Protein kinase D-mediated effects on the actin cytoskeleton of dendritic spines and on memory formation

PhD thesis

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Introduction

Almost 90 % of the excitatory synapses of the human brain terminate at the dendritic spines. The actin cytoskeleton determines predominantly the shape and the size of the dendritic spines (Hotulainen and Hoogenraad, 2010). During neuronal plasticity, the strength and the number of synaptic connections change according to the actual neuronal inputs. As it is generally accepted, extension of the dendritic spine head serves as the structural basis of learning and memory. During this process, the postsynaptic density (PSD) is extended, providing the stabilization of a higher amount of neurotransmitter receptors in the postsynaptic membrane. As a result, the efficiency of synaptic transmission is increased. Activity-dependent rearrangement of the dendritic spines is regulated by several proteins. There is intensive research ongoing to reveal their complex interactions.

Protein kinase D (PKD) was first described in 1994 (Johannes et al., 1994; Valverde et al., 1994) and belongs to a recently separated group of serine/threonine kinases. All three PKD isoforms (PKD1, PKD2 and PKD3) participate in many cellular functions. It has been described in non-neuronal cells that PKD controls gene transcription (Bossuyt et al., 2011), participates in the modulation of intracellular signal transduction (Wang et al., 2002) and in the realignment of the actin cytoskeleton (Olayioye et al., 2013). In addition, PKD isoforms play important role in the regulation of membrane recycling, as well (Hausser et al., 2005).

In the central nervous system, PKD is expressed in large amount already from embryonic development, but only a few PKD-dependent neuronal functions have been identified so far. Our research team has previously showed that PKD takes part in the maintenance of the integrity of the Golgi apparatus as well as in the preservation of the intact dendritic arborization (Czöndör et al., 2009). In addition, PKD may exert neuroprotective effect during elimination of the reactive oxygen species released in case of cerebrovascular accidents (Sánchez-Ruiloba et al., 2014). It is already proven that PKD plays important role in controlling membrane protein transport in neurons (Bisbal et al., 2008). However, no convincing evidence was available about the role of PKD in neuronal plasticity, therefore I focused on this topic in my doctoral work.

Main goals

During my doctoral study, I examined PKD-mediated regulation of neuronal plasticity using mouse embryonic hippocampal neuronal cultures and transgenic mice producing a dominant-negative mutant form of PKD in an inducible and tissue-specific manner. I aimed to answer the following questions in my study:

- Can the activation of PKD be demonstrated during the morphological changes of dendritic spines induced by different chemical stimulations?
- Through which signal transduction pathways does PKD regulate the morphological rearrangements of dendritic spines?
- How do the expression of a dominant-negative, mutant PKD form and the pharmacological inhibition of PKD activity change the morphology of the dendritic spines in hippocampal cell cultures?
- How does the dominant-negative, mutant form of PKD influence the shape of the dendritic spines in hippocampal pyramidal neurons, located within the CA1 and CA3 regions of the hippocampus?
- How does the expression of a dominant-negative mutant PKD affect general motor performance, cognitive memory and the hippocampus-related spatial learning of the experimental animals?

Applied methodology

- preparation and maintenance of primary hippocampal neuronal cultures
- Lipofectamine2000 based transfection of primary hippocampal neuronal cultures – expression of fluorescently labelled proteins

abbreviation	plasmids	reference
actin-EGFP	pEGFP-N1-actin	Clontech
EGFP	pEGFP-N1	Clontech
kdPKD-EGFP	pEGFP-N1-PKD1 K612W	Hausser et al., 2002
PKD activity reporter	pEGFP-C1-L-pS294-L	Czöndör et al., 2009
PKD activity reporter S/A	pEGFP-C1-L-pS294A-L	Czöndör et al., 2009

Table 1. Fluorescently labelled constructs used in transfection studies

- chemically induced long-term potentiation and long-term KCl treatment in hippocampal neurons
- immunocytochemical stainings carried out on fixed primary hippocampal neurons
- microscopic analyses:
  - visualising endogenous PKD activity by detecting PKD specific phosphorylation in neurons expressing a PKD activity reporter construct
  - fluorescent live cell imaging of transfected neurons to investigate the motility of filopodial dendritic protrusions

- quantitative analysis of phosphorylated cofilin levels within dendritic spine heads
- analysing the morphology and density of dendritic spines in transfected hippocampal neurons

- behavioural studies:
  - spontaneous motor performance was investigated using the open field test
  - novel object recognition test was used to compare memory formation and recall after 5h retention time
  - spatial orientation and learning abilities were measured in the radial arm maze and using the Morris water maze

Results

1. Endogenous PKD is active within the dendritic spines

Activity of PKD in the dendritic spines was examined with the use of the PKD activity reporter (Czöndör et al., 2009), suitable to visualize endogenous PKD-mediated phosphorylation events in fixed cells. PI4KIIIβ is a known target of PKD and gets selectively phosphorylated by PKD on S294, which can be identified with a phospho-specific antibody recognizing the phosphorylated site. We have examined the changes in PKD activity by inducing different types of plasticity in cultivated neurons. Prolonged depolarization[1] by excess KCl and chemically induced LTP by glycine (cLTP) are known to result in long-term, long-lasting increase of dendritic spine heads (Park et al., 2006; Grubb and Burrone, 2010).

The experiments performed with PKD activity reporter prove that PKD activity was rapidly increased at 5 and 30 minutes after the cLTP treatment, which was reduced by applying a PKD-specific inhibitor (kbNB 142-70). We confirmed that elevated PKD activity was inhibited by APV treatment applied during cLTP, showing that NMDA receptors also play a role in PKD activation.

In case of KCl-induced depolarization, PKD activity within the dendritic spines was elevated after 30 and 120 minutes, which was inhibited by the PKD-specific inhibitor (kbNB 142-70). The detailed elucidation of the depolarization-induced molecular mechanisms resulting in elevated PKD activity is still needed. Nevertheless, we assume that NMDA

[1] Potassium ion level raised to 25 mM increased the resting membrane potential (-28.8 ± 0.9 mV), leading to a prolonged depolarization block of neuronal activity. Long-term depletion of action potentials induces homeostatic upscaling in neurons, accompanied by distinct morphological changes (Wefelmeyer et al., 2016).

receptors are involved as MK-801 (a non-competitive antagonist of the NMDA receptors) significantly reduced KCl-induced PKD activity.

2. Inhibition of PKD activity increases the motility of dendritic filopodia *in vitro*

We have examined the motility of dendritic filopodia in living hippocampal neuronal cultures. We have analyzed the records with the help of the DFMA (Dendritic Filopodia Motility Analyzer) plugin running under the framework of NIH ImageJ/Fiji (developed by our research team; Tárnok et al., 2015).

PKD activity was inhibited via the expression of a dominant-negative PKD (kdPKD-EGFP) mutant as well as by treating the cultures with a PKD specific inhibitor (kbNB 142-70). Our results indicate that the motility of dendritic filopodia increased significantly upon impaired PKD activity.

3. PKD regulates the dynamics of the actin cytoskeleton by inactivating cofilin

It was described in non-neuronal cells that PKD promotes the stabilization of the actin cytoskeleton by regulating cofilin activity (Olayioye et al., 2013), known to influence rearrangements of the actin cytoskeleton (Mizuno, 2013). PKD indirectly increases the phosphorylation of the 3rd serine of cofilin (Ser3), leading to cofilin inactivation.

Phosphorylated Ser3 cofilin level was analyzed in the head of the dendritic spines with quantitative microscopy. Phosphorylated cofilin level was reduced in the head of the dendritic spines fixed 30 minutes after the onset of cLTP, when the F-actin system is in the condition of dynamic reorganisation (early LTP; Bosch et al., 2014). We observed a similar effect in the presence of the PKD-specific inhibitor, kbNB 142-70. In case of combining kbNB 142-70 treatment and cLTP, the effect was not additive, indicating that PKD activity is counterbalanced by other pathways during this initial, dynamic phase that shifts the cofilin cycle toward activation PKD inhibition did not increase further transient cofilin activity. After 2h, the level of phosphorylated cofilin in the heads of the dendritic spines has already increased, which is in compliance with the fact that actin skeleton is in the consolidation phase during this time (late LTP; Bosch et al., 2014). As expected, kbNB 142-70 significantly reduced the level of phosphorylated cofilin at this stage.

After 16 hours of KCl treatment, the level of phosphorylated cofilin has increased; this effect was prevented with kbNB 142-70. All of these findings confirm that PKD indirectly regulates the stabilization of F-actin network needed for the consolidation of morphological changes during LTP, and is responsible for the inactivation of cofilin.

4. PKD activity affects the distribution and morphology of the dendritic spines

We examined whether expression of kdPKD-EGFP exerting dominant-negative effect of endogenous PKD activity had any effect on the distribution and morphology of the dendritic spines. Morphological categorization of the dendritic spines was performed according to published methodology (Peters and Kaiserman-Abramof, 1970). Inhibition of PKD activity significantly reduced the density of the protrusions and caused characteristic changes in the distribution and morphology of the dendritic spine types. The proportion of the thin and long (so-called filamentous) dendritic spines increased and the frequency of the mushroom-shaped spine was reduced.

The experiments performed with the PKD activity reporter already proved that PKD is activated within the dendritic spines during KCl treatment. We aimed to elucidate whether PKD plays any role during the enlargement of the spine heads induced by long-term depolarization. 16-hour-long KCl treatment significantly increased the proportion of the mushroom-shaped spines, while the ratio of the filamentous spines was reduced. Applying kbNB 142-70 in itself reduced the ratio of the mushroom-shaped spines, although the difference was not significant. On the other hand, enlarging of the spine heads due to prolonged depolarization was prevented by the PKD-specific inhibitor. On this basis we can state that PKD plays a role during the activity-dependent enlargement of dendritic spine heads in the enlarging of the dendritic spine heads due to long depolarization.

5. Expression of the dominant-negative kdPKD-EGFP within the CA1 and CA3 hippocampal pyramidal neurons reduces the size of their dendritic spines

We have analysed the morphological consequences of the presence of a dominant-negative kdPKD-EGFP in the CA1 stratum radiatum and CA3 stratum lucidum of the hippocampus with electronmicroscopy, working in collaboration with Bence Rácz (Veterinary Faculty of Szent István University).

The area and perimeter of the dendritic spines expressing kdPKD-EGFP in the CA1 stratum radiatum were significantly smaller than those of the control animals. The area and the circularity of the dendritic spines, as well as the length of the PSD within the CA3 stratum lucidum were also significantly reduced compared to the control animals. Thus, we assume that PKD-mediated alterations in dendritic spine morphology take place similarly in cell cultures as well as within the intact brain tissue.

6. Expression of a dominant-negative PKD in the hippocampus leads to the selective impairment of hippocampus-dependent learning abilities

We have analyzed the behavioural conseqeunces of the dominant-negative PKD expression with the help of the AnimalTracker application running in the framework of NIH ImageJ/Fiji (developed by our research team; Gulyás et al., 2016).

In the open field test, we examined overall motor performance and exploration activity. No differences were found between the double transgenic and control animals in the place preference, neither within the examined motility parameters (ambulation distance, number of ambulatory segments and rearings, ambulation, rearing and immobility times).

The new object recognition test is a widely used method to analyse visual object recognition memory in rodents (Antunes and Biala, 2012). In doxycycline-induced double transgenic animals, kdPKD-EGFP expression was negligible in cerebral structures playing fundamental role in object recognition (perirhinal cortex, entorhinal cortex and inferior temporal cortex). Thus, it was not surprising that new object recognition was performed similarly by the control and the kdPKD-expressing animals, described by a similar discrimination index[2]. So we can state that kinase inactive PKD produced in the hippocampus does not affect recognition of the new objects.

The spatial orientation of the animals was compared using two classical spatial learning tests. In the Morris water test, the control animals spent significantly more time and performed longer swim path over the target area during the probe trials on the fifth and the tenth days compared to the double transgenice mice. While the control animals significantly improved their performance, transgenic mice were incapable of gaining better results. In the radial maze test, no differences were found between the animal groups neither regarding the running distance nor the time spent in the radial maze during the first week of training. From the beginning of the second week, however, control animals performed significantly better than transgenic mice. Thus, we proved that kinase-inactive PKD expression within the hippocampus deteriorates spatial learning abilities of the mice.

[2]A parameter often used in the new object recognition tests (quotient) Its calculation:

$$\mathrm{DI} = \frac{\text{time spent on sniffing the the new object} - \text{time spent on sniffing the old object}}{\text{time spent on sniffing the the new object} + \text{time spent on sniffing the old object}}$$

Conclusions

1. PKD is activated during activity-dependent enlargement of dendritic spines

The experiments performed with the PKD activity reporter proved that activity-dependent plasticity leads to endogenous PKD activation in the dendritic spines. Glycine-induced LTP mimicks the condition when the synaptic release of glutamate reaches such an extent that NMDA receptors become also active in addition to the AMPA receptors. As a consequence, calcium ions entering the cells will activate CaMKIIα with a subsequent phosphorylation and membrane transport of the AMPA receptors. On the other hand, PKA activation leads to morphological changes and extension of the spine head. The molecules and the signal transmission pathways between the NMDA receptors and the PKD activation are still unknown. Similar results were obtained in case of homeostatic upscaling, evoked by a prolonged depolarization block with KCl.

2. PKD promotes the stabilization of F-actin within the dendritic spines

Motility of the rapidly moving dendritic filopodia, which do not yet form stable synaptic connections, is predominantly determined by the dynamics of the actin cytoskeleton. Motility of the dendritic protrusions in our hippocampal cultures was increased upon inactivating endogenous PKD functions. Thus, PKD plays a role in the stabilization of dendritic filopodia.

Following LTP induction, the volume and distribution of the main molecular components of the dendritic spines with synaptic relations change characteristically (Bosch et al., 2014). During the initial phase of LTP formation (1-7 minutes), the head of the dendritic spine is temporarily enlarged, due to the depolimerization of F-actin. According to our results, PKD does not play important role in the regulation of actin cytoskeleton during this phase. It is likely that during the early LTP, several other proteins counterbalance the effects of PKD, directing dephosphorylation and activation of cofilin (Mizuno, 2013). During the later phase of LTP (up to 120 minutes), phosphorylated cofilin levels were reduced by kbNB 142-70, indicating that PKD plays a role in the regulation of actin dynamics during the consolidation phase of LTP. Our results confirmed that PKD exerted indirect effects through several auxiliary target proteins in the regulation of the dynamics of the actin cytoskeleton: it activates the PAK4-LIMK1/2-cofilin cascade while on the other hand, it inactivates SSH.

Based on the electron microscopy performed in collaboration with Bence Rácz, we have shown in the kdPKD-EGFP producing, double transgenic animals that kinase inactive PKD is found near the postsynaptic membrane, at the same place where cofilin is normally located.

3. Effects of kdPKD-EGFP expression on neuronal functions and memory formation

Electronmicroscopy confirmed the expression of the dominant-negative, mutant PKD form within the dendrites and the dendritic spines of the pyramidal cells. We performed electrophysiology to reveal the physiological consequences of kdPKD-EGFP expression. During the basal measurements before HFS stimulation, we did not experience any differences within the hippocampal pyramidal cells of the control and the double transgenic mice. This is in compliance with the fact that there was no difference between the two animal groups in the first week of the radial maze test, either, and indicated that PKD-mediated functions are not indispensable for the formation and maintenance of dendritic spines under normal conditions. However, when the animals had to use their long-term memory, accompanied with the morphological rearrangement of the dendritic spines, the double transgenic animals showed significantly worse performance. We confirmed this observation in our electrophysiological tests, too, since upon LTP formation, the amplitude of population spikes, measured from the brain slices of the double transgenic animals was significantly lower both in case of mono- and bisynaptic stimulations.

Taken together, we have shown that PKD participates in the regulation of plasticity and learning processes by stabilizing actin-turnover in the dendritic spines. Regulated exo- and endocytosis of the neurotransmitter receptors is obligate for the above processes in the neurons (Park et al., 2006). Experiments aiming at the investigation of the molecular pathways in the background of the endocytosis stimulating effect of PKD activity have yet to be performed in the future.

Publications related to the scientific topic of the dissertation

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