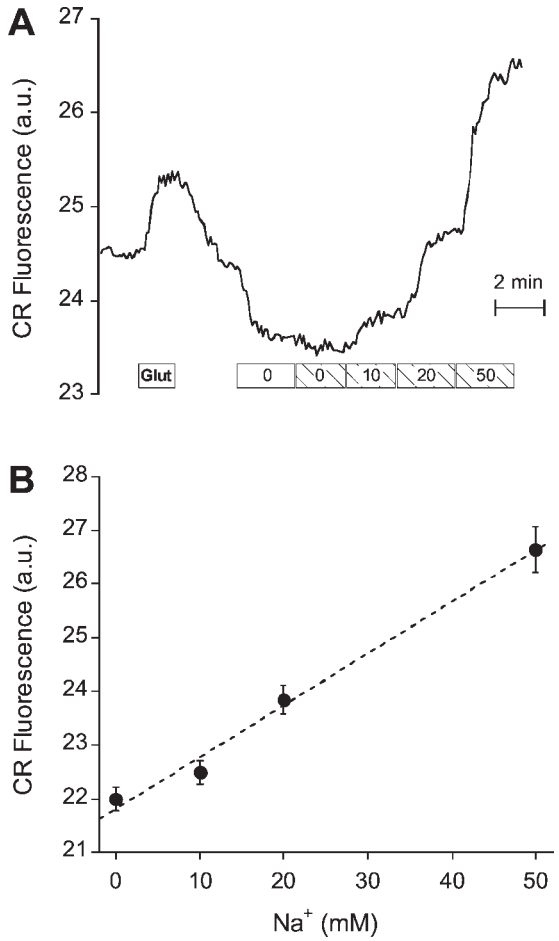

Fig. 4. Estimation of Na^+_{mit} concentration. (**A**) Example trace of CR fluorescence (arbitrary fluorescence units) measured in individual intact astrocytes showing the response to a first 200 μM glutamate application and the CR fluorescence change caused by a switch to zero Na^+ perfusion solution and the subsequent adjunction of ouabain and the ionophores monensin and gramicidin (hatched bars). The Na^+ concentration in the perfusion solution was then varied as depicted in the graph (see solution composition under material and methods). (**B**) Dependency of in situ CR fluorescence and applied bath Na^+ concentration. The raw CR plateau fluorescence values were measured for each applied Na^+ solution and for each individual cell of this series of experiments. The mean values ± SEM of a total of 57 cells from 8 independent experiments were plotted as a function of bath Na^+ concentration, and show a monotonic relationship between the two variables with a linear correlation $R = 0.995$ (dotted line).

(Fig. 4B). When applying each individual titration curve to its corresponding cell, the average resting Na^+_{mit} concentration was found to be 19.0 ± 0.8 mM, reaching 30.1 ± 1.2 mM during 200 μM glutamate application ($n = 57$ cells from 8 independent experiments). In comparison, calibration of Na^+_{cyt} changes measured with SBFI as described previously (Chatton et al., 2000) yielded a resting Na^+_{cyt} concentration of 13.2 ± 0.1 mM ($n = 12$ cells from 2 experiments) in the same batch of cells. It is difficult to evaluate how well Na^+_{mit} was clamped under these conditions; however, these values fall in the range of estimated Na^+_{mit} values reported in the literature (*see* Discussion).

Potential Pathways for Mitochondrial Na^+ Entry

The inner mitochondrial membrane, capable of sustaining a large transmembrane electrical gradient, is considered as the main barrier to the diffusion of ions, but nevertheless contains several potential pathways that could mediate the Na^+_{mit} response, including mitochondrial transporters, conductances, or permeability transition pore (Bernardi, 1999; Brierley et al., 1994).

We first tested whether activating mitochondrial cation conductances would indeed cause a Na^+_{mit} increase and developed a methodological approach to simultaneously monitor Na^+_{cyt} and Na^+_{mit} using SBFI and CR, respectively. Figure 5A shows that SBFI displays the typical staining of cytosol and nuclei observed in several cell types including astrocytes (Borin et al., 1993; Chatton et al., 2003), and CR staining is punctated and excluded from nuclei. The proper controls were performed to ensure the independency of the two fluorescent signals that show no spectral overlap (not shown).

A first 200 μM glutamate application led to an increase in Na^+ in the cytosolic and mitochondrial compartments with similar kinetics and without delay detectable given the temporal resolution of the measurement. We then used diazoxide, a compound known to open mitochondrial K_{ATP} channels that are found in the brain (Bajgar et al., 2001). Figure 5B shows that although diazoxide did not influence on Na^+_{cyt}, it induced a measurable Na^+_{mit} increase, indicating that even in absence of Na^+_{cyt} rise, opening of mitochondrial K_{ATP} channels could indeed cause an increase in Na^+_{mit}.

We then attempted to identify the pathway responsible for the glutamate-evoked Na^+_{mit} response. We first tested compounds described as pharmacological blockers of several mitochondrial conductances. The compounds were preincubated for up to 30 min before glutamate application to take into account their potential delayed access to mitochondrial membranes. However, none of them was found to be alone effective at preventing Na^+_{mit} increase induced by 200 μM glutamate. The compounds tested were RU-360 (10 μM, $n = 7$) a blocker of the mitochondrial Ca^{2+} uniporter; La^{3+} (200 μM, $n = 5$) a broad spectrum cation conductance blocker; cyclosporine A (5 μM, $n = 5$) and bongkrekic acid (10 μM, $n = 3$) two compounds known to block the mitochondrial permeability transition pore; glibenclamide (1, 10, 50 μM, $n = 12$), U37883A (100 μM, $n = 6$) and 5-hydrodroxydecanoate (500 μM, $n = 8$) blockers of mitochondrial K_{ATP} channels; and carbenoxolone (20 μM, $n = 4$) blockers of connexins, recently identified in mitochondrial membranes (Boengler et al., 2005) (not shown).

The possible involvement of mitochondrial Na^+/Ca^{2+} exchanger was tested by applying its inhibitor CGP-37157 (30 μM), which caused a ~20% inhibition of the glutamate-evoked Na^+_{mit} response (Fig. 5C). To directly probe for a role of intracellular Ca^{2+}, BAPTA-AM (50 μM, 30 min) was loaded into the cells in order to chelate intracellular free Ca^{2+}. We first verified that this maneuver efficiently prevented subsequent glutamate-evoked Ca^{2+}

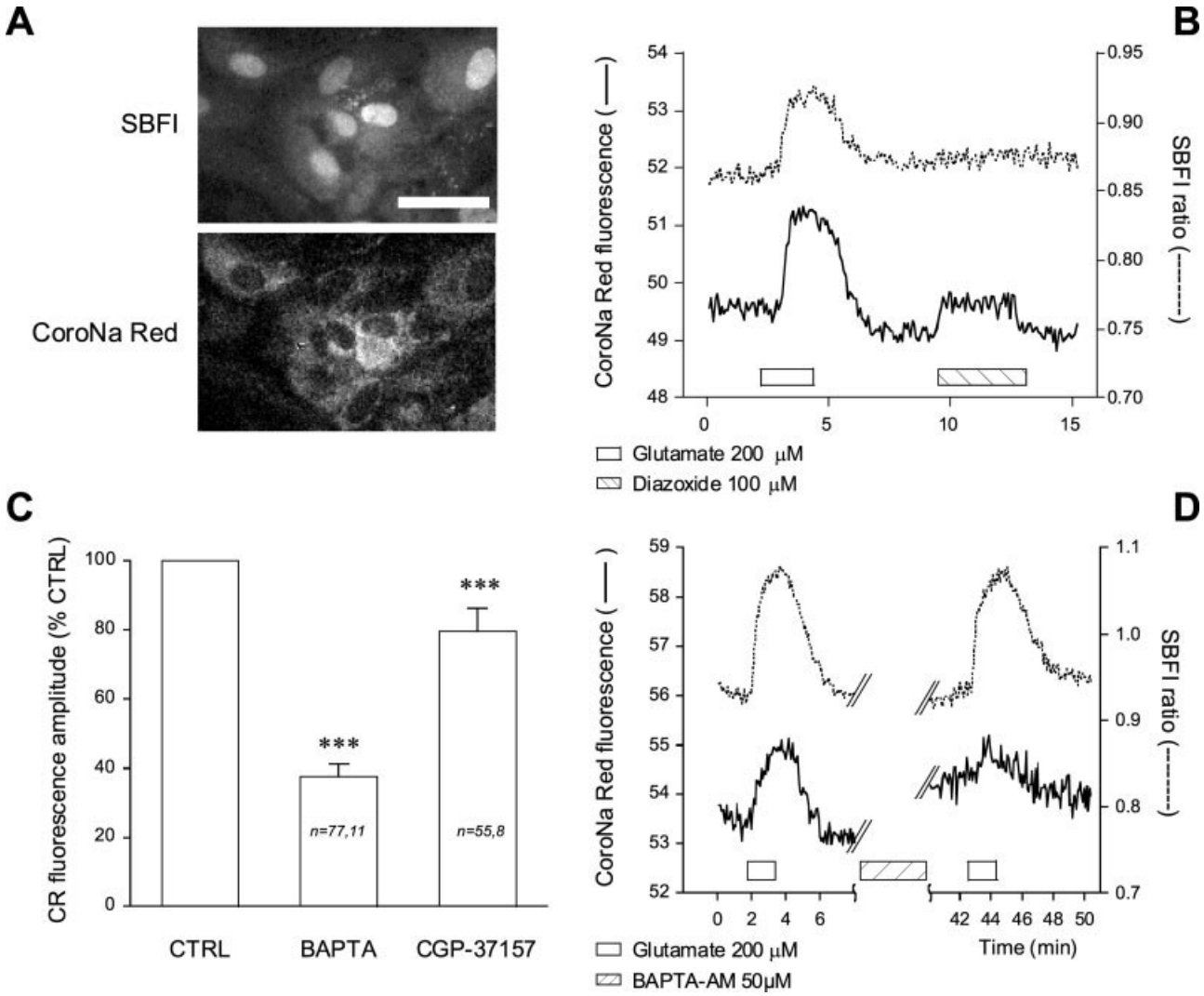

Fig. 5. Pathways for Na^+ entry in mitochondria. (**A**) Na^+_{mit} and Na^+_{cyt} were measured simultaneously by loading astrocytes with CR and SBFI, respectively. Scale bar, 20 μm. (**B**) A first glutamate (200 μM) application leads to a Na^+ response both in the cytosol (dotted line) and mitochondria (plain line). The responses appear to occur in the two compartments with a similar kinetics and without measurable delay. Application of the K_{ATP} channel opener diazoxide led to an increase in Na^+_{mit} without altering Na^+_{cyt}. Representative responses of one cell out of 56 from 7 experiments. (**C**) Bar graph of glutamate-evoked Na^+_{mit} response comparing the control condition with responses after BAPTA-AM treatment and CGP-37157 (30 μM) application. Data are mean amplitude of CR fluorescence increase measured in 77 cells from 11 experiments (BAPTA) and 55 cells from 8 experiments (CGP-37175). $^{***}P < 0.0001$ using paired *t*-test. (**D**) After a first glutamate pulse leading to a Na^+ response in the cytosol and mitochondria, BAPTA-AM (50 μM) was applied for 30 min and caused a strong (62%, see panel C) inhibition of the Na^+_{mit} response to glutamate, without decreasing the Na^+_{cyt} response (representative curve).

elevation (not shown). After a control glutamate application that led to Na^+ response in the cytosol and mitochondria, chelating Ca^{2+} strongly inhibited the Na^+_{mit} response without influencing Na^+_{cyt} response on the same cells as shown in the traces in Fig. 5D. It should be mentioned that the drift in the baseline CR signal after BAPTA application in this figure was not observed in all experiments and was less marked in cells loaded with CR alone. Overall, BAPTA inhibited the glutamate-evoked Na^+_{mit} response by 62.5% ± 3.7% (Fig. 5C).

We finally tested whether elevating Ca^{2+} would cause an increase in Na^+_{mit} by locally releasing Ca^{2+} using UV flash photolysis. Figure 6A shows that photoactivation of caged Ca^{2+} indeed caused a rapid rise in intracellular free Ca^{2+} measured using the Ca^{2+} probe Fluo-4 returning to the baseline in ~2 min. Interestingly, photoactivation of NP-EGTA also caused a Na^+_{mit} response (Fig. 6B) with a clearly distinct kinetics, but which also recovered within ~2 min. UV flashes applied on cells that had not been loaded with NP-EGTA evoked no Na^+_{mit} response (Fig. 6C). Taken together, these experiments show the prominent role of Ca^{2+} in the observed Na^+_{mit} increase, which is likely to involve mitochondrial Ca^{2+} exchangers.

Na^+_{mit} Regulation

The mitochondrial Na^+/H^+ exchanger was proposed to be the main mechanism preventing Na^+_{mit} concentration

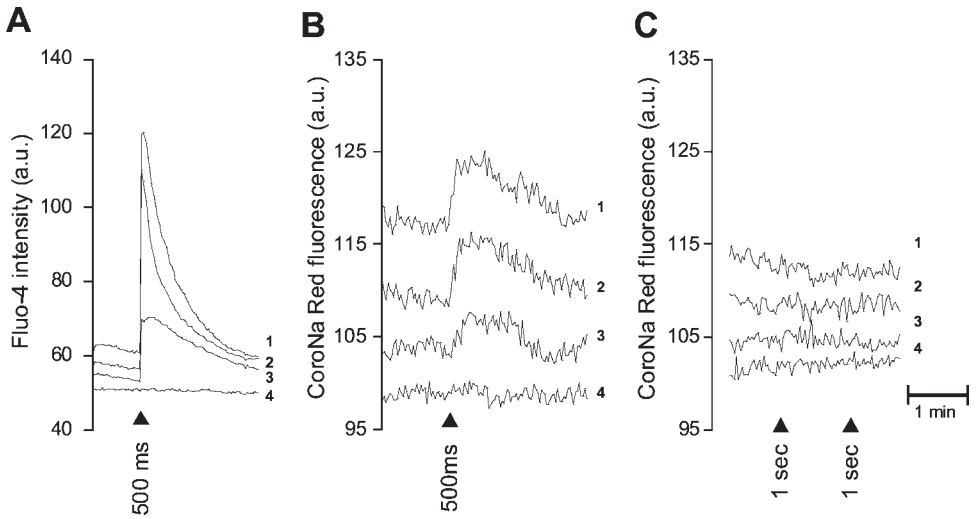

Fig. 6. Intracellular Ca^{2+} photorelease cause Na^+_{mit} responses. The caged Ca^{2+} compound NP-EGTA loaded in astrocytes was photoactivated in a group of 3–4 cells. (**A**) Effect of a 500-ms flash on cells loaded with Fluo-4 showing the rapid cellular Ca^{2+} rise and its recovery. (**B**) On cells loaded with CR, a 500-ms flash evoked a Na^+_{mit} response, whereas no response was seen for flashes as long as 1 s in cells containing no caged Ca^{2+} (**C**). For all panels, Cells 1–3 were located within the illuminated spot, whereas Cell 4 was outside the photoactivated spot (internal control). The protocols were repeated on 2–4 different coverlips and with up to 10 fields. The time scale for all plots is indicated in the graph C.

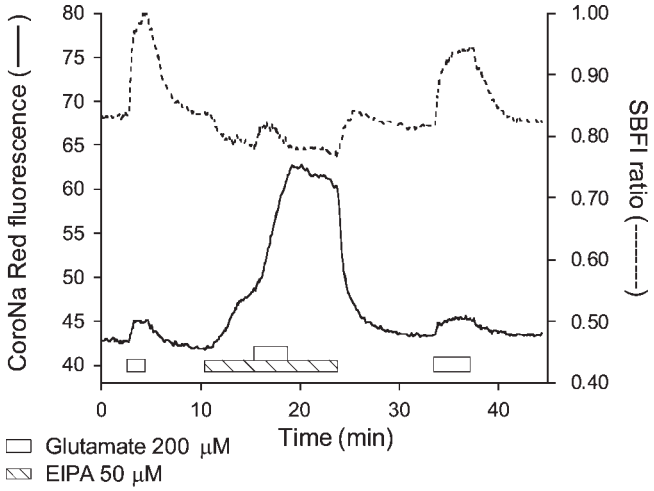

Fig. 7. Regulation of Na^+_{mit} by the mitochondrial Na^+/H^+ exchanger. A first 200 μM glutamate application evoked a Na^+ increase in both cytosolic and mitochondrial compartments. Application of EIPA, an inhibitor of cell membrane, and mitochondrial Na^+/H^+ exchangers had strikingly different effects on Na^+_{cyt} and Na^+_{mit}. In the presence of EIPA, Na^+_{mit} steadily increased and additional application of glutamate accelerated its rise, whereas Na^+_{cyt} rapidly stabilized to a lower level from which glutamate evoked a modest response. Upon glutamate washout, Na^+_{mit} rise ceased but it went back to baseline only after EIPA washout. A final control application of glutamate showed that the effects of EIPA were reversible. Representative responses of one cell out of 86 from 13 experiments.

buildup favored by the large electrochemical gradient. To test whether this is the case in situ we used the EIPA, an inhibitor of both plasma membrane and mitochondrial Na^+/H^+ exchangers (Sastrasinh et al., 1995). In this series of experiments, a first glutamate application led to the Na^+_{cyt} response described in detail previously (Chatton et al., 2000) and to a simultaneous Na^+_{mit} response (Fig. 7). When the Na^+/H^+ exchanger inhibitor EIPA was applied, Na^+_{cyt} rapidly decreased to a lower steady-state, compatible with the cessation of a cell membrane transport system using the transmembrane Na^+ gradient. On the contrary, at the same time Na^+_{mit} started to steadily increase, consistent with the inhibition of a transport system responsible of maintaining a low Na^+_{mit} level against the large electrochemical gradient favoring influx of Na^+ into mitochondria. When glutamate was applied, the slope of this Na^+_{mit} increase was abruptly accelerated by a factor of ~3 that only ceased when glutamate was washed out, whereas a cytosolic Na^+ response of low amplitude but regular kinetics was evoked. It is conceivable that with their Na^+ extrusion mechanism inhibited, mitochondria become a significant sink for cellular Na^+, explaining both the initial Na^+_{cyt} decline and the low amplitude response to glutamate. Both Na^+_{mit} and Na^+_{cyt} recovered to their respective basal levels after EIPA washout and a final control glutamate application led to a similar response as the first one, demonstrating the reversibility of effects of EIPA. Na^+_{mit} is therefore dynamically regulated in living astrocytes by the continuous activity of mitochondrial Na^+/H^+ exchangers.

DISCUSSION

Astrocytes express a high density of Na^+-coupled glutamate transporters, enabling them to play the essential role of clearing up synaptically released glutamate from the extracellular space (Danbolt, 2001). This extremely efficient transporter system endow them with the property of experiencing large amplitude intracellular Na^+ variations, rising to up to 30–40 mM from a resting

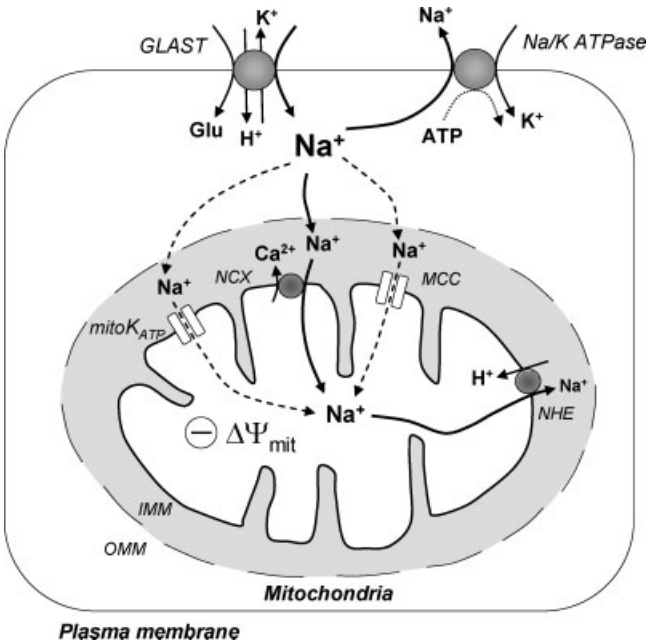

Fig. 8. Glutamate-induced Na^+ elevations in astrocytes. Schematic model of astrocytic Na^+ regulation in response to glutamate (Glu). The major cellular Na^+ influx pathways in response to glutamate application are due to Na^+-dependent glutamate transporters (GLAST). Glutamate also activates non-NMDA receptors that induce only minor Na^+ entry as compared to transporters (not shown). Cytosolic Na^+_{cyt} is mainly regulated by the Na^+/K^+-ATPase. Na^+ also enters mitochondria after crossing the highly permeable outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), driven by the highly negative electrical potential ($\Delta\psi_{mit}$). The mitochondrial entry of Na^+ involves its exchange with Ca^{2+} by the mitochondrial Na^+/Ca^{2+} exchangers (NCX). In addition, other entry pathways, including several mitochondrial cation conductances (MCC), or mitochondrial K_{ATP} channels ($mitoK_{ATP}$) may be involved. The mitochondrial Na^+/H^+ exchangers (NHE) is the main mechanism of Na^+ extrusion from mitochondria.

value of ~10 mM in the presence of physiological concentrations of glutamate (Chatton et al., 2000). The present study demonstrates that these robust Na^+ changes occurring in the cytosol of astrocytes propagate to the mitochondrial matrix with very similar kinetics. This was observed for glutamate concentrations compatible with values expected in the extrasynaptic space during activity (Dzubay and Jahr, 1999).

The study of Na^+_{mit} has been so far hampered by the lack of Na^+-sensitive probe with mitochondrial specificity. We show in the present study that the newly released fluorescent probe CR is selective for Na^+ and specifically localizes in mitochondria of astrocytes. Resting Na^+_{mit} concentration was estimated to be ~19 mM, *i.e.* moderately higher than the 13 mM cytosolic Na^+ concentration found in the present study, which falls in the range of values (10–15 mM) that we previously reported for astrocytes (Chatton et al., 2000, 2003). In response to glutamate application, Na^+_{mit} increased to ~30 mM, a value similar to the one observed in the cytosol. To our knowledge, Na^+_{mit} concentrations had not been reported before for astrocytes. However, studies using electron probe X-ray microanalysis of freeze-dried cryosections of CA3 hippocampal dendrites (Pivovarova et al., 2002) have reported resting mitochondrial Na^+ levels of 25 mmol/kg dry weight. When adjusted to the units used in the present study (*i.e.* ~11–12 mmol/L), these values are in a similar range as what we found for astrocyte mitochondria. In comparison, resting Na^+_{mit} was recently estimated to be ~50 mM in MDCK cells (Baron et al., 2005). Lower Na^+_{mit} values of 9.8 mM were estimated in intact cardiomyocytes (Yang et al., 2004) and even lower resting Na^+_{mit} concentration (~5 mM) were reported from experiments using SBFI in permeabilized ventricular cardiomyocytes (Donoso et al., 1992).

To enter the mitochondrial matrix, Na^+ has to cross two membranes in series (Fig. 8), the outer and the inner mitochondrial membranes. In resting conditions, the outer mitochondrial membrane is thought to present a high permeability to small molecules and ions, because of the presence of voltage-dependent anion channel (VDAC) also called mitochondrial porin (Shoshan-Barmatz and Gincel, 2003). Several mitochondrial transporters and conductances could mediate the Na^+ influx across the inner mitochondrial membrane that contains several cation conductances such as mitochondrial K^+ channels or Ca^{2+} channels with broad ion selectivity (Bernardi, 1999; Brierley et al., 1994). However, none of the pharmacological blockers of these cation conductances could convincingly prevent the Na^+_{mit} response to glutamate. One cannot exclude that some of the compounds used here on intact cells had a poor, if any, access to their mitochondrial target or that this Na^+ influx is simultaneously mediated by several entry pathways.

Another potential pathway for mitochondrial Na^+ entry is the mitochondrial Na^+/Ca^{2+} exchanger (Bernardi, 1999). Indeed, Na^+_{mit} has been so far attributed a main role in the regulation of intramitochondrial Ca^{2+}, through the activity of the mitochondrial Na^+/Ca^{2+} exchanger (Bers et al., 2003; Brierley et al., 1994). For this reason, Na^+_{mit} has attracted the attention of cardiac myocyte research because these cells experience large cytosolic Ca^{2+} changes that are transmitted to their mitochondria (Bers et al., 2003). In these cells, Na^+_{mit} overload has been reported following H_2O_2-induced oxidative stress (Yang et al., 2004). In axon terminals, it has been shown that mitochondria could act either as Ca^{2+} sinks (Scotti et al., 1998) or as Ca^{2+} reservoirs mobilized by cellular Na^+ influx using mitochondrial Na^+/Ca^{2+} as the route for mitochondrial Ca^{2+} efflux (Yang et al., 2003). We show in the present study that Ca^{2+}, probably by exchange with Na^+, is implicated in the Na^+ entry across the inner mitochondrial membrane. Indeed, the described inhibitor of this exchanger (CGP-37157) significantly diminished the Na^+_{mit} response to glutamate and chelating intracellular Ca^{2+} inhibited the Na^+_{mit} response by ~62%, without affecting the Na^+_{cyt} response to glutamate. The fact that CGP-37157 had a modest inhibitory effect of ~20% could indicate a limited access of the compound to the mitochondria in situ or could support the notion that more than one pathway contributes to the

influx of Na^+ into mitochondria. Another evidence that supports the involvement of Ca^{2+} is that rapid intracellular Ca^{2+} photorelease in astrocytes caused a Na^+_{mit} response.

With an electrical potential as negative as −150 to −180 mV, mitochondria tend to attract cations such as Na^+, which could accumulate to concentrations higher than 1 M (Bernardi, 1999) if no regulatory mechanisms were present. It has been proposed that resting cardiac myocytes maintain a low Na^+_{mit} (Donoso et al., 1992; Jung et al., 1992) that allow them to extrude Ca^{2+} ions from the mitochondrial matrix using the mitochondrial Na^+/Ca^{2+} exchanger activity. The mitochondrial Na^+/H^+ antiporter is the likely mechanism responsible for maintaining the Na^+ electrochemical gradient across the inner mitochondrial membrane (Bers et al., 2003; Brierley et al., 1994) by using the proton gradient actively generated by the respiratory chain (Mitchell, 1979). The mitochondrial Na^+/H^+ exchanger probably belongs to the NHE family of transporters, but presents different functional properties from its corresponding antiport in the plasma membrane (Brierley et al., 1994). In particular, it does not appear to be inhibited by the plasma membrane NHE inhibitor amiloride but is strongly inhibited by its analogue EIPA (Brierley et al., 1994; Sastrasinh et al., 1995). The present study provides evidence that in intact astrocytes, mitochondrial Na^+/H^+ dynamically prevents the excessive increase in Na^+_{mit} and maintains it in a dynamic steady-state with a resting level close to the Na^+_{cyt} concentration.

Astrocytes can generate massive Na^+_{cyt} increases caused by Na^+/glutamate uptake, one of the most prominent astrocytic functions (Danbolt, 2001). Besides preventing the excitotoxic buildup of interstitial glutamate during activity, this transport plays a pivotal role in coupling excitatory neuronal activity with energy metabolism (Magistretti et al., 1999). The increased Na^+_{cyt} activates the plasma membrane Na^+/K^+ ATPase, which more than doubles its activity (Chatton et al., 2000), along with its associated ATP consumption, leading to a substantial decrease in ATP levels (Chatton and Magistretti, 2005). The purpose of this ability of mitochondria to sense Na^+_{cyt} increases is yet to be clarified and could be different from that of regulating mitochondrial Ca^{2+} levels as proposed for other cell types. In particular, although astrocytes possess a substantial density of mitochondria, glutamate uptake enhances the formation of lactate from aerobic glycolysis (Pellerin and Magistretti, 1994). Evidence also supports this conclusion in acute hippocampal slices (Kasischke et al., 2004). One can speculate whether the increased Na^+ influx into mitochondria plays a role in modulating the metabolic response of astrocytes, as the associated activation of mitochondrial Na^+/H^+ antiporters, which occurs to the expense of the H^+ gradient generated by the respiratory chain, could weaken mitochondrial production of ATP. In contrast, Ca^{2+} that is shown in the present study to be intimately linked to the Na^+_{mit} response is known to stimulate mitochondrial oxidative phosphorylation (Gunter et al., 2004). Thus, the impact of Na^+_{mit} increases on the overall cellular energy metabolism and its relevance for the metabolic responses in the context of neurometabolic coupling has to be evaluated.

Little, if anything, was known on astrocytic Na^+_{mit} and on the contribution of mitochondria to the overall intracellular Na^+ homeostasis. By simultaneous in situ fluorescence imaging of mitochondrial and cytosolic Na^+, the present study identifies Na^+ as a signal dynamically transmitted to mitochondria and describes the mechanisms involved in its regulation.

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