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Research Report

Zinquin identifies subcellular compartmentalization of zinc in cortical neurons. Relation to the trafficking of zinc and the mitochondrial compartment

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ABSTRACT

Zinquin (Zn^{2+} selective fluorophore), when used to visualize intracellular Zn^{2+}, typically shows brightly fluorescent perinuclear endosome-like structures, presumably identifying Zn^{2+} containing organelles. In this study, zinquin identified numerous and widespread sites of Zn^{2+} compartmentalization in primary cultures of embryonic rat cortical neurons. Nuclear fluorescence, however, was absent. We labeled neuronal mitochondria with MitoTracker Green in the presence of zinquin and show that the fluorescent patterns of MitoTracker Green and zinquin were distinct and clearly different in both the perinuclear region and in processes. The mitochondrial compartment was much larger than the sum of the areas of zinquin fluorescence, as indicated by the small amount (<10% MitoTracker Green over zinquin) of overlap of MitoTracker Green on zinquin. Zinquin fluorescence was unaffected by carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) treatment. The zinquin fluorescent objects were generally spherical in shape with a average diameter of about 0.6 μm. Most fluorescent objects, nearly two thirds on average, appeared to be docked, but both anterograde and retrograde movements were observed by time lapse image analysis. Although some fluorescent objects moved as much as 1 μm in 5 min, typical movements were smaller, usually 0.5 μm or less. Colchicine treatment caused striking aggregation of MitoTracker Green most noticeable in the perinuclear region. Zinquin fluorescence similarly showed reduced distribution throughout the cytoplasm, suggesting that zinquin fluorescent structures were associated with microtubules. Treatment with cytochalasin D had little noticeable effect on either the pattern of zinquin and MitoTracker Green fluorescence or their coincidence. Thus, numerous Zn^{2+} sequestering organelles/structures are present in perinuclear regions and processes of cultured neurons and are sometimes found coincident with mitochondria. We demonstrated real time trafficking of sequestered Zn^{2+}, using zinquin fluorescence, apparently associated with an endosome-like compartment or protein complexes in the cytosol.

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1. Introduction

Zinquin, a Zn^{2+} selective fluorophore, has been used by many investigators in numerous mammalian cell types to visualize intracellular Zn^{2+} (Colvin et al., 2002; Haase and Beyersmann, 2002; Michalczyk et al., 2002; Ranaldi et al., 2002; Snitsarev et al., 2001; St Croi et al., 2002). The results of these studies typically show brightly fluorescent perinuclear endosome-like structures, presumably identifying Zn^{2+} containing organelles within the cytoplasm. Whereas the function of Zn^{2+} containing vacuoles in yeast is well defined (Devirgiliis et al., 2004; MacDiarmid et al., 2002; MacDiarmid et al., 2003), this is not the case for zinquin fluorescent structures found in mammalian cells. The goal of the present studies is to begin to characterize these structures in primary cultures of cortical neurons. Zinquin fluorescent structures are observed both in the soma and processes of cultured cortical neurons maintained in neurobasal media, suggesting that these structures have a role to play in normal Zn^{2+} homeostasis and trafficking (Colvin et al., 2002). In addition, these structures apparently accumulate significant Zn^{2+} when cells are under various stresses (Haase and Beyersmann, 1999, 2002; Pearce et al., 2000; Smith et al., 2002; St Croi et al., 2002; Tartler et al., 2000). Attempts to identify the transporters responsible for Zn^{2+} sequestration have met with mixed success (Kirschke and Huang, 2003; Michalczyk et al., 2002; Palmiter et al., 1996; Ranaldi et al., 2002).

The widespread distribution of zinquin fluorescence in cultured cortical neurons suggests that these structures could be associated with mitochondria. Many studies have shown a linkage between Zn^{2+} and mitochondrial function and dysfunction in cultured neurons. There is convincing evidence that when neuronal cytosolic concentrations of Zn^{2+} rise under pathological conditions, Zn^{2+} is taken up by mitochondria with derangements of mitochondrial structure and function (Dineley et al., 2003; Jiang et al., 2001; Sensi et al., 2003a, 2002, 1999, 2000; Wudarczyk et al., 1999). Such Zn^{2+}-dependent derangements in mitochondrial structure and function appear to contribute to cell death involving apoptotic pathways (Bossy-Wetzel et al., 2004; Budd et al., 2000). Thus, there is evidence for mitochondrial Zn^{2+} interactions under pathological conditions, but what is the evidence for the existence of endogenous Zn^{2+} in mitochondria that might be detectable by zinquin?

In elegant studies, from Sensi et al. (2003a), it was shown that RhodZin-3 (a Zn^{2+} selective fluorophore that accumulates in mitochondria) yields TPEN (tetrakis-(2-pyridylmethyl)ethylenediamine) sensitive punctate fluorescence in neurons. RhodZin-3 has a high affinity for Zn^{2+} (≈65 nM) suggesting that endogenous mitochondrial Zn^{2+} is in the nanomolar range (Sensi et al., 2003b). Recent studies (Malaiyandi et al., 2005) using isolated brain mitochondria have demonstrated pathways for the transport and accumulation of Zn^{2+}. In the present studies, we sought to find direct evidence to test the hypothesis that structures associated with zinquin fluorescence in cultured cortical neurons are associated with mitochondrial compartments. To accomplish this goal, we labeled neuronal mitochondria with the well characterized fluorophore MitoTracker Green in the presence of zinquin. To address the issue of Zn^{2+} trafficking in cultured cortical neurons, we observed the real time movements of zinquin fluorescent objects by time lapse image analysis.

2. Results

2.1. *The patterns of zinquin and MitoTracker Green fluorescence in living cortical neurons were distinct*

Confocal microscopy improves horizontal and particularly vertical resolution of fluorescent images when compared with conventional epifluorescence. Unfortunately, in our studies, zinquin fluorescence showed very low intensity in living cells and required increasing the pinhole diameter to a point where the thickness of the optical section and image resolution was no better than conventional epifluorescence. Thus, we found that the best quality images were obtained using conventional epifluorescence equipped with a high resolution, high sensitivity, cooled CCD monochrome camera (see Experimental procedures). Although using this camera provided excellent images, we still had background fluorescence from out of focus structures complicating our image analysis. To remove out of focus fluorescence in our images, we used the flatten background feature of MetaMorph image analysis software (as detailed in the Experimental procedures) for postprocessing, resulting in still better quality, more informative images.

When cortical neurons grown and maintained in neurobasal media were coincubated with zinquin ester and MitoTracker Green and subsequently observed by conventional epifluorescence, characteristic and distinct patterns of fluorescence were observed (Fig. 1) for each fluorophore. Fig. 1 shows representative images of the perinuclear region (A–C) and a region of just processes (D–F). All neurons examined for MitoTracker Green showed a densely fluorescent perinuclear region, matching the well known distribution of mitochondria in this region of living neurons. Profiles of individual mitochondria were easily observed at any focal plane. This is shown in Fig. 1A. In Fig. 1D, only processes are shown and individual mitochondria are clearly distinguishable. Zinquin showed numerous fluorescent spots with minimal diffuse or background fluorescence in the perinuclear region. The nucleus was virtually devoid of zinquin fluorescence (Fig. 1B). Most processes showed a similar pattern of punctate zinquin fluorescence as was seen in the perinuclear region (Fig. 1E). Zinquin fluorescence under these conditions had previously been shown to be sensitive to the addition of tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN), a membrane permeable metal chelator with high affinity for Zn^{2+} (Colvin et al., 2002). Also, when the neurons were exposed to 200 μM Zn^{2+} with 25 μM pyrithione (a Zn^{2+} ionophore), diffuse fluorescence in the cell body and nucleus increased dramatically (data not shown). Figs. 1C and F (perinuclear region and processes, respectively) show merged images of zinquin and MitoTracker Green fluorescence. Black arrows point to examples of regions where overlap occurred between the two fluorescent molecules, whereas white arrows point to examples of areas where zinquin fluorescence did not overlap with MitoTracker Green fluorescence. The fluorescent patterns of the two fluorophores were distinct and clearly different.