Ketamine effect on intracellular and mitochondrial calcium mobilization

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**ABSTRACT:** The suppressive effects of ketamine on intracellular calcium has been reported in a variety of cells although the mechanisms involved are not well understood. The aim of this work was to evaluate the ketamine effect on the mitochondrial Ca\textsuperscript{2+} accumulation and the cellular Ca\textsuperscript{2+} mobilization using FLUO4-AM and flow cytometry. The results showed that mitochondria from ketamine injected animals presented a lower ability to retain calcium at concentrations higher than 20 μM, as compared with controls (saline injected animals). In addition, ketamine showed a significant decreased KCl-induced intracellular calcium concentration. KCl increased calcium influx through cellular depolarization. According to the data presented herein, ketamine presents a clear inhibitory effect on cytosolic Ca\textsuperscript{2+} transport mechanisms, independently from their action on the calcium channel associated NMDA receptor.

The role of Ca\textsuperscript{2+} as a cell messenger is determinant in the regulation of neuronal metabolism, participating in neurotransmitter release, nerve excitability, cell differentiation and migration, synaptic plasticity, neurite growth, and neuronal apoptosis (Berridge, 1998; Blackstone and Sheng, 2002; Thomas \textit{et al}, 1996). The versatility of Ca\textsuperscript{2+} as an intracellular messenger is derived from the fluctuations of cytosolic Ca\textsuperscript{2+} concentrations, most of which are generated by regulated openings of Ca\textsuperscript{2+} permeable channels present in the plasma membrane and in different organelles. Under physiological conditions in the neuron, electrical pulses or receptor-mediated stimuli generate different Ca\textsuperscript{2+} signals with distinct spatial/temporal dimensions and subcellular localization. When the frequency and magnitude of these cytosolic calcium signals are altered, the physiological functions of neurons will be inevitably affected (Abramov \textit{et al}, 2004; Aley \textit{et al}, 2006; Bano and Nicoreta, 2007). The loss of balance between plasma membrane Ca\textsuperscript{2+} influx and Ca\textsuperscript{2+} export leads to either a sustained or decreased cytosolic Ca\textsuperscript{2+} concentration, thus inducing alterations in the normal intracellular Ca\textsuperscript{2+} pathways and in the mitochondrial function, being mitochondria important energy generators and Ca\textsuperscript{2+} signaling organelles.

The N-methyl-D-aspartate (NMDA) receptor plays an important role in neuronal developmental processes (Malenk\textsuperscript{a} and Nicoll, 1993; Perez-Ot\textsuperscript{a}no \textit{et al}, 2004), including proliferation, differentiation, synaptic plasticity, and regulation of intracellular Ca\textsuperscript{2+} levels in neurons (Dorsi \textit{et al}, 2015; Olney \textit{et al}, 2001; Wei and Xei, 2009). Ketamine, an intravenous anesthetic, exerts its function as a non-competitive NMDA receptor antagonist. Some studies have shown that ketamine can affect neuronal functions and induce neuronal apoptosis (Olney \textit{et al}, 2001, 2002). However, the effects of the ketamine on cell calcium transport and mitochondrial calcium...
accumulation and their evident consequences, such as the alteration in the magnitude of the mitochondrial and cytosolic Ca\(^{2+}\) signaling, still remain unclear as well as the mechanisms involved in the possible benefits or toxicity of this drug.

Expanding our knowledge of intracellular and mitochondrial calcium signals will contribute to the understanding of mechanisms associated with neuronal loss in different physiopathological conditions. Mitochondrial Ca\(^{2+}\) import occurs mainly through the voltage dependent anion-selective channel (VDAC) (Carafoli, 2003). The Ca\(^{2+}\) transport across the inner mitochondrial membrane is mediated by the uniporter (Carafoli, 2003), that mobilizes Ca\(^{2+}\) along the electrochemical gradient due to the negative mitochondrial membrane potential (ΔΨm) of 180 mV (Carafoli, 2003). Both the VDAC and the uniporter show Ca\(^{2+}\)-dependent activation which is relevant for the homeostatic control of cytoplasmic [Ca\(^{2+}\)]c (Carafoli, 2010; Bustamante and Lores-Arnaiz, 2010; Raluca et al., 2014). In the matrix, also, Ca\(^{2+}\) stimulates the Ca\(^{2+}\)-sensitive mitochondrial dehydrogenases to increase the H\(^+\) extrusion, which is important for the maintenance of the driving force for Ca\(^{2+}\) uptake and ATP production (Carafoli, 2003, 2010; Bustamante and Lores-Arnaiz; Raluca et al., 2014).

Using brain isolated mitochondria from ketamine-treated animals and Vero cell cultures (a fibroblast-like kidney cell line) treated with ketamine as study subjects, this study attempted to analyze the effect of this drug on the mitochondrial ability to accumulate calcium and on the cellular calcium transport response. In order to monitoring mitochondrial and cytosolic calcium mobilization, the calcium sensor fluorophore Fluo4AM was employed and the magnitude of the fluorescent signal was determined by flow cytometry.

The ability of mitochondrial calcium accumulation was evaluated in hippocampal mitochondria from ketamine injected animals. Ketamine (40 mg/kg i.p) was given to male S-D pups (ca. 21 days old) for 3 consecutive days and then sacrificed 24 hours after the injection; untreated animals received saline for 3 consecutive days as well. Brains were quickly removed, and hippocampal mitochondria isolated by differential centrifugation and suspended in MSH without EDTA buffer: 0.23 M mannitol, 0.07 M sucrose, 5 mM HEPES pH 7.4. For Ca\(^{2+}\) uptake measurements, 2.5 μg of mitochondrial fraction was washed with MSH, and loaded with 50 nM of an acetoxymethyl ester derivative of the green fluorescent indicator Fluo-4 (final concentration), in

**FIGURE 1.** A. Ketamine effect on mitochondrial calcium accumulation. Isolated hippocampal mitochondria from saline injected animals (control) and ketamine injected animals (40 mg/kg i.p) for 3 consecutive days, and, an aliquot of mitochondria from untreated animals exposed to 50 mM H\(_2\)O\(_2\) during 10 min (H\(_2\)O\(_2\)) were loaded with Fluo4AM, exposed to different external [Ca\(^{2+}\)] from 5 to 100 μM and incubated at 37° C for 20 min before acquirement by the cytometer. Each value represents the mean ± SD for 3 independent experiments. Data showed significant differences between control and ketamine or H\(_2\)O\(_2\) groups (*p< 0.05, Student’s t test for independent samples). B. Time dependent effect of ketamine on KCl-induced cell [Ca\(^{2+}\)] mobilization. Untreated cultured Vero cells (1x10^6) (control) were exposed or PBS and 300 μM ketamine respectively for 24 hs, (ketamine) harvested, suspended in buffer with 1 mM [Ca\(^{2+}\)], loaded with100 nM Fluo4AM at 37° C for 30 min and then exposed to 170 mM KCl. Kinetic Fluorescence was determined by measuring the fluorescence changes each minute, during 8 minutes, by flow cytometry. Data showed significant differences between control and ketamine groups in each time point (*p< 0.05, independent sample Student t test).
a reaction medium containing 1 ml of MSH buffer (0.23 M mannitol, 0.07 M sucrose, 5 mM HEPES) supplemented with 5 mM malate, 5 mM glutamate and 1 mM phosphate. Mitochondrial samples were incubated with different CaCl$_2$ concentrations in the range of 5-100 μM. For Ca$^{2+}$ deprivation experiments, CaCl$_2$ was omitted and 0.1 mM EGTA was added. The samples were incubated for 30 min in a shaking water-bath (37° C) and immediately acquired by the cytometer. Hydrogen peroxide (50 mM) was added to mitochondrial samples, and used as a criterion of damaged mitochondria with an impaired ability to acquire calcium.

As shown in Fig. 1A, brain hippocampal mitochondria from saline injected animals (control trace) showed a higher and sustained Ca$^{2+}$ fluorescent signal indicating an efficient ability to accumulate and retain Ca$^{2+}$ up to the highest Ca$^{2+}$ concentration (100 μM). The ketamine dose employed in this study, was low enough to avoid any systemic toxicity, and was chosen in accordance with earlier works (Huang et al., 2012; Ullah et al., 2012). In fact, the ability to acquire and retain Ca$^{2+}$ from the extracellular medium was markedly decreased in mitochondria from ketamine exposed rats (ketamine trace) as compared with mitochondria from saline injected rats. Mitochondria treated with a high concentration of hydrogen peroxide (50 mM) (H$_2$O$_2$; trace), showed the lowest fluorescence values indicating that oxidative-damaged mitochondria were not able to accumulate and retain Ca$^{2+}$. Indeed, Ca$^{2+}$ uptake by H$_2$O$_2$-damaged mitochondria was only observed at the lowest calcium concentration (5 μM).

Vero cell cultures were used to evaluate the ketamine effect on Ca$^{2+}$ influx by determination of the time dependent effect of ketamine on KCl-induced calcium mobilization. Cells were cultured and treated with 300 μM ketamine for 24 h; control cells received PBS only. Both ketamine-treated and control cells were harvested (1 x 10$^6$) and suspended in 1 ml HEPES buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 10 mM glucose and 10 mM Na-HEPES in presence of 1 mM Ca$^{2+}$), loaded with 100 nM Fluo4AM at 37° C for 30 min and followed by 170 mM KCl. Immediately after depolarization, calcium mobilization from the extracellular buffer to the cytoplasm was recorded by measuring fluorescence intensities every minute during as long as 8 minutes by flow cytometry in each sample.

The evaluation of the ketamine effect on the relative cellular calcium transport is shown in Fig 1B. After 5 days of culture, ketamine in a dose of 300 μM had no effect on cell viability (data not shown), but presented a decreased Ca$^{2+}$ influx after KCl incubation as observed in Fig 1B (ketamine). In fact, ketamine treated cells showed a lower fluorescence intensity under flow cytometry, as compared with the untreated control cells. These data indicate that Ca$^{2+}$ transport to the cytosol after cellular depolarization was inhibited by ketamine cell treatment. A fluorescence increase was observed for several seconds, followed by a slow decrease in both treated and untreated cells. The signal intensity was markedly decreased in ketamine treated cells showing a statistically significant difference as compared with untreated cells. Our results may be correlated with those of Chen et al. (2005) who observed that ketamine induced a time-dependent reduction of the bradykinin-enhanced intracellular calcium concentrations in endothelial cells from the human umbilical vein.

According to the data presented, ketamine presents an important inhibitory effect on cytosolic Ca$^{2+}$ transport mechanisms independent of the calcium channel associated NMDA receptor.

The two models proposed serve as suitable experimental systems for the study of cellular and mitochondrial Ca$^{2+}$ signals alterations induced by ketamine exposure, evaluating the responses by Fluo4AM and flow cytometry. In this study we could observe a clear inhibition of mitochondrial Ca$^{2+}$ accumulation in ketamine injected animals compared with the evident Ca$^{2+}$ accumulation in mitochondria from saline injected rats. Their ability to accumulate calcium was less affected than that of H$_2$O$_2$ damaged mitochondria where probably many of the calcium transport systems are impaired. Finally, the effect of non-toxic ketamine concentrations in Vero cells, showed that ketamine may be involved in the inhibition of different cell depolarizing mechanisms induced by KCl.

We conclude that the regulatory mechanisms of mitochondrial and cytosolic Ca$^{2+}$ signaling may constitute a central topic in the understanding of neuronal functions.

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