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Mitochondria are intracellular magnesium stores: investigation by simultaneous fluorescent imagings in PC12 cells

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Abstract

To determine the nature of intracellular Mg^{2+} stores and Mg^{2+} release mechanisms in differentiated PC12 cells, Mg^{2+} and Ca^{2+} mobilizations were measured simultaneously in living cells with KMG-104, a fluorescent Mg^{2+} indicator, and fura-2, respectively. Treatment with the mitochondrial uncoupler, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), increased both the intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) and the $[Ca^{2+}]_i$ in these cells. Possible candidates as intracellular Mg^{2+} stores under these conditions include intracellular divalent cation binding sites, endoplasmic reticulum (ER), Mg-ATP and mitochondria. Given that no change in $[Mg^{2+}]_i$ was induced by caffeine application, intracellular IP_3 or Ca^{2+} liberated by photolysis, it appears that no Mg^{2+} release mechanism thus exists that is mediated via the action of Ca^{2+} on membrane-bound receptors in the ER or via the offloading of Mg^{2+} from binding sites as a result of the increased $[Ca^{2+}]_i$. FCCP treatment for 2 min did not alter the intracellular ATP content, indicating that Mg^{2+} was not released from Mg-ATP, at least in the first 2 min following exposure to FCCP. FCCP-induced $[Mg^{2+}]_i$ increase was observed at mitochondria localized area, and vice versa. These results suggest that the mitochondria serve as the intracellular Mg^{2+} store in PC12 cell. Simultaneous measurements of $[Ca^{2+}]_i$ and mitochondrial membrane potential, and also of $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$, revealed that the initial rise in $[Mg^{2+}]_i$ followed that of mitochondrial depolarization for several seconds. These findings show that the source of Mg^{2+} in the FCCP-induced $[Mg^{2+}]_i$ increase in PC12 cells is mitochondria, and that mitochondrial depolarization triggers the Mg^{2+} release.

Keywords: Magnesium; Mitochondria; KMG; Photolysis; FCCP; Fluorescent imaging

1. Introduction

The magnesium ion, Mg^{2+}, is an important divalent cation in cells, serving to stabilize nucleic acid and protein structure [1,2], blocking a number of different ion channel types [3–6] and mediating Mg^{2+}-dependent enzymatic reactions as a cofactor [7–9], including ATP-related enzymatic reactions [10,11]. Given these important functions, it is evident that perturbations in the intracellular Mg^{2+} concentration, $[Mg^{2+}]_i$, could have serious implications for the proper physiological functioning of cells. Some age-related and neuronal diseases are known to be related to

Abbreviations: caged Ca^{2+}, nitrophenyl EGTA; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; KMG-104, 1-(2,7-difluoro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-4-oxo-4*H*-quinolizine-3-carboxylic acid; EPMA, electron probe microanalysis; IP_3, inositol 1,4,5-trisphosphate; Ψ_{Mt}, mitochondrial membrane potential; PD, Parkinson's disease

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a deficiency of Mg^{2+} [12–14]. For example, the $[Mg^{2+}]_i$ of rat smooth muscle and human red blood cells from hypertensives is lower than that of normotensive controls [15,16]. Patients with diabetes mellitus also have low serum $[Mg^{2+}]$ [17]. On this basis, it is important to know more about intracellular Mg^{2+} mobilizations in order to shed light on possible mechanisms or causes of these diseases.

$[Mg^{2+}]_i$ is usually maintained around 1 mM at rest in mammalian cells [7]. One of known Mg^{2+} mobilization mechanisms for the maintenance of $[Mg^{2+}]_i$ is Mg^{2+} exchange at the cell membrane. A Na^+/Mg^{2+} exchanger located on the cell membrane has been described for various cell types and it has been reported that $[Mg^{2+}]_i$ depends on the extracellular Na^+ concentration [18–21]. Mg^{2+} efflux is observed in response to an increase in the extracellular concentration of Na^+ or Ca^{2+} in rat liver plasma membranes [22,23]. The Na^+/Mg^{2+} exchanger is known to be inhibited by amiloride or imipramine [21,23], and it has been reported that the recovery of $[Mg^{2+}]_i$ to normal levels in PC12 cells was suppressed by imipramine after a transient increase induced by the mitochondrial uncoupler, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) [24]. While the main focus of these reports has been on Mg^{2+} translocation through the cell membrane, the issue of intracellular Mg^{2+} releasing points has received less attention.

The FCCP-induced increase in $[Mg^{2+}]_i$ has been described for a variety of cell types [24–26] and is also observed in cells bathed in Mg^{2+}-free media [26]. Given that $[Mg^{2+}]_i$ accounts for less than 5% of the total cellular Mg^{2+} (~20 mM) [7], these findings suggest that almost all intracellular Mg^{2+} exists in bound form or is sequestered within organelles, and that cells therefore possess endogenous Mg^{2+} releasing mechanisms [26]. The purpose of this study is to determine the source of Mg^{2+} in the FCCP-induced increase in $[Mg^{2+}]_i$ of PC12 cells.

FCCP also induces an increase in $[Ca^{2+}]_i$ [27,28]. Ca^{2+} is usually accumulated in the endoplasmic reticulum (ER) of cells, and released through ryanodine receptors and/or via ligand-gated inositol 1, 4, 5-trisphosphate (IP_3) receptors [29–31]. Ca^{2+}-induced Ca^{2+} release (CICR) mechanisms are also responsible for the release of Ca^{2+} from intracellular organelles. If a similar release mechanism exists for Mg^{2+}, then Ca^{2+} inducible stimuli might induce Mg^{2+} release through ion channel receptors. Another possibility concerns that of Mg^{2+} substitution. Many divalent cation binding sites are present in the cell, such as DNA and RNA [1,2]. EF-hand of troponin C has affinity for both Mg^{2+} and Ca^{2+}, and the binding is competitive [32,33]. Furthermore, it has been reported that most of the proteins which interact with Ca^{2+} can also interact with Mg^{2+} [34]. A strong Ca^{2+} increase may preferentially interact with binding sites and liberate Mg^{2+} from those binding sites in the cell.

ATP synthesis in cells is inhibited by FCCP treatment. When ATP, which binds strongly to Mg^{2+} and mainly exists as an Mg-ATP complex in cells, decomposes to ADP with energy release, free Mg^{2+} will be released to the cytosol. Therefore, ATP depletion by FCCP could induce an increase in $[Mg^{2+}]_i$.

It has been revealed by electron probe micro analysis (EPMA) that mitochondria contain Mg^{2+} [35,36]. Mitochondria are also known as Ca^{2+} stores [37–39], and FCCP induces an increase in $[Ca^{2+}]_i$ via Ca^{2+} release from these organelles. Therefore, Mg^{2+} release from mitochondria is plausible upon FCCP-induced mitochondrial membrane depolarization.

Possible candidates for Mg^{2+} release points include the ER, intracellular divalent binding sites, Mg-ATP, and mitochondria. In this regard, we have examined here Mg^{2+} storage and release properties of PC12 cells, and have made simultaneous Mg^{2+}-Ca^{2+} measurements to investigate Mg^{2+} mobilization mechanisms in these cells.

2. Materials and methods

2.1. Chemical reagents and cell culture

Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), and fetal bovine serum (FBS) were purchased from GIBCO (MD, USA). The free acid form of fura-2, its acetoxymethyl ester form (fura-2-AM), nitrophenyl EGTA-AM (caged Ca^{2+}-AM), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), caged IP_3 and MitoFluor™ Red 589 were from Molecular Probes (OR, USA). Oligomycin was from Calbiochem (CA, USA). Poly-D-lysine (PDL), nerve growth factor (NGF), carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), and other reagents were from Sigma (MO, USA). The free acid and AM forms of 1-(2,7-difluoro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-4-oxo-4*H*-quinolizine-3-carboxylic acid (KMG-104) were developed and synthesized as highly selective fluorescent Mg^{2+} indicators [40,41]. PC12 cells [42] were obtained from RIKEN Tsukuba Institute; cells were cultured at 37°C in DMEM containing heat-inactivated serums (10% HS, 5% FBS), 25 U/ml penicillin and 25 μg/ml streptomycin, under a humidified atmosphere with 5% CO_2. For experimental use, cells (passage number 5–9) were cultured on glass coverslips coated with PDL, and differentiated by culturing with 50 ng/ml NGF-containing serum-free medium for 5–7 days.

2.2. KMG-104 is a novel Mg-specific fluorescent indicator

KMG-104, which has a fluorescein chromophore, is an up-to-date version of the KMG series of Mg^{2+} probes [40,41,43]. The fluorescence intensity of KMG-104 increases largely with increasing $[Mg^{2+}]$ and shows no response to Na^+ and K^+, the main intracellular cations. The K_d for Mg^{2+} is about 2.1 mM, which is near to the physiological concentration of Mg^{2+} [40,41]. The most outstanding point of this indicator is its selectivity for Mg^{2+}