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Zinc modulation of calcium activity at the photoreceptor terminal: A calcium imaging study

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ABSTRACT

There is abundant experimental evidence that zinc ions (Zn^{2+}) are present in the synaptic vesicles of vertebrate photoreceptors, and that they are co-released with glutamate. Here we show that increasing the concentration of extracellular zinc (2 μM–2 mM) suppresses the entry of calcium into the synaptic terminals of isolated salamander double cones. The resultant dose-dependent curve was fit by an inverse Hill equation having an IC50 of 38 μM, and Hill coefficient of 1.1. Because there is currently no reliable way to measure the concentration of extracellular zinc, it is not known whether the zinc released under normal circumstances is of physiological significance. In an attempt to circumvent this problem we used zinc chelators to reduce the available pool of endogenous zinc. This enabled us to determine how the *absence* of zinc affected calcium entry. We found that when intra- or extra-cellular zinc was chelated by 250 μM of membrane-permeable TPEN or 500 μM of membrane-impermeable histidine, there was a significant rise in the depolarization-induced intracellular calcium level within photoreceptor terminals. This increase in internal [Ca^{2+}] will undoubtedly lead to a concomitant increase in glutamate release. In addition, we found that blocking the L-type calcium channels that are expressed on the synaptic terminals of photoreceptors with 50 μM nicardipine or 100 μM verapamil abolished the effects of zinc chelation. These findings are a good indication that, when released *in vivo*, the zinc concentration is sufficient to suppress voltage-gated calcium channels, and reduce the rate of glutamate release from photoreceptor terminals.

1. Introduction

Zinc is one of the most ubiquitous and important trace elements in biological systems, and it has proven indispensable to the growth and development of all forms of life (Hambidge, 1981; Vallee, 1988). Its importance stems largely from the fact that zinc is an integral and essential component of scores of enzymes and thus participates in a broad range of metabolic functions (Vallee and Auld, 1990), as well as playing a significant role in translation and transcription of the genetic message (Vallee and Falchuk, 1981; O'Halloran, 1993).

Less well known is the physiological significance of Zn^{2+} located in the synaptic terminals of glutamatergic neurons. Experimental study of the role of ionic zinc has been severely impeded by the fact that Zn^{2+} was considered a quantitatively immeasurable trace element, and by a lack of analytical methods for its detection and localization. With the availability of sensitive and reliable methods for histochemical imaging, the presence of "chelatable" or "free" zinc was shown to be within the synaptic vesicles of glutamatergic nerve terminals in the hippocampus (Aniksztejn et al., 1987; Ketterman and Li, 2008), in specific layers

Abbreviations: AM, acetoxymethyl; AOI, area of interest; IC_{50}, concentration giving half maximum response; OPL, outer plexiform layer; SEM, standard error of the mean; TPEN, N,N,N′,N′-tetrakis(2 pyridylmethyl)ethylenediamine.

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of the cerebral cortex and other regions of the CNS (Frederickson and Danscher, 1990; Sensi et al., 2009), and in the synaptic terminals of photoreceptors in the vertebrate retina (Wu et al., 1993; Qian et al., 1997; Ugarte and Osborne, 1998). Moreover, the presence of the vesicle-associated zinc transporter 3 (ZnT-3, Cole et al., 1999) in the synaptic region of photoreceptors (Redenti and Chappell, 2004) suggested that ionic zinc is co-localized with glutamate within the vesicles of the synaptic terminal. This has since been confirmed, and there is now evidence that the co-release of glutamate and ionic zinc is a calcium-dependent process (Frederickson and Bush, 2001; Gee et al., 2002; Redenti et al., 2007).

The inability to determine the concentration of extracellular Zn^{2+} at post-synaptic sites has clouded an evaluation of its physiological significance as an effective participant in neuronal activity. We have attempted to circumvent the uncertainty regarding ion concentration by analyzing the effects induced by *removal* of endogenous zinc from synaptic sites; we were then able to compare results obtained under "normal" conditions with those observed after zinc chelation. Using this approach in conjunction with calcium-imaging microscopy of photoreceptor cells, we could determine changes in intracellular calcium when zinc chelation occurs either inside or outside the cell.

2. Methods

All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and were approved by the Institutional Animal Care and Use Committee of the Marine Biological Laboratory Woods Hole, MA in accordance with its guidelines.

2.1. Cell dissociation and dye loading

Tiger salamanders (*Ambystoma tigrinum*), obtained from Kons Scientific (Germantown, WI) and Charles Sullivan (Nashville, TN), were used in this study. The salamanders were kept at 4–8 °C on a 12 h light–dark cycle. Prior to tissue extraction, animals were anesthetized with 0.1% tricaine methanesulfonate (MS-222, Argent Chemical, Redmond, WA) and decapitated. Eyes were enucleated under ambient light, the cornea and lens were removed, and the retina was isolated. The retinal tissue was enzymatically dissociated by gentle agitation in Ringer solution containing 12 U/ml papain (Worthington Biochemical, Lakewood, NJ) activated with 5 mM L-cysteine (Calbiochem, La Jolla, CA), and adjusted to pH 7.4. It was then triturated through a fire-polished Pasteur pipette to obtain isolated neurons. Cells were plated on 35 mm #1.5 glass bottom dishes (In Vitro Scientific, Sunnyvale, CA), coated with 1 mg/ml lectin from *Canavalia ensiformis* (Sigma Aldrich, St. Louis, MO), and allowed to adhere for 10–20 min. The cells were then immersed for 30 min at 10 °C in the membrane permeable calcium indicator dye Fluo-4, AM (Molecular Probes, Inc., Eugene, OR), dissolved in high quality 99.7% anhydrous dimethylsulfoxide (DMSO, Acros Organics, Fairlawn, NJ) which was diluted with Ringer to a 0.5–1 μM working concentration. The Ringer solution contained (in mM): NaCl (111), KCl (3), $CaCl_2$ (2), $MgCl_2$ (1), Dextrose (10), HEPES (5), (all from Fisher Scientific, Pittsburgh, PA), pH 7.7. After incubation, during which time Fluo-4 AM was internalized and the acetoxymethyl (AM) esters were cleaved to form the free Fluo-4, the external Fluo-4 AM solution was washed off and replaced with normal Ringer solution for calcium imaging. All other chemicals and solutions (nicardipine hydrochloride, verapamil hydrochloride, L-histidine, N,N,N′,N′-Tetrakis (2-pyridylmethyl)ethylenediamine (TPEN)) were purchased from Sigma–Aldrich (St Louis, MO).

2.2. Ca^{2+} imaging

Isolated, dye-loaded photoreceptor cells were imaged using a Zeiss Axiovert 200M microscope with a 40× Plan Neofluor 0.6 NA dry objective (Carl Zeiss Vision GmbH, Munich, Germany). Images were collected continuously with a Zeiss AxioCam MRm CCD camera and displayed with AxioVision 4.8.2 software. A Zeiss HXP 120 mercury halide light source with a built-in shutter was used for dye excitation. Images were collected every second with an exposure time of 150 ms. The double cone somas and their bulging synaptic terminals were selected as areas of interest (AOI) and the change in average fluorescence signal intensity of the selected area was plotted over time. Peak intensities at every drug application were compared graphically and statistically. Drugs were delivered via a pressurized perfusion system (ALA-VM8, ALA Scientific, Farmingdale, NY) with fast pinch- or solenoid valve-controlled solution changes allowing all test solutions to be delivered for a period of 10 s. The solutions were oxygenated by using an oxygen tank as the pressure source for perfusion. The system allowed accurate replication of the perfusion rate (1 ml/min) between experiments, and the volume of the delivery manifold was ~5 μl.

2.3. Data analysis

Statistical analysis of calcium recordings was performed using GraphPad Prism software, version 5.04. All trials started with a control application of 30 mM KCl alone and AOI peak intensities for every cell were normalized to the first application of KCl. A control group of cells ($n = 18$) received only KCl treatments at regular intervals, while treatment groups received KCl with drugs at the same intervals following the initial (control) KCl pulse. A 1-way ANOVA test, performed on all treatment groups, showed overall significance values of $p < 0.05$.

Subsequently, each peak in the treatment group was compared to its sister peak in the control group using a two-tailed unpaired student *t*-test. Error bars shown in the figures are ±SEM.

Since photo-bleaching caused a small time-dependent decline in the fluorescence signal (see Fig. 1C), we were able to construct an average decay curve of peak fluorescence intensity with time (not shown) based on repeated applications of 30 mM KCl alone. This enabled us to correct for changes in fluorescent intensity due to bleaching, and any reduction in fluorescent signal could be attributed solely to drug application. Decay coefficients were only used for fitting the zinc dose–response of Fig. 2D.

3. Results

3.1. Depolarization-induced calcium influx in photoreceptor terminals

Photoreceptors, like other neurons, release their transmitter when depolarized. This vesicular release is triggered by calcium entry through voltage-gated calcium channels, and numerous studies have confirmed the presence of L-type calcium channels on the photoreceptor terminals of salamander (Steele et al., 2005) and other vertebrate species (Morgans et al., 2005; Mercer et al., 2011). In this way, synaptic release of glutamatergic vesicles is tightly coupled to an increase of calcium levels in the terminal. To monitor calcium level changes in photoreceptor terminals, we repeatedly applied 30 mM KCl to depolarize photoreceptors, and recorded the temporal changes in intracellular calcium by means of the fluorescent intensity changes of Fluo-4. Fig. 1A shows a number of time