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Inositol-1,4,5-trisphosphate 3-kinase A regulates dendritic morphology and shapes synaptic Ca^{2+} transients

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ABSTRACT

Inositol-1,4,5-trisphosphate 3-kinase-A (itpka) accumulates in dendritic spines and seems to be critically involved in synaptic plasticity. The protein possesses two functional activities: it phosphorylates inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and regulates actin dynamics by its F-actin bundling activity. To assess the relevance of these activities for neuronal physiology, we examined the effects of altered itpka levels on cell morphology, $Ins(1,4,5)P_3$ metabolism and dendritic Ca^{2+} signaling in hippocampal neurons. Overexpression of itpka increased the number of dendritic protrusions by 71% in immature primary neurons. In mature neurons, however, the effect of itpka overexpression on formation of dendritic spines was weaker and depletion of itpka did not alter spine density and synaptic contacts. In synaptosomes of mature neurons itpka loss resulted in decreased duration of $Ins(1,4,5)P_3$ signals and shorter $Ins(1,4,5)P_3$-dependent Ca^{2+} transients. At synapses of itpka deficient neurons the levels of $Ins(1,4,5)P_3$-5-phosphatase (inpp5a) and sarcoplasmic/endoplasmic reticulum calcium ATPase pump-2b (serca2b) were increased, indicating that decreased duration of $Ins(1,4,5)P_3$ and Ca^{2+} signals results from compensatory up-regulation of these proteins. Taken together, our data suggest a dual role for itpka. In developing neurons itpka has a morphogenic effect on dendrites, while the kinase appears to play a key role in shaping Ca^{2+} transients at mature synapses.

1. Introduction

Inositol-1,4,5-trisphosphate 3-kinase-A (itpka) is the F-actin binding and neuron specific isoform of the itpk family [1,2]. Itpka mRNAs are present in hippocampus, cerebellum and neocortex [3] and the respective proteins accumulate in dendritic spines [4]. Both, itpka and $Ins(1,4,5)P_3$-5-phosphatase catalyze the metabolism of the Ca^{2+}-mobilizing second messenger inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) to inositol-1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) and inositol-1,4-bisphosphate ($Ins(1,4)P_2$), respectively. The most active $Ins(1,4,5)P_3$-5-phosphatase is inpp5a [5,6], exhibiting a higher basal specific enzyme activity than itpka (25 $\mu mol_* mg^{-1}{}_* min^{-1}$ [6], vs. 5–10 $\mu mol_* mg^{-1}{}_* min^{-1}$ [7,8]). Thus, under basal conditions dephosphorylation of $Ins(1,4,5)P_3$ occurs faster than phosphorylation by itpka.

In addition to its enzymatic function, itpka bundles actin filaments leading to induction or elongation of cellular protrusions after overexpression of itpka [9–12]. The physiological function of itpka is not completely understood but the protein seems to be involved in the regulation of long-term potentiation (LTP) and/or long-term depression (LTD) as its levels increase during spatial learning training [13] and itpka-deficient mice exhibit increased LTP in the CA1 region of the hippocampus [14] and decreased LTP in the dentate gyrus [15]. However, the molecular mechanism controlling itpka-mediated regulation of synaptic plasticity has not yet been elucidated. Here we examined the effect of different itpka levels on morphology and on $Ins(1,4,5)P_3$-dependent Ca^{2+} signaling in developing and mature hippocampal dendritic spines.

2. Experimental procedures

2.1. Vectors

In mRFP-itpka, the coding region of itpka cDNA (nucleotide 12 to 1398, NCBI accession number NM_002220.1) was cloned into a modified pEGFP-C1 vector (Clontech, Mountain View, CA, USA), in which the enhanced green fluorescent protein (EGFP) cDNA has been exchanged for a corresponding region encoding monomeric red fluorescent protein (mRFP). pEGFP-γ actin was purchased from Clontech/Takara (Saint-Germain-en-Laye, France).

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2.2. Animals, protein preparation and Western blotting

The itpka knock out mouse line (*itpka*$^{-/-}$) was provided by Hee-Sup Shin (Pohang, Korea). Animals were genotyped by both PCR and by Western blotting using a goat polyclonal itpka antiserum (Santa Cruz, sc-11206, Santa Cruz, CA, USA). Western blot analysis was performed as described [10] with antibodies against itpka, Ins (1,4,5)P_3-5-phosphatase (inpp5a) (Sigma-Aldrich, HPA012285, Steinheim, Germany), sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump-2 (Santa Cruz, sc-8094) actin (Sigma-Aldrich, A-2066) and Hsc70 (Santa Cruz, sc-7298, Santa Cruz, CA, USA), respectively.

2.3. Cell culture, transfection, immunocytochemistry and Golgi-Cox staining

Rat and mouse primary neurons were grown in NEUROBASAL™ medium (Invitrogen, Darmstadt, Germany). Transfection of rat primary neurons was performed eight days after plating (at DIV8 (days in vitro)) and the cells were fixed at DIV9 and DIV21, respectively [16]. Immunocytochemistry was performed as described [16] with primary antibodies directed against MAP2 (Sigma-Aldrich, 1:200 dilution), drebrin (Abcam, dilution 1:200) and vGlut (Abcam, dilution 1:200), respectively, followed by Alexa Fluor®568 or Alexa Fluor®468 coupled secondary antibodies (Invitrogen, Darmstadt, Germany). Images were captured with a confocal microscope (LSM 510, Zeiss MicroImaging, Göttingen, Germany). Golgi-Cox staining of mouse brains was performed as described [17].

2.4. Measurement of Ins(1,4,5)P_3 metabolism in hippocampal lysates and synaptosomes

Hippocampi from 15 week old wild type (wt) and *itpka*$^{-/-}$ mice were isolated. Three hippocampi each were pooled, pottered in lysis buffer (20 mM Hepes pH 7.5, 0.5% NP-40, 5 mM EDTA, 0.3 M NaCl, 1 mM DTT, 1 mM PMSF), sonified twice for 20 s and frozen in liquid nitrogen. After thawing, samples were centrifuged for 10 min at 1.500×*g* and 4 °C, and the protein concentration of the supernatant was determined by using the Bio-Rad Protein Assay Kit (BIO-RAD, Munich, Germany). For analysis of Ins(1,4,5)P_3 metabolism at synapses, synaptosomes were isolated as described [18]. One ml of synaptosome fraction was lysed with 0.1 ml 10 x lysis buffer (see above) and its protein concentration was determined as described above. Two-hundred and fifty μg protein of each hippocampal and synaptosome lysate were employed for measuring metabolism of 8 μM Ins(1,4,5)P_3 in 1.6 ml reaction buffer and aliquots sampled (150 μl) were analyzed by MDD-HPLC. The assay conditions were exactly the same as described [11].

2.5. Ca^{2+} imaging in neuronal cultures

Specific phospholipase C (PLC)-induced Ca^{2+} transients were measured in hippocampal neurons at DIV17. Formation of spines in these cells was controlled by drebrin labeling of parallel samples (see Fig. 4A). To specifically stimulate PLC-mediated production of Ins (1,4,5)P_3, metabotropic glutamate receptors were activated by 200 μM 3.5 dihydroxyphenylglycine (DHPG, Sigma-Aldrich, Steinheim, Germany). In addition, ionotropic glutamate receptors were inhibited by 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM, Sigma-Aldrich, Steinheim, Germany) and DL-2-amino-5-phosphopentanoic acid (AP5, 50 μM, Sigma-Aldrich, Steinheim, Germany) and the measurements were performed in Ca^{2+}-free medium in order to avoid calcium influx from the extracellular space.

Cells were loaded with Fura 2-AM (Invitrogen, Darmstadt, Germany) and transferred to a recording chamber, where they were constantly perfused with physiological bath solution (160 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose) at 2.5 ml/min. Prior to the experiment, the physiological bath solution was switched to the same solution lacking $CaCl_2$ and supplemented with 0.5 mM EGTA. The cells were visualized with an Axioskop 2F microscope (Zeiss, Göttingen, Germany) equipped with a 63 × objective (Zeiss) and an iXon CCD Camera (Andor Technology, Belfast, North Ireland). Fura-2 measurements were carried out with alternating excitation wavelengths of 380 and 340 nm at a 0.25 Hz sequential frame-rate using a Polychrome V monochromator device controlled by TILLvisION Software (Till Photonics, Munich, Germany). The 1002×1004 pixel arrays were symmetrically binned 2 times. For analysis, regions of interest (ROIs) were defined from baseline fluorescence images. ROIs were arranged around morphologically identified dendritic protrusions (1–2 μm in diameter). The data were corrected for bleaching by dividing the fluorescence obtained at 340 nm excitation wavelength by the one obtained at 380 nm. Relative changes in intracellular Ca^{2+} were quantified by calculating ΔF/F in %, were F is the baseline fluorescence measured before DHPG application and ΔF is the change in fluorescence intensity in the presence of DHPG. Using Axograph X Software (Dr. John Clements, http://axographx.com) we analysed the peak amplitude of Ca^{2+} transients and fitted the rise and decay phase with the sum of two exponential functions. The distances between the two flanks of the exponentials at 50 and 30% of the peak amplitude were used as a measure of Ca^{2+} signal duration as described [11].

2.6. Statistical analysis

Statistical comparisons of normalized values were made using Student's *t*-test for unpaired samples. Relative concentrations of Ins (1,4,5)P_3 over time were tested by linear regression. *P*-values ≤ 0.05 were taken to indicate significant differences between groups.

3. Results

3.1. Itpka levels increase during rat brain development

To analyze itpka levels during brain development, Western blots were performed from lysates of rat hippocampus, neocortex and cerebellum of late embryonic and postnatal rat brains. As shown in Fig. 1A, the itpka concentration increased with age in all three brain areas, whereby in the hippocampus itpka was already present at E19. To quantify these results, itpka specific luminescence was measured and normalized against the corresponding actin signal intensity (results are termed nITPKA value) (Fig. 1B). Normalization of the neocortical and cerebellar nITPKA values against the hippocampal nITPKA value (arbitrarily set to 100%) showed that at P28 ITPKA levels in the neocortex and cerebellum are about 60% and 35% of the hippocampal kinase concentration, respectively. This result was confirmed by analyzing P28 extracts from all three tested brain regions (Fig. 1C).

As itpka has been shown to accumulate in the postsynaptic density (PSD) [4], protein levels in PSD fractions from hippocampi of different developmental states were examined (Fig. 1D). Distinct from whole hippocampal lysates, itpka was first detectable in P21 PSD samples, and its concentration further increased in PSD fractions from P84 mice. Similar to itpka, inpp5a also accumulates in PSDs of the adult brain, whereas in developing neurons (P1, P3, P7) only low amounts of the protein were detected (Fig. 1E). Thus, in neurons two Ins(1,4,5)P_3-metabolizing enzymes coexist in the postsynaptic compartment of excitatory synapses.

3.2. Overexpression of itpka induces formation of dendritic protrusions in primary rat hippocampal neurons

To further assess the cellular function of itpka in hippocampal neurons the kinase was overexpressed in primary neurons. Protein