SPONTANEOUS HIPPOCALCIN TRANSLOCATION IN HIPPOCAMPAL NEURONS

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A research team, headed by Dr. ***Pavel Belan****, uses electrophysiological and imaging techniques to study synaptic transmission and Ca^{2+}-dependent signaling in the hippocampus. Dr. Pavel Belan graduated form Moscow Institute of Physics and Technology in 1985. In the same year he has began to work at the Bogomoletz Institute of Physiology where he defended his Ph.D. thesis in Biophysics in 1991. After an initial postdoctoral work at Roche Institute of Molecular Biology (USA), he obtained further training at the University of Liverpool (UK). In 1998, he received a faculty appointment at Bogomoletz Institute of Physiology. Dr. Belan received his Doctor of Science degree in Biophysics at Bogomoletz Institute of Physiology in 2005 and in the same year he was appointed as a leading scientist in the Department of General Physiology of the CNS of this Institute.*

Dr. Belan is particularly well known for his research and expertise in fields of calcium signaling in different types of excitable and non- excitable cells and of synaptic transmission in the hippocampus. Dr. Belan is an active member of the Society for Neuroscience (USA), Physiological Society (UK), Ukrainian Biophysical and Physiological societies. He is in the Board of Ukrainian Society for Neuroscience. Dr. Belan has been awarded by many national and international grants. Dr. Belan has established a number of world-wide scientific contacts. Results, obtained by Dr. Belan and his co-authors have been published in high-rating scientific journals such as Journal Physiology, Journal of Biological Chemistry, Cell etc. Currently Dr. Belan teaches graduate students at Kiev Branch of Moscow Institute of Physics and Technology and at Taras Shevchenko National University.

The photo shows a student of P.G. Kostyuk, Prof. P. Belan.

Introduction

Hippocalcin is a Ca^{2+}-binding protein, which belongs to the family of neuronal Ca^{2+} sensors (Burgoyne, 2007). It is highly expressed in the hippocampus but physiological role and molecular mechanisms underlying its action in this part of

the brain have not been investigated in detail. The molecular mechanism, by which hippocalcin operates, is thought to be a Ca^{2+}/myristoyl switch (Burgoyne, 2007). Hippocalcin is N-terminally myristoylated and myristoyl group is sequestered in the Ca^{2+} free form of the protein and following Ca^{2+}-binding a substantial conformational change allows extrusion of the lipophilic myristoyl group (Ames et al., 1997) resulting in the protein translocation from cytosol to membranes. It is obvious that neurons might use this property of hippocalcin in signal transduction processes (Kobayashi et al., 1993; O'Callaghan et al., 2003). Hippocalcin as well as other neuronal Ca^{2+} sensor proteins are discussed to play many roles in calcium-dependent signal transduction of physiological and pathological processes in the central nervous system. In spite of clear importance of hippocalcin-dependent processes in neuronal functioning hippocalcin translocation during intrinsic neuronal activity in living hippocampal neurons have not been investigated yet. Recently we have observed spontaneous translocation of hippocalcin tagged by Yellow Fluorescent Protein (HPCA-YFP) in dendrites of hippocampal neurons growing in primary cultures (Markova et al., 2008). In the present short report we have studied spatio-temporal profiles of this translocation.

Methods

Tissue cultures. Hippocampi from newborn rats were enzymatically dissociated with pronase E. Cell suspension at the initial density of $3\text{-}5 \times 10^5$ cells per cm^2 was plated on glass coverslips coated with laminine and poly-L-ornithine. Cells were maintained in feeding solution consisted of minimal essential medium, 10% horse serum and other necessary additives in humidified atmosphere containing 5% CO_2 at 37 °C.

Plasmids. EYFP-tagged hippocalcin (HPCA-YFP) was made as described previously (O'Callaghan et al., 2002).

Transient transfection. Hippocampal neurons were transfected after 5-9 days in culture using the DNA-calcium phosphate precipitation method essentially as described by a supplier (ProFection Mammalian Transfection System, Promega). All cultures were used for the experiments in 2-5 days after transfection.

HPCA translocation and $[Ca^{2+}]_i$ imaging. Time lapse video imaging of transiently transfected hippocampal neurons was performed using TILL Photonics imaging system (TILL Photonics, Germany) running under TILLvision software. An acquisition rate of Imago CCD camera was varied to precisely record fast changes in HPCA-YFP fluorescence and typically was in a range of 0.1-1 Hz.

For $[Ca^{2+}]_i$ imaging neurons were loaded with a ratiometric calcium indicator fura-2 (Invitrogen, USA).

Monochromator based excitation allowed to measure fura-2 (at excitation wavelengths of 355 and 380 nm), HPCA-YFP (at excitation wavelengths of 490 nm) and CFP (at excitation wavelengths of 435 nm) fluorescence using the same set of optical elements.

Results

In the rat hippocampus, hippocalcin mRNA and immunoreactivity first appeared in the CA3 pyramidal cells on embryonic day 19 and postnatal day 1, respectively (Saitoh et al., 1994). At the same time a high level of hippocalcin expression and its localization to both neuronal soma and processes were observed in primary hippocampal cultures beginning with 4-6 DIV (Palmer et al., 2004). Thus, the hippocampal cultured neurons used in this study (6-12 DIV) should express hippocalcin and have the whole set of endogenous molecular mechanisms involved in hippocalcin trafficking, subcellular localization, and possible translocation during Ca^{2+}-dependent activation.

Spontaneous activity is observed in both the hippocampus and primary hippocampal cultures during early postnatal development. This activity should result in fast (rising time substantially less than a second) elevation of $[Ca^{2+}]_i$. Based on our previous results [7] showing that hippocalcin can perform a calcium-myristoyl switch in non-neuronal cells and translocate in less than a second we specifically searched for spontaneous localized elevations in HPCA-YFP fluorescence occurring in a time window of 0.5-2.0 sec.

Real-time imaging of HPCA-YFP fluorescence performed at acquisition rates between 0.5 to 2.0 Hz revealed many sites, especially on the plasma membrane, where HPCA-YFP fluorescence was gradually changed within a dendritic tree and axon of transfected neurons (data not shown). However, close inspection of these sites showed that the observed changes were mainly due to neuronal process movement rather than due to changes in HPCA-YFP fluorescence. Therefore, in the most following experiments we co-transfected hippocampal neurons with plasmids carrying genes of HPCA-YFP and cyan fluorescent protein, CFP, and used the latter as a control for morphological re-arrangement. A customized routine written in TILLvision software has been used to calculate relative changes in HPCA-YFP fluorescence against CFP fluorescence background and to determine the sites of genuine changes of HPCA-YFP fluorescence.

Fig. 1 represents an example of fast spontaneous elevations in HPCA-YFP fluorescence observed in several local sites within a dendritic tree of cultured hippocampal neuron. These transient elevations had a rising time of 1-5 sec and a decay time of 5-10 sec. They repeatedly occurred in the same places within a dendritic tree and each of them was spatially restricted to the area of 1-5 μm. Transient amplitudes were in a range from several up to 100% percent of initial level of fluorescence. In all cases of these fast transient elevations HPCA-YFP fluorescence was completely reversed to its initial value. At the level of optical resolution of our equipment we could not resolve if the increases in fluorescence occurred in the plasma membrane or in some intradendritic compartments. At the same time no changes in CFP fluorescence were observed in the sites under study indicating genuine increase in HPCA-YFP fluorescence rather than morphological re-ar-

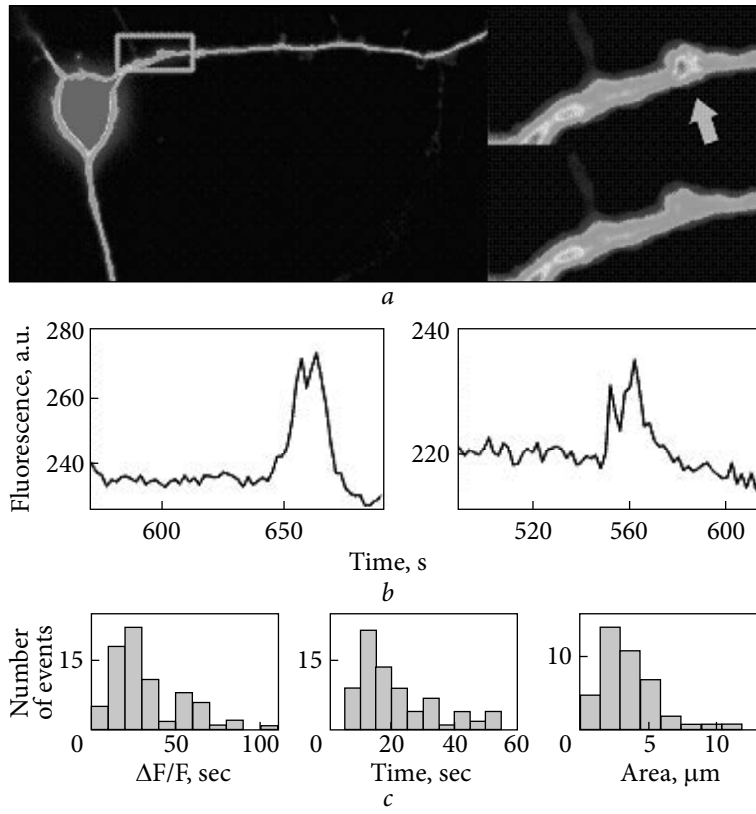

Fig. 1. Properties of short hippocalcin-YFP translocation: *a* — time-lapse fluorescent images of axon of neuron co-transfected with HPCA-YFP and CFP plasmids. HPCA-YFP fluorescence is show in green whereas CFP fluorescence is show in blue. Time courses of changes in HPCA-YFP and CFP fluorescence along a yellow curve depicted in the upper image are shown in *b*. *b* — Spatio-temporal profiles of HPCA-YFP (*1*), CFP (*2*) changes and their overlay (*3*). Horizontal color arrows indicate places from which time course of changes in HPCA-YFP fluorescence are present in *c*. Colors of arrows in *b* match colors of curves in *c*. Ratio of HPCA-YFP fluorescence along white vertical lines (right before and at a maximum of long translocation) is shown in *d*

rangement (Fig. 1). Phenomenological properties of HPCA-YFP translocation are shown in Fig. 1, *c-e*.

A robust increase in HPCA-YFP florescence in some sites coincided with moderate but significant decrease in the neighboring (10-20 μm) sites indicating longitudinal rather than transverse translocation of HPCA-YFP (data not shown). An average total value of fluorescence in the ROI covering the whole area of fluorescence changes was practically constant during the spontaneous event (data not shown) indicating that genuine translocation of HPCA-YFP was a reason of the observed spontaneous fluorescence changes. Some translo-

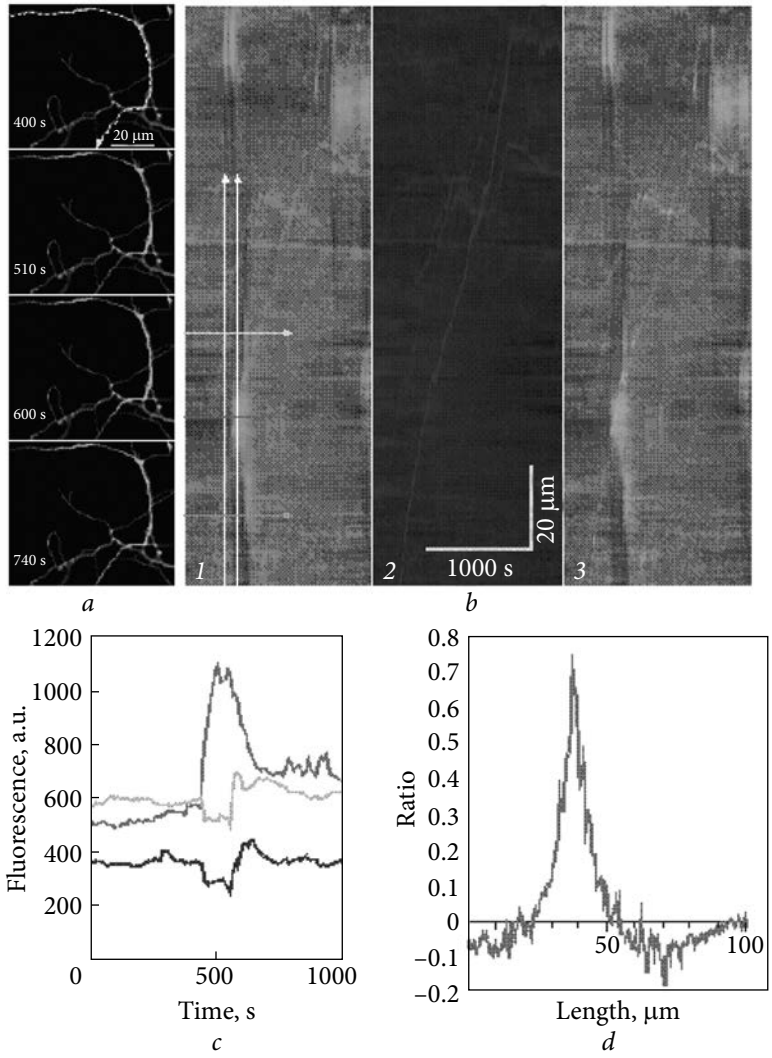

Fig. 2. Spatio-temporal profiles of long HPCA-YFP translocation: *a* — time-lapse fluorescent images of axon of neuron co-transfected with HPCA-YFP and CFP plasmids. HPCA-YFP fluorescence is show in green whereas CFP fluorescence is show in blue. Time courses of changes in HPCA-YFP and CFP fluorescence along a yellow curve depicted in the upper image are shown in *b*. *b* — Spatio-temporal profiles of HPCA-YFP (*a*), CFP (*b*) changes and their overlay (*c*). Horizontal color arrows indicate places from which time course of changes in HPCA-YFP fluorescence are present in *c*. Colors of arrows in *b* match colors of curves in *c*. Ratio of HPCA-YFP fluorescence along white vertical lines (right before and at a maximum of long translocation) is shown in *d*

cation events took place synchronously in remotely situated regions of dendritic tree indicating that a fast synchronization, possibly due to action potentials, underlies this fast translocation.

Besides observing the fast translocation we have also observed another type of HPCA-YFP behavior. It was long (sometimes longer than 100sec) plateaus, during which protein concentration in translocation sites could be increased 2-3 times. Fig. 2 represents an example of such HPCA-YFP behavior. It is clearly seen that HPCA-YFP was temporally and robustly collected several times within several sites of a particular branch of neuronal dendritic tree (Fig. 2, *b*). At the same time no changes in CFP fluorescence was observed in these sites (Fig. 2, *c*) indicating protein-specific translocation. Spatio-temporal properties of long translocation are shown in Figure 2 *d* and *e*. During this type of translocation events HPCA-YFP was collected to translocation sites from a region covering up to 50-100 μm along neuronal process. This type of translocation was relatively rear compared to the fast translocation and never occurred in a synchronous way, indicating that different molecular mechanisms are engaged in generation of fast and long translocation.

Most probably that biophysical molecular mechanisms underlying both types HPCA-YFP translocation are similar at the level after $[Ca^{2+}]_i$ increase and are as follows. Ca^{2+}-myristoyl switch (Ames et al., 1997; O'Callaghan et al., 2003) taking place after $[Ca^{2+}]_i$ elevation all over the segment resulted in initial hippocalcin translocation (from the cytosol to plasma membranes) only in a part of the segment where hippocalcin affinity to the plasma membrane was higher than in other parts (Haynes et al., 2006). It led to a decrease in concentration of cytosolic hippocalcin in this part of the segment, diffusion 'sticky' (Ca^{2+} bound) hippocalcin molecules from neighboring parts of the segment and their additional insertion in the site of translocation. Thus, the main part of hippocalcin translocated to a 'hot' site did it via diffusion in the cytosol and, therefore, the rising phase of translocation transients can be fully explained by cytosolic diffusional delay which in a line with our observation. At the same time the decaying phase should be mainly determined by slow dissociation of membrane-bound hippocalcin rather than by hippocalcin diffusion from a site of translocation.

Thus, we have shown that:

1. Hippocalcin may spontaneously translocate to many sites within a dendritic tree of cultured hippocampal neurons during intrinsic neuronal activity.

2. Hippocalcin signaling takes place in diffusionally restricted dendritic compartments that work as independent units.

3. Two different patterns of observed spontaneous HPCA-YFP translocation may contribute to different signaling process within the hippocampal neurons.

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