Essential Role of Aralar in the Transduction of Small Ca\(^{2+}\) Signals to Neuronal Mitochondria*

Received for publication, July 5, 2005, and in revised form, October 26, 2005. Published, JBC Papers in Press, November 3, 2005, DOI 10.1074/jbc.M507270200

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Aralar, the neuronal \(Ca^{2+}\)-binding mitochondrial aspartate-glutamate carrier, has \(Ca^{2+}\) binding domains facing the extramitochondrial space and functions in the malate-aspartate NADH shuttle (MAS). Here we showed that MAS activity in brain mitochondria is stimulated by extramitochondrial \(Ca^{2+}\) with \(S_0.5\) of 324 nM. By employing primary neuronal cultures from control and aralar-deficient mice and NAD(P)H imaging with two-photon excitation microscopy, we showed that lactate utilization involves a substantial transfer of NAD(P)H to mitochondria in control but not aralar-deficient neurons, in agreement with the lack of MAS activity associated with aralar deficiency. The increase in mitochondrial NAD(P)H was greatly potentiated by large [\(Ca^{2+}\)] signals both in control and aralar-deficient neurons, showing that these large signals activate the \(Ca^{2+}\) uniporter and mitochondrial dehydrogenases but not MAS activity. On the other hand, small [\(Ca^{2+}\)] signals potentiate the increase in mitochondrial NAD(P)H only in control but not in aralar-deficient neurons. We concluded that neuronal MAS activity is selectively activated by small \(Ca^{2+}\) signals that fall below the activation range of the \(Ca^{2+}\) uniporter and plays an essential role in mitochondrial \(Ca^{2+}\) signaling.

\(Ca^{2+}\) signaling in mitochondria is mainly achieved through the entry of \(Ca^{2+}\) across the \(Ca^{2+}\) uniporter, a highly selective \(Ca^{2+}\) channel whose identity remains unknown (1, 2). Because of its apparent low affinity for \(Ca^{2+}\), the fact that there is a substantial mitochondrial \(Ca^{2+}\) uptake despite modest changes in average [\(Ca^{2+}\)], is explained by the strategic location of mitochondria close to the endoplasmic reticulum \(Ca^{2+}\) release channels, or plasma membrane \(Ca^{2+}\) channels, and therefore to microdomains of very high [\(Ca^{2+}\)], (3–6).

In mitochondria, \(Ca^{2+}\) activates pyruvate, isocitrate, and \(\alpha\)-ketoglutarate dehydrogenases, resulting in an increase in the mitochondrial NADH/NAD ratio (7). The \(Ca^{2+}\) uniporter-mitochondrial dehydrogenases signaling pathway is very relevant for cell and neuronal function (8–13). However, whether this is the only mechanism to transduce \(Ca^{2+}\) signals to mitochondria has never been assessed. In fact, in studies where changes in NAD(P)H, [\(Ca^{2+}\)], and [\(Ca^{2+}\)] have all been measured in parallel, it was found that mitochondrial NAD(P)H levels did not closely match those of [\(Ca^{2+}\)] (12, 14–17).

The identification of aralar (aralar1 (18, 19)) as the brain isofrom of the \(Ca^{2+}\)-dependent aspartate-glutamate mitochondrial carrier (AGC)\(^{5}\), SLC25A12 (20) opens up the possibility of an alternative way to transduce \(Ca^{2+}\) signals to neuronal mitochondria. Indeed, the \(Ca^{2+}\) binding domains of aralar face the mitochondrial intermembrane space, and increases in [\(Ca^{2+}\)], result in the activation of aspartate-glutamate exchange, in the absence of calcium entry in mitochondria (20).

The AGCs are one of the transporters responsible for the malate-aspartate NADH shuttle (MAS). Because of the electrogenic nature of Asp/Glu exchange (20, 21), the AGC reaction is irreversible under physiological conditions and thus a potential site for regulation. The first aim of this work was to explore the potential of aralar as a brain AGC isoform to regulate MAS activity in brain at low \(Ca^{2+}\) concentrations, below those required for the function of the mitochondrial \(Ca^{2+}\) uniporter. MAS activity in brain mitochondria was found to have \(Ca^{2+}\) activation properties adequate for this purpose.

The second aim of this work was to study the role of the aralar-MAS pathway in the supply of reducing equivalents to neuronal mitochondria. Aralar is expressed postnatally in rat and mouse brain, and it is located in neurons. Both MAS activity and aralar expression are acquired in parallel during neuronal maturation (22, 23). Aralar is important for neuronal function as underscored by the finding that alterations in aralar gene and protein are associated with central nervous system diseases such as Mohr-Tranebaerg syndrome, in which there is an impaired targeting of aralar to mitochondria (24), and autism (25). Aralar null mice also exhibit prominent motor coordination defects along with deficient myelination (26).

We have employed primary neuronal cultures from control and aralar-deficient mice (26) and two-photon excitation microscopy imaging of NAD(P)H to monitor the transfer of reducing equivalents from cytosol to mitochondria. We show that MAS is the main pathway to transfer reducing equivalents to neuronal mitochondria. High [\(Ca^{2+}\)] signals activate the \(Ca^{2+}\) uniporter-mitochondrial dehydrogenases signaling pathway, whereas small [\(Ca^{2+}\)] signals selectively activate MAS activity in neurons. We conclude that the aralar-MAS pathway plays an

\(^{5}\) The abbreviations used are: AGC, aspartate/glutamate carrier; MAS, malate-aspartate NADH shuttle; MSK, malate/sucrose/K\(^+\); medium; LM, lactate + malate; IP\(_3\), inositol 1,4,5-trisphosphate; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid; ANOVA, analysis of variance; mit/cyt, mitochondrial/cytosolic; RR, ruthenium red; \(\alpha\)-KG, \(\alpha\)-ketoglutarate dehydrogenase; \(\alpha\)-KG, \(\alpha\)-ketoglutarate.
essential role to transduce small \([\text{Ca}^{2+}]\), signals into neuronal mitochondrial energization.

**MATERIALS AND METHODS**

**Animals**—Male Wistar rats (3 months old) were used to prepare rat brain and skeletal muscle mitochondria. SVJ129/C57BL mice carrying a deficiency for aralar expression, Aralar\(^{-/-}\), Aralar\(^{+/+}\), and Aralar\(^{+/+}\), which had been obtained from Lexicon Genetics Inc. (The Woodlands, TX) (26), were used to obtain primary neuronal cultures.

**Genotypes**—Genotype was determined by PCR using genomic DNA obtained from tail or embryonic tissue samples (Nucleospin tissue kit, Macherey-Nagel). The following primers were used for genomic DNA amplification: sense primer mAra3-GATGTGAGAACTCACCAGTGT-3\(^{-}\) that detects only mutated alleles, sense primer mAra int 13F1 (5\'-GTGTTCTCTAGAACTGCTGAGG-3\(^{-}\)) that detects wild-type alleles, and antisense primer mAra int 13B (5\'-ACCCACACGGCTGTACAGC-3\(^{-}\)) that detects both wild-type and mutant alleles (data not shown). PCR mixtures were preincubated at 94 °C for 5 min, followed by 35 cycles of DNA amplification at 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 60 s; the process was finished with an extension at 72 °C for 5 min. Whole rat brains were homogenized in 250 mM sucrose, 25 mM Hepes, 100 mM potassium glutamate, 100 mM sucrose, 10 mM Tris, 0.1% BSA, pH 7.4 (mannitol/sucrose/KCl, 0.5 mM EDTA, 100 mM KCl, 0.1% BSA, 0.4 mg/ml papain, and 6 mM glucose) and then mechanically dissociated, in the presence of DNase, by using glass pipettes of different sizes. Cerebral cortices were removed free of meninges, cut into small pieces, dissociated, in the presence of DNase, by using glass pipettes of different sizes. Cultures were suspended (16 mg of protein/ml) in a glutamate-containing medium (100 mM potassium glutamate, 100 mM sucrose, 10 mM Tris, 0.1% BSA, pH 7.4) for 10 min at 0 °C to allow for glutamate accumulation in mitochondria. Then the suspension was diluted 1:10 in 320 mM sucrose, 1 mM EDTA, 0.1% BSA, 10 mM Tris, pH 7.4, and centrifuged (10,000 \(\times\) g, 5 min). Glutamate-loaded mitochondria were washed with the same medium and resuspended (7 mg of protein/ml) in an Asp-loading medium (150 mM KCl, 10 mM MOPS, 0.1% BSA, pH 7.4). Asp loading was started by adding 3 mM oxaloacetate, and after 3 min at 28 °C, the incubation was stopped (0 °C, 5 min of centrifugation at 10,000 \(\times\) g). Asp-loaded mitochondria were washed with Asp-loading medium and resuspended in MSK to assay MAS activity.

**Ca\(^{2+}\) uptake** in brain and muscle mitochondria preparations was measured in the presence of 0.1 \(\mu\)M Ca\(^{2+}\)-Green 5N (excitation 506 nm, emission 532 nm) in modified MSK (without EDTA and supplemented with 1 mM MgCl\(_2\)). Experiments were performed at 25 °C under constant stirring, with ADP (0.2 mM), glutamate (5 mM), and malate (5 mM) as respiratory substrates. Measurements were started by the addition of mitochondria (to a final concentration of 0.5 mg/ml protein; not shown). After 4 min of preincubation, a first addition of Ca\(^{2+}\) (50 \(\mu\)M) was made, followed by sequential additions of 20 \(\mu\)M Ca\(^{2+}\) as indicated. Where appropriate, 200 nM ruthenium red was added.

**Neuronal Cultures**—Cortical neuronal cultures were prepared from 16-day-old mouse embryos, following the procedure used for rat embryos (23, 30) modified as follows. Mouse embryos were obtained from crosses between SVJ129/C57BL6 Aralar\(^{-/-}\) mice, and nonbrain tissue was used for determination of DNA genotype of every embryo. Because the number of Aralar\(^{-/-}\) embryos doubled that of Aralar\(^{+/+}\), Aralar\(^{+/+}\) mice were employed as controls, unless indicated otherwise.

Cerebral cortices were removed free of meninges, cut into small pieces, and enzymatically dissociated in phosphate-buffered saline containing 1% BSA, 0.4 mg/ml papain, and 6 mM glucose and then mechanically dissociated, in the presence of DNase, by using glass pipettes of different pore size. Dissociated cells were collected by centrifugation (800 \(\times\) g, 5min) and seeded in medium containing 20% horse serum for 3 h. After this time, medium was completely replaced with serum-free B27-supplemented Neurobasal medium (31). The culture medium was partially replaced every 2nd day. Cells were plated at a density of 1 \(\times\) 10\(^5\) cells/cm\(^2\) on poly-L-lysine- and laminin-coated pretreated glass cover slips for the determination of \([\text{Ca}^{2+}]\), (30), or on glass coverslips sealed at the bottom of plastic wells (4-well LabTek cover slides, NUNC, Roskilde, Denmark), for two-photon microscopy. The different cell types in these neuronal cultures were characterized with specific antibodies as described earlier (30, 32). Under the present culture conditions, neurons represented more than 90% of the total cell population. Cultures were used for experimentation between 9 and 11 days *in vitro*.

**Two-photon Microscopy in Neuronal Cultures**—Neuronal cultures, seeded on 4-well LabTek cover slides, were washed once and incubated in glucose-free HCSS (1 mM CaCl\(_2\), 120 mM NaCl, 0.8 mM MgCl\(_2\), 25 mM Hepes, 5.4 mM KCl, pH 7.4) for 1 h before experimentation. When Ca\(^{2+}\)-free conditions were needed, cells were washed once and maintained with Ca\(^{2+}\)-free HCSS in the presence of 100 \(\mu\)M EGTA. Two-photon excitation microscopy was performed by using Olympus IX70 inverted microscope with an \(\times\)60 Plan Apo NA 1.4 objective, coupled to a Bio-Rad Radiance 2000 MP confocal/multiphoton microscopy system. An infrared multiphoton laser (Coherent Mira 690–1000 nm) provided excitation of intrinsic NADH fluorescence. The laser set-
tings used for imaging were as follows: 4 milliwatts of power, with a neutral filter set to reduce the excitation light in 80%, and long wave at 735 nm with 150-fs pulses. Images were collected with a 390–460 nm emission filter. Cells were maintained at 37 °C with a temperature-controlled microscopic stage. Additions (0.1 ml) were made as a bolus to the cell chamber (initial volume, 0.5 ml).

Images (averages of three frames, 512 × 512 pixels per frame, 0.4 μm/pixel) were taken every 5 or 10 s for about 2–5 min. Image analysis was carried out with MetaMorph (Universal Imaging). Mitochondrial and cytosolic intensities were determined in individual cells or groups of cells following procedures used in islet beta cells (33, 34). Because of their movement in and out of the focal plane, a particular mitochondria may not be imaged at all time points. Thus, the analysis was carried out only on those mitochondria that stood in the focal plane at each time point. Selected regions (with 4–10 cells) or individual cells were outlined, and intensity thresholds were set that highlighted the bright areas shown to correspond to in-focus mitochondria (from Mitotracker labeling, see below). With these thresholds, which may underestimate the mitochondrial fluorescence but avoid contamination from the cytoplasmic compartment, the intensity of these bright areas was calculated. To estimate the cytoplasmic fluorescence, intensity thresholds were set in order to include the dim areas within cells and to exclude mitochondrial fluorescence. This probably entails an overestimation of the cytosol because of the dense neurite network where neurites and background are hard to tell apart.

Changes in NAD(P)H fluorescence were then calculated as mitochondrial/cytosolic (mit/cyt) NAD(P)H ratios or as normalized fluorescence values (cyt/cyt<sub>i</sub>, mit/mit<sub>i</sub>). By using a more restrictive estimation of the cytosol, mit/cyt NAD(P)H ratios were increased, but the differences between genotypes or stimuli were maintained.

In each case, measurements to determine changes in NAD(P)H autofluorescence were performed in at least three independent experiments. Statistical significance was calculated for every experimental condition (genotype and/or stimuli applied) by using time course data sets of mit/cyt NAD(P)H fluorescence ratios and one-way analysis of variance (ANOVA). Thereafter, time courses for mit/cyt NAD(P)H fluorescence ratios obtained from different genotypes or stimuli were compared by a two-factor ANOVA test. The statistically significant results of the previous comparisons are indicated in the figure legends. In addition, data at the indicated time points were used to calculate the reducing equivalent transfer ratio (quotient between maximum and initial mit/cyt NAD(P)H ratios) and were analyzed for significance (unpaired two-tailed t test or Bonferroni’s test).

To label mitochondria with MitoTracker, neurons were first incubated with lactate and malate to reduce mitochondrial NAD(P)H. After capturing the NAD(P)H fluorescence images, they were then incubated bated with lactate and malate to reduce mitochondrial NAD(P)H. After

The activity of the glycerol phosphate NADH shuttle (36). To study the variations of Ca<sup>2+</sup> concentrations in mitochondria in single neurons, individual cells were outlined manually, and the variations in fluorescence were expressed as F/F<sub>0</sub>, where F is the emitted fluorescence at any given time, and F<sub>0</sub> is the fluorescence of the same zone at time = 0.

RESULTS

Ca<sup>2+</sup> Activation of the Malate-Aspartate NADH Shuttle in Brain Mitochondria—MAS activity in brain mitochondria, measured as a decrease in NADH in the incubation medium, was dependent upon Glu addition, and under our assay conditions, it did not require Asp-preloading of mitochondria (Fig. 1, A and B). MAS activity was completely absent in brain mitochondrial fractions from Aralar<sup>−/−</sup> mice (26). In addition, respiration on glutamate plus malate was extremely reduced in skeletal muscle mitochondria with no compensatory increase in respiration with pyruvate plus malate (26). Similarly, the lack of citrin, the liver AGC isoform, is not accompanied by compensatory increases in the activity of the glyceral phosphate NADH shuttle (36).

To study Ca<sup>2+</sup> activation of MAS, shuttle activity was measured after the addition of calibrated Ca<sup>2+</sup> loads in the presence of 200 nm ruthenium red (RR) to block Ca<sup>2+</sup> uptake through the Ca<sup>2+</sup> uniporter. Under these conditions, any activation of the shuttle can be attributed to the effect of Ca<sup>2+</sup> on the external face of the inner mitochondrial membrane, where the Ca<sup>2+</sup> binding domains of aralar are located (20).

Fig. 1, C and D, shows that MAS activity increased with Ca<sup>2+</sup>. The maximal activation was about 3.1-fold (from 26.7 ± 5.28 to 85.18 ± 10.4 nmol × mg protein<sup>−1</sup> × min<sup>−1</sup>, in the absence of Ca<sup>2+</sup> or at saturating Ca<sup>2+</sup> concentrations, respectively) with an S<sub>0.5</sub> of 324 ± 114 nm. A similar increase in activity (2.55 ± 0.57-fold) was observed when the assay was carried out with aspartate-preloaded mitochondria. The S<sub>0.5</sub> value for Ca<sup>2+</sup> activation is substantially lower than that of the Ca<sup>2+</sup> uniporter of mitochondria (apparent K<sub>m</sub> about 10–20 μM (2, 37)), suggesting that Ca<sup>2+</sup> activation of the shuttle may contribute to NADH reduction in mitochondria at Ca<sup>2+</sup> concentrations where the Ca<sup>2+</sup> uniporter is still inactive.
Aralar Transduces Small Ca\(^{2+}\) Signals to Mitochondria

At the subcellular level, autofluorescence was found to concentrate in bright punctate regions stained with Mitotracker (Fig. 2B) that were presumed to be mitochondria. The LM-induced increases in fluorescence of mitochondria, and the neighboring cytoplasm, are represented in Fig. 2, C–F. As observed, the addition of LM resulted in an increase in the two regions, with individual variation of the responses among cells (Fig. 2, C and E). This was expected because lactate entering the cells via neuronal monocarboxylate carriers (38) would immediately increase the cytosolic NADH/NAD level through the lactate dehydrogenase reaction. However, mitochondria had the greatest increase in NAD(P)H fluorescence (note the different scales). We have not quantified the actual increase in NAD(P)H in mitochondria or cytosol, because it may involve both free but mostly bound NAD(P)H, and the fluorescence of these two forms is very different, about 10-fold higher when bound (39, 40).

In order to test that cytosolic and mitochondrial fluorescence signals were adequately separated in the imaging experiments, we studied the response to rotenone, which has selective effects on mitochondrial NAD(P)H. Fig. 2G shows that rotenone addition results in a very large increase in normalized mitochondrial fluorescence, with no changes in cytoplasmic fluorescence, indicating that contamination between mitochondrial and cytosolic signals is below detection in our current set up.

Lack of Increase in Mitochondrial NAD(P)H in Response to Lactate in Aralar-deficient Neurons—To study the role of aralar, the response to LM addition was studied in control and aralar-deficient neuronal cultures derived from littermate embryos in Ca\(^{2+}\)-free media and in the presence of 1 mM Ca\(^{2+}\). Fig. 3A shows the changes in the mit/cyt NAD(P)H fluorescence ratio in response to LM (added at the arrow) in Ca\(^{2+}\)-free media. The changes in normalized mitochondrial fluorescence ratio are shown in Fig. 3B. Control neurons showed varied responses to LM and reached a plateau at about 1 min after LM addition (Fig. 3A). In contrast, neurons from aralar-deficient mice showed a very modest rise in both the mit/cyt and normalized mitochondrial fluorescence ratio (Fig. 3, A and B). These changes were essentially the same in the presence of Ca\(^{2+}\). In fact, the reducing equivalent transfer (see 'Materials and Methods') was not statistically different in the presence of 1 mM Ca\(^{2+}\) or in Ca\(^{2+}\)-free media both in control (n = 30–180 neurons, p > 0.6) and aralar-deficient neurons (n = 30–180, p > 0.8). [Ca\(^{2+}\)]\(_{i}\) was not modified by LM addition either in the absence or presence of Ca\(^{2+}\) (results not shown), indicating that MAS activity is activated solely by the rise in cytosolic NADH obtained upon lactate entry.

Lactate gives rise to pyruvate through lactate dehydrogenase. Therefore, the increase in mitochondrial NAD(P)H may be due to NADH shuttle activity and to the entry and oxidation of pyruvate in mitochondria. To determine the contribution of lactate-derived pyruvate oxidation in the increase in mitochondrial NAD(P)H, we have studied the responses to pyruvate plus malate in control and aralar-deficient neurons. Two and 10 mM (not shown) pyruvate (plus 5 mM malate) induced concentration-dependent increases in the mit/cyt NAD(P)H fluorescence ratio, which were similar in control and aralar-deficient neurons (Fig. 3C).

Because the responses to pyruvate and the internal pyruvate concentrations observed 2 min after LM addition (nmol/5 × 10\(^6\) cells) were the same in control (0.79 ± 0.09) and aralar-deficient (1.08 ± 0.18) neurons, the blunted response to LM caused by aralar deficiency is clearly due to a decreased transfer of reducing equivalents from cytosolic NADH to mitochondria. Although a small and nonsignificant increase in cytosolic NAD(P)H fluorescence has been observed in aralar-deficient neurons (results not shown), its contribution to the blunted response to LM is probably a minor one, as the changes in mitochondrial/cytosolic or

Two-photon Measurement of Lactate-induced Changes in NAD(P)H in Cultured Neurons—Primary cortical neuronal cultures from Aralar\(^{-/-}\) mouse embryos used as control were equilibrated with glucose-free HCSS for 1 h and then switched to glucose- and Ca\(^{2+}\)-free HCSS, 100 μM EGTA, to start autofluorescence imaging. After acquiring 2 images (one every 10 s), 20 mM lactate + 5 mM malate (LM) was added to the incubation chamber, and fluorescence was recorded for 2–5 min.

The total fluorescence of the neurons (average of 30 cells) increased markedly after LM addition (Fig. 2, A and B), and this increase was not observed when vehicle was added (Fig. 2A), indicating that it was LM-dependent. There were hardly any changes in total, mitochondrial, or cytosolic fluorescence in vehicle-exposed neurons (Fig. 2A and data not shown), indicating that photobleaching in these experiments was very low.

![FIGURE 1. Ca\(^{2+}\) activation of the MAS activity in brain mitochondria. A and B, MAS activity in rat brain mitochondrial preparations subjected to aspartate-preloading (B) or under control conditions (A) assayed in the presence (+ Asp) or absence (No Asp) of 5 mM aspartate. 5 mM glutamate (Glut) is added where indicated. The presence of Asp in the external medium reduced the time lag required to obtain the maximal slope (compare traces No Asp and + Asp in A and B) but did not modify the final activity obtained (compare final slopes in A and B). Asp-preloading did not change the activity of the shuttle (compare traces + Asp in A and B). C, MAS activity at the free Ca\(^{2+}\) concentrations (μM) indicated. Traces in A–C correspond to representative experiments. D, kinetics of Ca\(^{2+}\)-activation of MAS activity. Data are mean ± S.E. of three experiments performed in triplicate. E, Ca\(^{2+}\)-activation of malate-aspartate NADH shuttle and mitochondrial dehydrogenases. See "Discussion" for details. AcCoA, acetyl-CoA; CU, Ca\(^{2+}\)-uniporter; Glut, glutamate; Isoc, isocitrate; αKG, α-ketoglutarate; Mal, malate; OAA, oxalacetic acid; OGC, α-ketoglutarate/malate carrier; Pyr, pyruvate; SuccCoA, succinyl-CoA; step 1, aspartate aminotransferase (AAT); step 2, malate dehydrogenase (MDH); step 3, pyruvate dehydrogenase (PDH); step 4, isoocotate dehydrogenase (IDH); step 5, α-KGDH.](image-url)
normalized mitochondrial fluorescence ratios are the same (Fig. 3, A and B).

Reducing equivalent transfer at 70 s after LM addition in Ca\(^{2+}\)-free medium (quotient between mit/cyt NAD(P)H ratios at 70 s and initial values) was 1.76 ± 0.03 in control and 1.3 ± 0.01 in aralar-deficient neurons (mean ± S.E. of 72–180 neurons, \(p < 0.0001\); ANOVA followed by unpaired two-tailed \(t\) test). Similar results were obtained in the presence of 1 mM Ca\(^{2+}\) (reducing equivalent transfer was 1.82 ± 0.11 and 1.29 ± 0.16, in control and aralar-deficient neurons, \(n = 30–100\) neurons, \(p < 0.05\), unpaired \(t\) test). The contribution to this increase of pyruvate (lactate-derived) oxidation can be estimated from the values in aralar-deficient neurons, and it represents about 40% of the increase, with MAS activity corresponding to the remaining 60%. It is unlikely that glycerol 3-phosphate shuttle activity contributes significantly to this process, because it is relatively small in neurons (41). It donates electrons to complex III, and any change in mitochondrial NAD(P)H level from that activity would depend on reverse electron transport. Thus, MAS activity is a major pathway for lactate-dependent reduction of mitochondrial NAD(P)H in control neurons.

**Regulation of Mitochondrial NAD(P)H Response by Large Ca\(^{2+}\) Signals**—By having established that lactate-induced increase in reducing equivalent transfer to mitochondria is mostly due to MAS activity, we sought to determine the role of \([\text{Ca}^{2+}]_i\) in the regulation of shuttle activity.

To this end, we have studied the increase in mitochondrial NAD(P)H in neurons in response to two types of Ca\(^{2+}\) signals as follows: large \([\text{Ca}^{2+}]_i\) signals brought about by depolarization-dependent activation of voltage-operated Ca\(^{2+}\) channels in the presence of millimolar external Ca\(^{2+}\) concentrations, and small \([\text{Ca}^{2+}]_i\) signals, generated in a Ca\(^{2+}\)-free medium by Ca\(^{2+}\) mobilization via activation of IP\(_3\) receptors. In the first case, in addition to Ca\(^{2+}\)-mediated MAS activation, it was expected that voltage-operated Ca\(^{2+}\) channel-dependent Ca\(^{2+}\) entry would result in a large increase of intracellular Ca\(^{2+}\) concentration, which in turn would stimulate Ca\(^{2+}\)/Ca\(^{2+}\) release from the endoplasmic reticulum, further increasing the intracellular Ca\(^{2+}\) concentration.

**FIGURE 2.** NAD(P)H autofluorescence measured by two-photon excitation microscopy in neuronal cell cultures. Neuronal cell cultures from embryonic cortex of control, Aralar\(^{+/−}\), mice were preincubated in glucose-free HCSS, 1 mM CaCl\(_2\), for 1 h at 37 °C and then switched to Ca\(^{2+}\)- and glucose-free HCSS (100 μM EGTA). The initial autofluorescence of the cells revealed only a few bright spots per cell that corresponded to in-focus mitochondria. A, total NAD(P)H autofluorescence in 30 individual cells was registered before (20 s) and after 20 mM lactate plus 5 mM malate (LM) or vehicle (Ca\(^{2+}\)-free HCSS; \(n = 15\) cells) addition, as indicated by the arrow. Images were acquired every 10 s. B, NAD(P)H images of selected neurons immediately before (\(t = 0\)) and after 90 s of stimulation with LM and after MitoTracker Red CMXRos staining. Bar represents 20 μm. C-F, changes in normalized \(F/F_0\) mitochondrial and cytoplasmic NAD(P)H autofluorescence in eight individual cells (C and E) and mean ± S.E. values of 30 cells (D and F). LM addition is indicated by the arrow. G, changes in normalized mitochondrial (mit/mit\(_0\)) and cytoplasmic (cyt/cyt\(_0\)) fluorescence ratio in neuronal cultures exposed to 2 μM rotenone at \(t = 0\).
The mit/cyt NAD(P)H fluorescence ratio obtained in the presence of KCl (Fig. 4, D and E) was significantly larger than that obtained by LM addition itself both in control and aralar-deficient neurons (p ≤ 0.0001). Reducing equivalent transfer within 40–70 s after LM or LM + KCl addition was 1.89 ± 0.09 and 3.35 ± 0.35, respectively, in control neurons (n = 30–100, p < 0.0001) or 1.34 ± 0.16 and 2.77 ± 0.4 in aralar-deficient neurons (n = 30–100 neurons, p < 0.0019). Furthermore, the time course of the response to LM + KCl was the same in control and aralar-deficient neurons (p = 0.397).

To verify that the lack of difference between wild-type and aralar-deficient neurons was not due to NAD(P)H being maximally reduced in both cases, we compared the increases in mitochondria/cytosol NAD(P)H ratios obtained with LM plus high K+ with the NAD(P)H signals in mitochondria obtained in response to rotenone. By inhibiting electron flow through complex I, the addition of 2 μM rotenone plus LM induced a progressive increase in reducing equivalent transfer to mitochondria, as observed previously (13). The quotient between final and initial mit/cyt NAD(P)H ratio obtained after 2 min incubation with rotenone was 8.32 ± 0.62 (n = 36–90 neurons), i.e. substantially larger than those observed with LM plus high K+.

The increase in mitochondrial NAD(P)H obtained with LM plus high K+ is expected to result from the additive effects of Ca2+ activation of MAS activity and Ca2+ activation of mitochondrial dehydrogenases. The lack of differences in mitochondrial NAD(P)H increase between wild-type and aralar-deficient neurons under conditions appropriate for Ca2+ uniporter signaling indicates that MAS activity contributes very little, if at all, to this process, suggesting that MAS activity may be inhibited under conditions allowing mitochondrial Ca2+ uptake.

By studying MAS activity in isolated mitochondria in the presence or absence of 200 nM RR (Fig. 4F), it was observed that Ca2+ activation of MAS was abolished if no RR was present. At this RR concentration, Ca2+ uptake in brain mitochondria was fully inhibited (Fig. 4G). These results indicate that Ca2+ activation of MAS is blocked if the Ca2+ uniporter is active and Ca2+ is allowed to enter the mitochondria. We believe that this is because of the effect of mitochondrial Ca2+ on the affinity for α-KG of α-KGDH. This Krebs cycle dehydrogenase and the α-KG-malate carrier (OGC) compete for the substrate α-KG (Fig. 1E), so that Ca2+ activation of α-KGDH activity results in an increased affinity for α-KG and a decrease in α-KG efflux from mitochondria that would immediately oppose MAS activation, as shown by O’Donnell et al. (43, 44).

**Regulation of Reducing Equivalent Transfer to Mitochondria by Small Ca2+ Signals**—Lalo and Kostyuk (45) showed that ATP activation of metabotropic P2Y receptors present in neonatal neurons in a Ca2+-free medium results in small [Ca2+]i transients. To study the effect of small [Ca2+]i signals on MAS activity, neurons incubated in Ca2+-free HCSS plus 100 μM EGTA were exposed to LM together with 100 μM ATP or different Ca2+-mobilizing agonists. In our neuronal cultures, ATP-induced [Ca2+]i transients were much smaller than those obtained with high K+, with departures of ≤100 nM from resting values that lasted 1 min at most (Fig. 5A); these transients were not accompanied by any detectable increase in [Ca2+]mit in control or aralar-deficient neurons (Fig. 5B). Therefore, [Ca2+]mit variations are below the detection limit of rhod-2 (dissociation constant (Kd)) determined in situ in 1.3 μM mitochondria (46), which is somewhat lower than that of fura-2 (Kd 0.2 μM). However, this small Ca2+ signal resulted in a remarkable potentiation of LM-dependent increase in mitochondrial NAD(P)H fluorescence in control neurons (Fig. 5, C and D), which was notably absent in aralar-deficient neurons (Fig. 5, C and E). In control neurons, reducing equivalent transfer at 80 s after LM + ATP addition was 2.88 ± 0.076, and

![FIGURE 3. Lactate- and pyruvate-induced NAD(P)H responses in control and aralar-deficient neurons. A, control (closed symbols) and aralar-deficient neuronal cultures (open symbols) derived from littermate embryos were preincubated in glucose-free HCSS, 1 mM CaCl2 for 1 h at 37°C, switched to Ca2+- and glucose-free HCSS (100 μM EGTA), and exposed to 20 mM lactate plus 5 mM malate (LM) (added at the arrow). The changes in mitochondrial/cytosolic NAD(P)H fluorescence were computed within 18 regions containing 4–10 neurons/region. In controls, but not in aralar-deficient neurons, mit/cyt fluorescence ratios were greater than that at t = 0 (p < 0.0001, one-way ANOVA; from 60 s after LM addition onward, p < 0.05, Bonferroni’s test). Time course data sets of mit/cyt NAD(P)H ratios from control and aralar-deficient neurons were compared (p < 0.0001, two-factor factorial ANOVA). B, time course data sets of normalized mitochondrial (mit/mit0) fluorescence ratios from control and aralar-deficient neurons were also analyzed (p < 0.0001, two-factor factorial ANOVA). C, aralar-deficient and control neuronal cultures derived from littermate embryos were preincubated as above and exposed to 2 mM pyruvate + 5 mM malate (PM) (squares) added at the arrow, and the changes in mitochondrial/cytosolic NAD(P)H fluorescence ratios were computed.
Aralar Transduces Small Ca\textsuperscript{2+} Signals to Mitochondria

FIGURE 4. Effect of high K\textsuperscript{+} depolarization on lactate-induced increase in mitochondrial NAD(P)H fluorescence and malate-aspartate shuttle activity. A, NAD(P)H fluorescence images from control (Ctr) and aralar-deficient (KO) neurons incubated in glucose-free HCSS, 1 mM Ca\textsubscript{2+} before (Ctr–KCI and KO–KCI) and 60 s after LM + 60 mM KCl (Ctr + KCI and KO + KCI) administration. Bar represents 20 \mu m. B and C, [Ca\textsuperscript{2+}]\textsubscript{i} imaging (B) and mitochondrial Ca\textsuperscript{2+} dynamics (C) of the responses to LM + KCl in fura-2 (B) or rhod-2-loaded (C) cortical neuronal cultures from control (Ctr) and aralar-deficient (KO) mice. LM + KCl were added at the arrows. Each trace represents recordings from a single neuron. D and E, increase in mitochondrial/cytosolic NAD(P)H fluorescence ratio in control (D, closed symbols) or aralar-deficient (E, open symbols) neurons from littermate embryos after the addition of LM + KCl (the arrow indicates the time of addition). Each trace corresponds to a group of cells. In both genotypes, LM + KCl induced a significant increase in mitochondrial/cytosolic NAD(P)H fluorescence ratios, as compared with their corresponding LM time courses (p < 0.0001, two-factor factorial ANOVA). F, MAS activity in mouse brain mitochondrial fractions measured at different free Ca\textsuperscript{2+} concentrations in the presence (open bars) or absence (closed bars) of 200 \mu M ruthenium red (RR). Data are means of two independent experiments performed in triplicate. Differences from 0 Ca\textsuperscript{2+} were significant where indicated. *, p < 0.01; **, p < 0.001; ***p < 0.0001 (unpaired two-tailed t test).

FIGURE 5. Effect of small Ca\textsuperscript{2+} signals on lactate-induced increase in mitochondrial NAD(P)H fluorescence. A, [Ca\textsuperscript{2+}]\textsubscript{i} imaging of fura-2-loaded cortical neuronal cultures from control (Ctr) and aralar-deficient (KO) mice, exposed to LM + ATP in Ca\textsuperscript{2+}-free HCSS in the presence of 100 \mu M EGTA. LM + ATP were added at the arrows. Each trace represents the [Ca\textsuperscript{2+}]\textsubscript{i} from a single neuron. B, mitochondrial Ca\textsuperscript{2+} dynamics of the responses to LM + ATP in rhod-2-loaded cortical neuronal cultures from control (Ctr) and aralar-deficient (KO) mice. C, NAD(P)H fluorescence images corresponding to control (Ctr) and aralar-deficient (KO) neurons before (Ctr–ATP and KO–ATP) or 80 s after LM + ATP addition (Ctr + ATP and KO + ATP). Bar represents 20 \mu m. D–E, control (D) or aralar-deficient neurons from littermate embryos (E) were preincubated in glucose-free HCSS, 1 mM Ca\textsubscript{2+} for 1 h at 37°C and switched to Ca\textsuperscript{2+}- and glucose-free HCSS with 100 \mu M EGTA and LM (circles) or LM + 100 \mu M ATP (LM + ATP, squares) added at the arrow. Results correspond to mitochondrial/cytosolic NAD(P)H fluorescence ratios (means ± S.E. values) of 72–180 and 50–120 cells in control and aralar-deficient groups, respectively. Differences in mit/cyt NAD(P)H fluorescence ratios between incubations in the absence or presence of ATP were significant in control cells (D; p < 0.0001, two-factor factorial ANOVA) but not in aralar-deficient (E) neurons. F, changes in mitochondrial/cytosolic NAD(P)H fluorescence ratio in control neurons after the addition of 2 mM pyruvate + 5 mM malate, 2 mM pyruvate + 100 \mu M ATP, or 10 mM pyruvate + 5 mM malate (Pyr 10), or 10 mM pyruvate + 100 \mu M ATP (Pyr 10 + ATP), in Ca\textsuperscript{2+}-free HCSS in the presence of 100 \mu M EGTA (the arrow indicates the time of addition).
that obtained in the absence of ATP was 1.99 ± 0.15 (p < 0.0001), whereas ATP did not modify the responses to LM in aralar-deficient cells (1.38 ± 0.10 versus 1.63 ± 0.15, in the absence or presence of ATP, p = 0.16). Other Ca\(^{2+}\)-mobilizing agonists, such as carbachol (50 μM) or thapsigargin (1 μM), also potentiated in a significant way the response to LM in control neurons (two-factor factorial ANOVA, p < 0.0001). Reducing equivalent transfer at 80 s after LM addition was increased in the presence of carbachol (2.68 ± 0.2 versus 2.07 ± 0.15, n = 72–180 neurons, p = 0.02) and thapsigargin (2.35 ± 0.13 versus 1.92 ± 0.17, n = 44–110 neurons, p = 0.03).

Because lactate-derived pyruvate contributes to NAD(P)H generation, we have tested whether the effects of ATP involve Ca\(^{2+}\)-stimulated pyruvate metabolism rather than Ca\(^{2+}\)-stimulated MAS activity. Fig. 5F shows that the small \([Ca^{2+}i]\), signals triggered by ATP had no effect on the increase in mitochondrial NAD(P)H induced by pyruvate. Therefore, these results clearly showed that the neuronal aralar-MAS pathway is selectively activated by small Ca\(^{2+}\) signals, below the threshold for Ca\(^{2+}\) uniporter activation.

**DISCUSSION**

Our results show that brain mitochondria have a Ca\(^{2+}\)-dependent malate-aspartate NADH shuttle activated by low extramitochondrial Ca\(^{2+}\) concentrations, with an \(S_{0.5}\) for Ca\(^{2+}\) of 324 nM. In neurons, this property is exploited to transduce Ca\(^{2+}\) signals to mitochondria via the aralar-MAS pathway, by transferring NAD(P)H reducing equivalents from the cytosol. Quite remarkably, we find that small \([Ca^{2+}i]\), signals activate MAS activity under conditions where Ca\(^{2+}\) uptake in mitochondria is not observed. Moreover, these same \([Ca^{2+}i]\), signals do not activate the pyruvate-dependent reduction of NAD(P)H in mitochondria, a process dependent on the activity of the Ca\(^{2+}\) uniporter. We have observed that synaptosomal mitochondria start taking up Ca\(^{2+}\) at a global \([Ca^{2+}i]\), of about 350–400 nM (27). Clearly, Ca\(^{2+}\) signaling through the aralar-MAS pathway in neuronal mitochondria, with an \(S_{0.5}\) of 324 nM, would be only significant below these Ca\(^{2+}\) concentrations, which is exactly what we found. Our results show for the first time that mitochondria respond to \([Ca^{2+}i]\), signals with a substantial increase in NAD(P)H through a Ca\(^{2+}\) uniporter-independent pathway.

The Ca\(^{2+}\) uniporter-dehydrogenase signaling pathway results in a large activation of mitochondrial dehydrogenases (Ca\(^{2+}\) activation of about 4–5-fold for pyruvate dehydrogenase and α-KGDH (47, 48). However, Ca\(^{2+}\) activation of MAS is smaller (3-fold activation in brain mitochondria). Moreover, in the mitochondrial matrix three dehydrogenases are activated by Ca\(^{2+}\), whereas only one (malate dehydrogenase) is activated by the aralar-MAS pathway. It was very surprising to find that the magnitude of the response via the aralar-MAS pathway is very large in neurons, in fact not far from that evoked by the activity of the Ca\(^{2+}\) uniporter-dehydrogenases pathway.

**Ca\(^{2+}\) Activation of Malate-Aspartate NADH Shuttle in Neurons—** Lactate is produced by astrocytes and taken up by neurons that use it as an oxidizable substrate, particularly during periods of high activity (49–52). When neurons are supplied with lactate, cytosolic NAD(P)H levels increase, and reducing equivalents are immediately transferred to mitochondria via the aralar-MAS pathway. In the absence of aralar, mitochondrial NAD(P)H levels show a very small increase, which may be attributed to mitochondrial metabolism of lactate-derived pyruvate. Thus, during lactate utilization in resting conditions, most of the transfer of reducing equivalents to mitochondria (about 60%) is carried out by MAS activity.

Small \([Ca^{2+}i]\), signals evoked by agonists that, by themselves, do not stimulate mitochondrial Ca\(^{2+}\) uptake and dehydrogenase activity selectively activate the aralar-MAS pathway so that reducing equivalent transfer to mitochondria is now strongly potentiated. ATP and carbachol applied in Ca\(^{2+}\)-free media behave this way in neurons and result in about 3-fold MAS activation (the difference between reducing equivalent transfer values in control and aralar-deficient neurons, at 50–70 s after LM addition in the absence or presence of ATP). This activation is close to the maximal activation of MAS observed in isolated mitochondria (about 3-fold). However, the ATP-evoked \([Ca^{2+}i]\), transients only reach 200 nM at best, clearly below the Ca\(^{2+}\) concentrations required to fully activate MAS in brain mitochondria (about 1 μM; Fig. 1). This implies a close proximity between mitochondrial aralar and the IP₃ receptors, the Ca\(^{2+}\) release sites activated by ATP, so that localized Ca\(^{2+}\) concentrations, higher than those of the bulk cytosol, could exist and result in full activation of MAS. However, these local Ca\(^{2+}\) concentrations would still be unable to activate the Ca\(^{2+}\) uniporter-dehydrogenase pathway.

Neurotransmitters (glutamate and acetylcholine) and/or neuro-modulators (histamine, adenosine, and neurotrophin-3) acting on G-protein-coupled receptors or Trk receptors activate phospholipase C isoenzymes resulting in different dynamics of IP₃ production and diverse \([Ca^{2+}i]\), transients (53–56). This variability may arise from the engagement of different G-proteins and phospholipase C isoforms in a cell-specific manner. IP₃-dependent Ca\(^{2+}\) mobilization is critically involved in neuromodulation, as it controls excitability, transmitter release, and gene expression (53–55) in a cell- and receptor-specific way. Agonists that evoke IP₃ production may produce quite different \([Ca^{2+}i]\), and \([Ca^{2+}]_{mito}\) transients, depending on the specific receptor being activated and agonist concentration (57, 56), and some may produce small \([Ca^{2+}i]\), transients that selectively activate the aralar-MAS pathway in neuronal mitochondria. The energization obtained by Ca\(^{2+}\) activation of MAS can be utilized to produce more ATP in mitochondria, as found in studies with aralar-overexpressing cells (42). Indeed, preliminary data indicate that lactate supply during mild hypoglycemia increases ATP levels in neurons and even more so in the presence of nonhydrolyzable ATP agonists, but none of these effects were observed in aralar-deficient neurons. Further consequences of this energization have yet to be explored, but it is tempting to suggest that it could prime mitochondria for subsequent energy-requiring processes such as metabolite transport, etc., that may be involved in the final response to the neuromodulator.

Activity-dependent changes in the NAD(P)/NAD(P)H ratio have been detected long ago in different cell types (58). In neurons and synaptosomes, high K\(^+\) depolarization leads to an initial transient drop in NAD(P)H that is followed by an increase in NAD(P)H (8). Similar changes are produced by neural activity (12, 13, 52, 59, 60). These changes have been attributed to physiological increases in \([Ca^{2+}i]\), and Ca\(^{2+}\) (and possibly ADP) entry in mitochondria leading to the following: 1) increased respiration and mitochondrial NADH oxidation, and 2) Ca\(^{2+}\) activation of mitochondrial dehydrogenases resulting in NADH reduction, whereas Shuttleworth et al. (13) found that they were Ca\(^{2+}\)-independent. In our experimental conditions, the transient decrease in mitochondrial NAD(P)H was not observed, possibly because the imaging rate was too slow, and lactate was added together with high K\(^+\). However, a rapid increase in mitochondrial NAD(P)H in response to high K\(^+\) stimulation was clearly shown, and this was not modified by aralar deficiency, in agreement with the notion that it involves the Ca\(^{2+}\) uniporter signaling pathway.

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B. Pardo and J. Satrústegui, unpublished observations.
Indeed, our results show that MAS activity in neuronal mitochondria is upregulated under conditions where high [Ca$^{2+}$], signals drive Ca$^{2+}$-unipporter activity. This is probably because of competition for substrate between α-KGD of Krebs cycle and the α-KG-malate transporter (OGC) of MAS. The two reactions compete for α-KG by virtue of their apparent $K_m$ values. The $K_m$ value for α-KG of the OGC on the matrix side of the carrier is 1.5 mM (61) and that of α-KGD is around 0.2 mM (47). Activation by Ca$^{2+}$ of α-KGD increases its affinity for α-KG (7, 47), causing a reduction in OGC activity (43) and an inhibition of MAS activity (44). Although this effect is present within the whole time window of our experiments (about 1.5 min), it could disappear when the activation by Ca$^{2+}$ of α-KGD comes to an end. Thus, it is possible that after the decay of [Ca$^{2+}$]$_{mit}$ transients, MAS activity could prolong the increase in mitochondrial NAD(P)H induced by high [Ca$^{2+}$], signals and thus contribute to ATP synthesis and recovery of the resting state, conditions that rely on neuronal lactate utilization (62).

It should be noted that the increase in mitochondrial NAD(P)H obtained through the aralar-MAS pathway does not require the presence of Ca$^{2+}$ in the mitochondrial matrix, an event that contributes to cell death in a number of cell types (63), including neurons (64). It may also provide an alternative mechanism to supply mitochondrial NADH under oxidative stress conditions, where α-KGD is selectively inhibited (65). Lactate, and not the mitochondrial matrix, an event that contributes to cell death in a number of cell types (63), including neurons (64). It may also provide an alternative mechanism to supply mitochondrial NADH under oxidative stress conditions, where α-KGD is selectively inhibited (65). Lactate, and not glucose, is the major neuronal energy substrate after an insult, and overexpression of a lactate transporter in neurons enhances neuronal resistance to excitotoxicity (66), suggesting that the aralar-MAS pathway might also enhance survival to glutamate excitotoxicity.

Acknowledgments—We thank Dr. A. Martinez-Serrano and Dr. J. M. Cuevas for helpful advice and critical reading of the manuscript. We also thank Barbara Sesé, Inmaculada Ocaña, and Juliana Sánchez Garcia for excellent technical assistance.

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