Functional analysis of calcium channel-mediated exocytosis in synaptic terminals by FM imaging technique

Feng GU¹, Li FU¹, Yong-Jie MA²

¹Department of breast pathology, ²Central Laboratory of Oncology Department, Tianjin Medical University Cancer Institute and Hospital, Key Laboratory of Breast Cancer Prevention and Therapy of the Ministry of Education, Key Laboratory of Cancer Prevention and Therapy of Tianjin, Tianjin 300060, China.

Abstract: Objective Presynaptic voltage-gated Ca^{2+} channels mediate rapid Ca^{2+} influx into the synaptic terminal which triggers synaptic vesicle exocytosis and neurotransmitter release. The FM 1-43 dye was firstly introduced as a fluorescence probe by Betz and his colleagues in 1992, and has been used to monitor exocytosis, endocytosis and endosomal traffic in a variety of cell types. The present study aims to investigate the feasibility of applying the FM 1-43 dye in the functional analysis of calcium channel-mediated exocytosis in synaptic terminals. **Methods** The hippocampi were isolated from embryos of pregnant rats, and hippocampal neurons were then transfected with Ds-Red conjugated plasmid. The neurons were then loaded with 8 $\mu\text{mol/L}$ FM 1-43 and 47 mmol/L KCl for 90 s after transfection. After that, 90 mmol/L KCl was employed to induce FM dye destaining, which was recorded by FM imaging system. **Results** The neuron synaptic terminals of rat hippocampus could be effectively stained by the FM 1-43 dye. Besides, the destaining of the labeled neuron terminals was in accordance with the transmitter release, which could be blocked by the application of nifedipine (inhibitor for L-type calcium channel). **Conclusion** The FM imaging technique is an advanced and effective method for analyzing synaptic vesicle exocytosis and neurotransmitter release, and can be applied in various synaptic functional studies.

Keywords: calcium channel; FM 1-43; hippocampus; neuron

1 Introduction

Neurons are highly polarized, and the structural, functional and molecular differences between their axonal and somatodendritic domains determine their capabilities to receive, process and transmit information. Signal transmission between neurons is achieved via synapses^[1,2], the highly specialized structure of neurons. Presynaptic voltage-gated Ca^{2+} channels mediate rapid Ca^{2+} influx into the synaptic

terminal, which triggers synaptic vesicle exocytosis and neurotransmitter release. Multiple types of Ca^{2+} channels are expressed in neurons^[3,4], including N-type, P/Q-type, L-type, R-type and T-type, among which L-type Ca^{2+} channel plays a predominant role in supporting chemical neurotransmission in synapses^[5-11].

Markers that label neuron terminals in an activity-dependent fashion have been useful tools in neurological research. Although several hydrophilic fluorescent dyes were found to stain motor nerve terminals in the 1980s^[12,13], they do not work well in most animals for unknown reasons. Instead, the styryl dye FM 1-43 can stain the motor nerve terminals in frog, snake and mouse, and the stain intensity depends on the amount of released transmitter^[14]. In addition,

Corresponding author: Yong-Jie MA
Tel: +86-22-23340123-5225; Fax: +86-22-23558945
E-mail: yongjiemagu@yahoo.com.cn
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it has been reported that the stained terminals can be destained in the dye-free medium, and the destaining process also depends on the transmitter release. It has been suggested that the dye labels the membranes of recycled synaptic vesicles, therefore it may be a useful tool for optical studies of vesicle exocytosis and recycling, and for labeling living terminals in a broad range of preparations according to their levels of functional activity.

In this study, the FM 1-43 dye was used to label rat hippocampal neuron terminals, and it was found that the neuron terminals could be stained effectively by FM 1-43, and the destaining process was consistent with the transmitter release, which could be blocked by application of nifedipine (inhibitor for L-type calcium channel). The FM imaging technique using FM 1-43 dye is an advanced method for analyzing synaptic vesicle exocytosis and neurotransmitter release, and is feasible and effective in various synaptic functional studies.

2 Materials and methods

2.1 Hippocampal neuronal culture Hippocampal neuronal culture was carried out as previously described^[1]. Briefly, the hippocampi were isolated from rat embryos at embryonic day 18 and digested with trypsin (Invitrogen) and DNase I (Sigma). Glial cells were seeded in the 24 well plate 1 day before seeding the hippocampal neurons. Neurons were plated on glass coverslips coated with poly-L-lysine (Sigma) and cultured on glial cell monolayer in neurobasal medium supplemented with glucose, B-27 and sodium pyruvate, with a density of 60–80 neurons/cm². Glial cell growth had being inhibited by 4 µmol/L AraC (Sigma) from the day of glial cell seeding.

2.2 FM dye imaging technique FM dye imaging technique was carried out as previously described^[1,15]. A coverslip was mounted in a custom-made, low-volume laminar perfusion chamber on the stage of an inverted microscope. This permitted continuous perfusion at approximately 1 mL/min while imaging through the coverslip to which the cells adhered. Cells were perfused with a modified Tyrode solution containing 119 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl₂, 2 mmol/L MgCl₂, 25 mmol/L Hepes and 30 mmol/L glucose. And 10 µmol/L CNQX and 50 µmol/L APV were also added to reduce

spontaneous activity and prevent recurrent excitation during the stimulation. All the imaging experiments were performed at room temperature. Stock solution of FM 1-43 (500 µmol/L) in water was stored at 4 °C. When incubated for 48 h after transfection, the synaptic vesicles were loaded with FM 1-43 dye by co-treatment with 8 µmol/L FM dye and 47 mmol/L KCl prepared in the Tyrode solution for 90 s. This depolarized the cells and stimulated endocytosis and consequent dye uptake into the vesicles. Then the excess dye was washed away by dye free solution. FM dye destain was performed by adding Tyrode solution containing 90 mmol/L KCl. Images were acquired every 2 s with a cascade 650 camera and MetaFlour 5.0 imaging software using 25 ms exposure to minimize photo bleaching. Fluorescence was visualized with Nikon Inverted Fluorescence Microscope (Nikon, Inc, Tokyo, Japan). In all experiments, more than 10 isolated buttons (about 1 µm²) were analyzed and apparent synaptic clusters were also avoided according to previous report^[1].

2.3 Neuronal transfection For transfection, conditioned medium was removed and the neurons were washed once in minimum essential medium (MEM) and incubated for 30 min with the calcium-phosphate DNA precipitates formed in HEPES-buffered saline. After that, the cells were re-cultured in conditioned medium and analysis was performed 2–4 days after transfection.

3 Results

On day 5 of primary culture, hippocampal neurons were transfected with Ds-Red conjugated vector and the red fluorescence was expressed 48 h after transfection. Morphology of transfected pyramidal neurons and transfected neurons in CA1 or CA3 subfield was observed clearly using inverted fluorescence microscope (Fig. 1A, B; Fig. 2C).

As shown in Fig. 2A, synaptic buttons were loaded with FM dye 1-43 (8 µmol/L) by exposure to Tyrode solution containing 47 mmol/L KCl. After the following application of 90 mmol/L KCl, approximately 96% of the FM 1-43 loaded synaptic buttons showed obvious destaining (Fig. 2B). Individual analysis of the buttons revealed that the kinetics of FM 1-43 dye destaining fit with double-exponential function, with a major fast component and a minor slow component (Fig. 2E),

Fig. 1 Primary cultured hippocampal neurons were transfected with Ds-Red conjugated vector. **A:** a representative of transfected CA2 pyramidal neuron. **B:** a representative of transfected neuron in CA1 or CA3 subfield. Scale bar: 10 μ m.

Fig. 2 Results of FM imaging system using cultured hippocampal neurons. **A:** synaptic boutons of Ds-Red transfected hippocampal neurons were stained by FM dye in loading process; **B:** synaptic boutons destained after 90 mmol/L KCl stimulation; **C:** the same hippocampal neuron expressing Ds-Red protein; **D:** merged photograph of A and C; **E:** kinetics of FM dye imaging system. Destaining process was recorded for more than 150 s. The fluorescence intensity on the application of 90 mmol/L KCl was considered as the reference intensity and 37 boutons were calculated in this experiment. Data were from 8 independent experiments. Scale bar: 20 μ m.

which was similar to previously reported results^[16]. More than 10 buttons were included in each independent experiment. FM dye destaining was very fast during the first period of about 20 s, which corresponded to the readily release of FM 1-43 dye from the vesicles. After the 20 s, the destaining was much slower, which depended on the recruitment of vesicles from the reserve pool. FM dye destaining lasted for almost 100 s after 90 mmol/L KCl stimulation. In the present experiment, the FM dye destaining process was recorded for 120 s or 150 s.

In the present study of presynaptic Ca^{2+} channel function, the fast component that strongly correlated with the rate of Ca^{2+} influx after depolarization was mainly analyzed.

Moreover, the depolarization-induced destaining was reversibly inhibited by 10 $\mu\text{mol/L}$ nifedipine, the specific blocker for L-type Ca^{2+} channel, which confirmed the essential role of voltage-gated Ca^{2+} channel in observed FM 1-43 signals (Fig. 3), and this was consistent with the known sensitivities of voltage-gated Ca^{2+} channels to nifedipine. The continuous reduction in the FM 1-43 signal in the presence of nifedipine resulted from the combined effects of spontaneous vesicle fusion and photo bleach.

Fig. 3 Treatment with L-type Ca^{2+} channel blocker nifedipine inhibited KCl-induced FM destaining. Open triangle represented the nifedipine group. Open circle represented the control group. FM destaining process began to be recorded on the application of 90 mmol/L KCl, when the fluorescence intensity was considered as the reference intensity. FM destaining process was recorded for 120 s. Data were from 5 independent experiments.

4 Discussion

The key property of FM 1-43 dye as a fluorescence probe was firstly introduced by Betz and his colleagues in 1992^[14].

Since then, it has been used to monitor exocytosis, endocytosis and endosomal traffic in a variety of cell types. At the central nervous system synapses, the FM 1-43 dye has been used as an optical marker for studying vesicle dynamics using a light microscope^[16-19]. The photoconversion of FM 1-43 has provided valuable information about the vesicle number in a readily releasable pool^[20].

The mechanisms of FM 1-43 staining and its persistence may be a combination of its high affinity for lipid membrane and the inability to penetrate membrane completely, which could be supported by the FM 1-43 structure—the formations of one hydrophobic tail and one hydrophilic moiety at the opposite ends, respectively^[21].

FM dye is not only used in labeling neuron terminals, but also applied in cancer studies. It has been reported that the FM 1-43 dye technique could be used in studying the membrane endocytic activities in strongly metastatic and weakly metastatic prostate cancer cells^[22]. In these cancer cells, the FM 1-43 dye labels the plasma membrane first, and then the vesicular membranes. Meanwhile, little FM 1-43 dye is released, suggesting that the exocytotic route only plays a minor role in the dye release from these cells.

By using FM imaging system, we demonstrate that the FM imaging technique is an advanced method to analyze synaptic vesicle exocytosis and neurotransmitter release, consistent with previous reports. The styryl dye FM 1-43 is a useful tool for further cell biological studies on the endocytosis and exocytosis in neurons and other types of cells.

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References:

- [1] Anton M, Ilya B. Synaptic targeting of N-type calcium channel in hippocampal neurons. *J Neurosci* 2002, 22 (16): 6939-6952.
- [2] Crang AM, Banker G. Neuronal polarity. *Annu Rev Neurosci*

- 1994, 17: 267-310.
- [3] Anton M, Thomas CS, Ilya B. Association of neuronal calcium channels with modular adaptor proteins. *J Biol Chem* 1999, 274 (35): 14453-24456.
- [4] Tsien RW, Ellinor PT, Horne WA. Molecular diversity of voltage-dependent Ca channels. *Trends Pharm Sci* 1991, 12: 349-354.
- [5] Williams ME, Brust PF, Feldman DH, Patthi S, Simerson S, Maroufi A, *et al.* Structure and functional expression of an ω -conotoxin-sensitive human N-type calcium channels. *Science* 1992, 257: 389-395.
- [6] Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, *et al.* Nomenclature of voltage-gated calcium channels. *Neuron* 2000, 25: 533-535.
- [7] Mori Y, Friedrich T, Kim M-S, Mikami A, Nakai J, Ruth P, *et al.* Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* 1991, 350: 398-402.
- [8] Takahashi T, Momiyama A. Different types of calcium channels mediate central synaptic transmission. *Nature* 1993, 366: 156-158.
- [9] Wheeler DB, Randall A, Tsien RW. Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science* 1994, 264: 107-111.
- [10] Dunlap K, Luebke JI, Turner TJ. Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurosci* 1995, 18: 89-98.
- [11] Li K, Xu E. The role and the mechanism of gamma-aminobutyric acid during central nervous system development. *Neurosci Bull* 2008, 24(3): 195-200.
- [12] Lichtman JW, Wilkinson RS. Properties of motor units in the transversus abdominis muscle of the garter snake. *J Physiol* 1987, 393: 355-374.
- [13] Lichtman JW, Wilkinson RS, Rich MM. Multiple innervation of tonic endplates revealed by activity-dependent uptake of fluorescent probes. *Nature* 1985, 314: 357-359.
- [14] Betz WJ, Mao F, Bewick GS. Activity dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J Neurosci* 1992, 12(2): 363-375.
- [15] Waters J, Smith SJ. Vesicle pool partitioning influences presynaptic diversity and weighting in rat hippocampal synapses. *J Physiol* 2002, 541: 811-823.
- [16] Betz WJ, Bewick GS. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science* 1992, 255: 200-203.
- [17] Murthy VN, Steven CF. Reversal of synaptic vesicle docking at central synapses. *Nat Neurosci* 1999, 2(6): 503-507.
- [18] Ryan TA, Reuter H, Smith SJ. Optical detection of a quantal presynaptic membrane turnover. *Nature* 1997, 388: 478-482.
- [19] Klingauf J, Kavalali ET, Tsien RW. Kinetics and regulation of fast endocytosis at hippocampal synapses. *Nature* 1998, 394: 581-585.
- [20] Schikorski T, Steven CF. Morphological correlates of functionally defined synaptic vesicle populations. *Nat Neurosci* 2001, 4 (4): 391-395.
- [21] Nobutoshi H, Timothy AR, Stephen JS, Joann B, Richard T. Visualizing recycling synaptic vesicles in hippocampal neurons by FM 1-43 photoconversion. *Proc Natl Acad Sci U S A* 2001, 98(22): 12748-12753.
- [22] Mycielska ME, Schäfer M, Fraser SP, Djamgoz MB, Bashford CL. A study of membrane activity in rat prostate cancer cells: an evaluation of the FM1-43 dye technique. *Biosci Rep* 2000, 20 (1): 31-40.

利用 FM 成像方法检测突触前膜钙离子通道介导的神经递质释放

谷峰¹, 付丽¹, 马勇杰²

天津医科大学¹附属肿瘤医院乳腺病理研究室,²肿瘤研究所中心实验室, 教育部乳腺癌防治重点实验室, 天津市乳腺癌防治重点实验室, 天津 300060

摘要: 目的 突触前膜上的钙离子通道介导钙离子内流, 从而实现突触小泡的胞吐以及神经递质释放。1992年Betz等首次发现FM 1-43染料可以作为一种标记荧光用于科学研究。目前, FM 1-43染料已被广泛用于检测各种细胞类型的胞吞和胞吐过程。本研究旨在探讨将FM 1-43染料用于检测突触前膜钙离子通道介导的神经递质释放的可行性。方法 取孕鼠进行原代大鼠神经元的培养, 利用带有Ds-Red标记的质粒进行转染, 48小时后经8 $\mu\text{mol/L}$ 的FM 1-43染料和47 mmol/L的KCl孵育90秒, 然后用90 mmol/L的KCl再次孵育, 其间用FM 1-43成像技术记录代表突触的亮点荧光衰减的过程。结果 FM 1-43染料能有效标记大鼠海马神经元突触末端, 刺激后被标记部位的荧光强度衰减的过程与神经递质的释放过程相一致, 并且这个过程可以被L型钙离子通道抑制剂硝苯地平抑制。结论 FM 1-43成像技术能够检测钙离子介导的神经递质的释放过程, 是一种非常先进并切实有效的检测神经元突触末端神经递质释放过程的手段和方法。

关键词: 钙通道; FM 1-43染料; 海马; 神经元