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Ca^{2+} regulation of mitochondrial function in neurons☆

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ABSTRACT

Calcium is thought to regulate respiration but it is unclear whether this is dependent on the increase in ATP demand caused by any Ca^{2+} signal or to Ca^{2+} itself. $[Na^+]_i$, $[Ca^{2+}]_i$ and $[ATP]_i$ dynamics in intact neurons exposed to different workloads in the absence and presence of Ca^{2+} clearly showed that Ca^{2+}-stimulation of coupled respiration is required to maintain $[ATP]_i$ levels. Ca^{2+} may regulate respiration by activating metabolite transport in mitochondria from outer face of the inner mitochondrial membrane, or after Ca^{2+} entry in mitochondria through the calcium uniporter (MCU). Two Ca^{2+}-regulated mitochondrial metabolite transporters are expressed in neurons, the aspartate–glutamate exchanger ARALAR/AGC1/Slc25a12, a component of the malate–aspartate shuttle, with a Kd for Ca^{2+} activation of 300 nM, and the ATP-Mg/Pi exchanger SCaMC-3/Slc25a23, with $S_{0.5}$ for Ca^{2+} of 300 nM and 3.4 μM, respectively. The lack of SCaMC-3 results in a smaller Ca^{2+}-dependent stimulation of respiration only at high workloads, as caused by veratridine, whereas the lack of ARALAR reduced by 46% basal OCR in intact neurons using glucose as energy source and the Ca^{2+}-dependent responses to all workloads (veratridine, K^+-depolarization, carbachol). The lack of ARALAR caused a reduction of about 65–70% in the response to the high workload imposed by veratridine, and completely suppressed the OCR responses to moderate (K^+-depolarization) and small (carbachol) workloads, effects reverted by pyruvate supply. For K^+-depolarization, this occurs in spite of the presence of large $[Ca^{2+}]_{mit}$ signals and increased reduction of mitochondrial NAD(P)H. These results show that ARALAR-MAS is a major contributor of Ca^{2+}-stimulated respiration in neurons by providing increased pyruvate supply to mitochondria. In its absence and under moderate workloads, matrix Ca^{2+} is unable to stimulate pyruvate metabolism and entry in mitochondria suggesting a limited role of MCU in these conditions. This article was invited for a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

In muscle, exercise causes fluctuations in workload involving changes in ATP consumption rates due to muscle contraction. Exercise is coupled to about 100-fold increases in oxygen consumption rates [1,2]. Neurons are also subject to changes in workload, as a result of graded changes in the ionic composition of the cytosol which follow the opening of various types of ionic channels. To restore the resting state, neurons consume vast amounts of ATP in pumping these ions out of the cell or into intracellular organelles [3]. As in muscle, this is tightly associated with ATP production by the cell.

In neurons using glucose oxidation as the main metabolic pathway, an increase in workload is necessarily associated with increased glucose oxidation and increased ATP production by mitochondria. The classical principles of chemiosmotic coupling dictate that increased ATP production by mitochondria is coupled to increased oxygen consumption in the respiratory chain and increased substrate supply to mitochondria. However, this is not the only mechanism governing changes in mitochondrial function caused by changes in the workload. It has also

Abbreviations: MAS, malate/aspartate shuttle; MCU, mitochondrial calcium uniporter; OCR, oxygen consumption rate; PTP, permeability transition pore; ANT, adenine nucleotide translocase; AdN, adenine nucleotides

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become apparent that Ca^{2+} regulation of mitochondrial function plays an important role in maintaining ATP homeostasis.

In excitable cells, Ca^{2+} regulates cell function both by activation of ATP consumption (contraction, movement, ion transport, Ca^{2+} pumps) [4,5] and by activating ATP production through stimulation of OXPHOS and other means such as stimulation of glycogen breakdown (glycogen phosphorylase kinase is Ca^{2+} dependent) [6].

Ca^{2+} regulation of oxidative phosphorylation is thought to occur thanks to two different mechanisms: Ca^{2+} entry in mitochondria or Ca^{2+}-activation of metabolite transport. Calcium entry in mitochondria involves the recently identified mitochondrial calcium uniporter (MCU) complex, composed by the calcium channel proteins MCU [7,8] and MCUb, a dominant negative component of the oligomeric channel [9], together with regulatory subunits MICU1 [10], MICU2 [11] MCUR [12] and EMRE [13]. Ca^{2+} entry in mitochondria is followed by the activation of three matrix dehydrogenases and complex V, F_1F_0-ATP synthase [14–16]. In skeletal muscle mitochondria Ca^{2+} increases the activity of all complexes of the mitochondrial respiratory chain, in addition to complex V, although the mechanisms responsible for this increase are yet unknown [2]. The generation of a MCU-KO mouse has confirmed that MCU is essential in Ca^{2+}-induced increase in respiration of isolated skeletal muscle mitochondria [17].

Ca^{2+} activation of mitochondrial metabolite transporters occurs by the action of Ca^{2+} on the external side of the inner mitochondrial membrane rather than in the matrix. There are two types of such mitochondrial transporters, the aspartate–glutamate carriers (AGCs) and the ATP-Mg/Pi transporters (APCs or SCaMCs) [18–23]. Ca^{2+}-activation of these transporters does not require Ca^{2+} entry in mitochondria [19, 24–28].

By acting on both of these targets, through matrix Ca^{2+} and through stimulation of metabolite transport, Ca^{2+} may activate simultaneously both NADH supply to the respiratory chain and ATP synthesis resulting in metabolic homeostasis, i.e., increase in workload under conditions where the ATP/ADP and NADH/NAD levels remain constant [16], or only change in microdomains sensed by the appropriate targets.

ARALAR/AGC1 is the mitochondrial transporter of aspartate–glutamate present in brain and it is a component of the malate–aspartate NADH shuttle (MAS). Activation by extramitochondrial Ca^{2+} of ARALAR-MAS results in an increase in NADH production in neuronal mitochondria [25] with a requirement of about 300 nM Ca^{2+} to obtain half maximal activation of MAS. Ca^{2+}-activation of respiration of brain mitochondria on glutamate + malate had a similar requirement [29,30]. Gellerich et al. [31] have proposed that Ca^{2+}-activation of ARALAR functions as a "gas pedal" to increase pyruvate formation, thus amplifying the effects of ARALAR in terms of mitochondrial matrix NADH production and respiration. Under conditions of increased workload, such amplifying effect of Ca^{2+} on ARALAR may be required to fully activate substrate supply to mitochondria.

SCaMC-3 is the main mitochondrial ATP-Mg/Pi carrier present in brain and liver [20,21,32]. Activation by Ca^{2+} of the tumor cell SCaMC-1 or the liver cell ATP-Mg/Pi carrier SCaMC-3 results in the electroneutral uptake of either $[ATP\text{-}Mg]^{2-}$ or $HADP^{2-}$ against Pi^{2-} [28,33]. Work from Aprille's group [34,35, reviewed in 36] and Amigo et al. [32] have shown that adenine nucleotide accumulation in rat liver mitochondria through the ATP-Mg/Pi carrier SCaMC-3 results in a progressive increase in state 3 respiration. Compared to ARALAR/AGC1, SCaMC-3 requires much higher Ca^{2+} concentrations to obtain half maximal activation of ATP-Mg/Pi transport, about 3.4 μM [32], in the range of that of the mitochondrial calcium uniporter.

2. Calcium regulation of mitochondrial function is required to maintain cytosolic ATP levels upon changes in workload

Although changes in neuronal mitochondrial function in response to an increase in workload have been described before [37,38], the control of respiration by Ca^{2+} in intact neurons is still largely unknown. Hayakawa et al. [39] have described rapid Ca^{2+} dependent changes in oxygen consumption in response to high KCl in cultured Purkinje neurons, but Mathiesen et al. [40] have found no evidence for a role of cytosolic Ca^{2+} in activity-dependent rises in cerebral metabolic rate of oxygen in cerebellar Purkinje neurons in the intact brain. A confounding variable in these and other studies relates to the coincidence of the Ca^{2+}-mechanism with the classical mechanism activating mitochondrial respiration, i. e., ATP demand. Indeed, any Ca^{2+} signal involves ATP consumption in order to reestablish pre-stimulation values, and the role of Ca^{2+} versus ADP-stimulation of respiration needs to be established.

This problem has been addressed by analyzing the role of Ca^{2+} as a signaling molecule versus an inducer of ATP demand in neurons subject to different workloads: veratridine, high K^+ depolarization, and carbachol.

Veratridine induces a robust increase in $[Na^+]_i$ and $[Ca^{2+}]_i$, a large increase in $[Ca^{2+}]_{mit}$ in intact cortical neurons, and a large increase in respiration (Fig. 1). In a Ca^{2+}-free medium the increase in $[Na^+]_i$ was the same, but that of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$ was abolished. The increase in respiration was also much lower. Cytosolic ATP dropped after veratridine addition in these neurons, but the drop was larger in a Ca^{2+}-free than in a Ca^{2+}-containing medium (Fig. 1E–G). These results showed that the smaller respiratory response to veratridine in Ca^{2+}-free medium is not due to a smaller ATP demand, but to the absence of a Ca^{2+}-regulatory mechanism.

A similar strategy was employed to establish the role of Ca^{2+} in the response to KCl and carbachol. As for veratridine, the increase in respiration caused by KCl or carbachol was strongly reduced in Ca^{2+}-free medium, which also abolished K^+-induced $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$ signals, and carbachol-induced $[Ca^{2+}]_i$ signals. Not surprisingly, K^+ or carbachol-induced drop in cytosolic ATP was smaller or unchanged in Ca^{2+}-free conditions, and this could explain by itself the smaller increase in respiration in a Ca^{2+}-free medium. However, incubation with BAPTA-AM, which maintained the workload but blocked Ca^{2+} signaling also resulted in a lower respiratory response to these agents indicating that Ca^{2+}-regulation is required to increase respiration and maintain cytosolic ATP levels in response to any workload [41].

3. Role of the mitochondrial calcium uniporter (MCU) and the Ca^{2+}-regulated mitochondrial carriers in OCR stimulation in response to different workloads

Having found that Ca^{2+} regulation of respiration is required to maintain ATP levels in response to workload in neurons using glucose

Fig. 1. Changes in cytosolic and mitochondrial Ca^{2+}, cytosolic ATP, oxygen consumption and cytosolic Na^+ in primary neuronal cultures in response to veratridine. A, B, Changes in $[Ca^{2+}]_{cyt}$, in Fura-2 loaded neurons and C, D changes in $[Ca^{2+}]_{mit}$ in neurons transfected with Mit-GEM-GECO1 probe, obtained by stimulation with 50 μM veratridine (Ver or Verat) in 2 mM Ca^{2+} (A, C) or Ca^{2+}-free medium (B, D). Recordings from at least 60 cells per condition and two independent experiments were used for $[Ca^{+2}]_i$ (A, B) and a minimum of 15 cells and 8 independent experiments for $[Ca^{2+}]_{mit}$ imaging (C, D). Individual cell recordings (gray) and average (thick black trace) were shown. E–G, Cytosolic ATP in neurons transfected with cyt-GO-ATeam1 probe stimulated with veratridine in 2 mM Ca^{2+} medium (E), Ca^{2+}-free medium plus 100 μM EGTA (F) and comparison of the two conditions (G). H, Veratridine-induced stimulation of oxygen consumption rate (OCR) in *aralar* WT neurons under the mentioned Ca^{2+} conditions: 2 mM Ca^{2+} medium (filled circles or black bars), Ca^{2+}-free medium plus 100 μM EGTA (empty circles or white bars). OCR was measured using a Seahorse XF24 Extracellular Flux Analyzer. The sequential injections at different time points of veratridine (Ver, 50 μM) and the metabolic inhibitors oligomycin (Oli, 6 μM), 2,4-dinitrophenol (DNP, 0.5 mM) and antimycin A/rotenone (A/R, 1.0 μM both) are indicated by dashed lines. I, J, Stimulation of respiration (indicated as percentage of basal values) and respiratory control ratio (RCR) at the assayed conditions ($n = 9$–11 experiments, $^*p < 0.05$, $^{***}p < 0.001$, Student's t-test). RCR in non-stimulated state is represented with horizontal lines for each experimental condition. K–M, Changes in $[Na^+]i$, in individual SBFI-loaded neurons by stimulation with 50 μM veratridine in 2 mM Ca^{2+} medium (K) or Ca^{2+}-free medium (L), and comparison of mean values (M).
Taken from Llorente-Folch et al. [41].

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