

PhD thesis

**THE ROLE OF PROTEIN KINASE D IN NEURONAL TRANSPORT
PROCESSES AND IN DENDRITIC DEVELOPMENT**

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Introduction

The PKD family has recently become a separate family among the serine-threonine kinases (Van Lint et al., 2002). Recent findings revealed a role for the three mammalian PKD isozymes (PKD1, PKD2 and PKD3) in very diverse cellular functions including immune responses, apoptosis, cell proliferation, Golgi organisation and plasma membrane directed transport (Rykx et al., 2003). PKD localised at the Golgi apparatus plays a key role in the fission of the vesicles (Liljedahl et al., 2001) and in polarised epithelial cells, PKD is selectively involved in basolateral membrane protein transport (Yeaman et al., 2004).

All 3 isoforms of PKD are expressed in the central nervous system already at embryonic stages in mice (Oster et al., 2006), however, just few aspects are known about the role of PKD in neurons. As neurons need a complex and strictly regulated protein transport machinery to establish and maintain the highly polarised morphology and function, it is a feasible idea that PKD located at the Golgi membrane can regulate membrane supply and directed neuronal protein transport, as well. Some data have already indicated the involvement of PKD in dendritic growth (Horton et al., 2005) as well as in directed delivery of dendritic membrane proteins (Bisbal et al., 2008), however, the contribution of PKD to the maintenance of the polarised structure and function of neurons is far from well understood.

Main goals

In order to reveal the role of PKD in diverse neuronal processes, we used mouse embryonal hippocampal neuronal cultures and analysed the effects of overexpressing various, fluorescently labelled proteins. In the presented experiments we aimed:

1. To investigate the localisation of endogenous PKD activity during neuronal development by expressing a PKD activity reporter in primary hippocampal neuronal cultures.
2. To unravel the intracellular localisation and possible interactions of fluorescently labelled wild type and mutant PKD proteins in mature hippocampal neurons by colocalisation studies.
3. To analyse PKD-mediated effects on neuronal Golgi structure and dendritic arborisation in mature neurons upon expression of kinase inactive and constitutive active PKD proteins.
4. To investigate the axonal transport of PKD, as well as its possible effect on the transport of diverse axonal proteins with the aid of fast live cell imaging recordings.

Applied methods

- preparation and cultivation of primary hippocampal neuronal cultures
- Lipofectamine based transfection of primary hippocampal neuronal cultures – expression of fluorescently labelled wild type and mutant PKDs, diverse dendritic and axonal proteins
- immunocytochemical stainings carried out on fixed primary hippocampal neurons
- microscopic analyses

Morphological analysis:

- Analysis of *Golgi morphology* in differently transfected hippocampal neurons
- Quantitative analysis of *dendritic arborisation* in differently transfected hippocampal neurons

Intracellular localisation:

- Visualising *endogenous PKD activity* by detecting PKD specific phosphorylation of a PKD activity reporter construct
- Comparison of the intracellular distribution of diverse, fluorescently labelled dendritic proteins upon *cotransfection* with various PKD constructs

Live cell imaging:

- Analysis of the mobility of overexpressed, fluorescently labelled proteins by *fluorescence recovery after photobleaching (FRAP)*
- Investigation of diverse types of axonal transport by *dual color, fast live cell imaging*
- Following changes in the intracellular distribution of PKD constructs upon activation

Results

1. Endogenous PKD activity is polarised during neuronal development

In order to visualise endogenous PKD activity, EGFP-tagged PKD activity reporter construct was expressed in hippocampal neurons. PKD-mediated phosphorylation of the transfected construct was detected by an antibody specific for a serine known to be phosphorylated specifically by PKD.

Investigations on transfected neurons during different developmental stages revealed that PKD activity is ubiquitously distributed in every compartment of young, non-polarised neurons but diminish from the axon in parallel with neuronal development. In mature neurons, PKD-mediated phosphorylation of the activity reporter construct was restricted to the somatodendritic compartment while being absent from the axon.

With the aid of a Golgi targeted PKD activity reporter we could also show that endogenous PKD is highly active at the Golgi apparatus.

2. Wild type and mutant PKD constructs show different intracellular localisation in mature neurons and influence the localisation of coexpressed dendritic proteins

Diverse, fluorescently labelled PKD constructs were expressed in mature hippocampal neurons. Wild type PKD1 (wtPKD1-EGFP) and constitutive active PKD1 (caPKD1-EGFP) were ubiquitously distributed in the cytoplasm of transfected neurons and were slightly enriched at the Golgi compartment. In contrast, kinase inactive PKD1 (kdPKD1-EGFP) showed relatively low cytoplasmic expression and was rather concentrated in discrete spots in the perinuclear region and in the dendrites of transfected neurons. The enrichment of kdPKD1-EGFP at Golgi structures was elevated compared to wild-type or constitutive active PKD expressing neurons. Furthermore, FRAP analysis revealed a much lower mobility of kdPKD1-EGFP within the cytoplasm as it was detected in case of wtPKD1-EGFP.

Cotransfection experiments between fluorescently labelled PKD variants and dendritic marker proteins indicated that PKD activity can influence the transport and/or the localisation of certain dendritic proteins. Altered PKD activity led to changes in the intracellular localisation of the Kv4.2 ion channel subunit, while the distribution of PSD-95 or NMDA receptor subunit 1 was not affected by elevated or decreased PKD activity.

3. PKD activity regulates Golgi structure and dendritic arborisation of mature neurons

Golgi morphology was analysed by immunostaining both cis- and trans-Golgi network with GM130 and VAMP4 antibodies respectively, in EGFP, wtPKD1-EGFP, kdPKD1-EGFP and caPKD1-EGFP transfected neurons. In a large portion of kdPKD1-EGFP transfected neurons, Golgi complex was disrupted into several small fragments containing both cis- and trans-Golgi elements. In contrast, wtPKD1-EGFP and caPKD1-EGFP did not disturb the filamentous structure of the neuronal Golgi apparatus. Quantitative analysis of Golgi morphology from 3 independent cultures showed a significantly higher ratio of neurons possessing dispersed Golgi complex upon kdPKD1-EGFP expression compared to neurons transfected with wtPKD1-EGFP, caPKD1-EGFP or EGFP.

The time-course of Golgi dispersal in kdPKD1-EGFP transfected neurons was investigated by analysing Golgi morphology 12, 18 or 24 hours after transfection and showing significantly increased ratio of transfected neurons with dispersed Golgi apparatus in parallel with post-transfection time. As kdPKD1-EGFP average expression level increased in proportion with ongoing post-transfection time, these results indicate a strong correlation between Golgi structure dispersal and the expression level of kdPKD1-EGFP.

The effect of PKD on dendritic morphology and rearrangement was investigated by analysing the extent of dendritic branching with a modified Sholl analysis in EGFP, wtPKD1-EGFP, kdPKD1-EGFP and caPKD1-EGFP transfected neurons. The presence of kdPKD1-EGFP led to a significant decrease in the dendritic arborisation, while constitutively active PKD1 increased the extent of dendritic tree in mature neurons. Investigating the Golgi morphology and the dendritic arborisation within the same kdPKD1 transfected cells revealed a strong correlation between the onset of Golgi apparatus disruption and the extent of impaired dendritic arborisation.

4. Axonal transport of PKD - live cell imaging observations

Despite the lack of endogenous PKD activity in the axon of mature neurons, immunostaining with a monoclonal PKD1 antibody revealed the presence of PKD in the axons. Furthermore, fast live cell imaging recordings indicated the transport of wtPKD1-EGFP within the axons. According to our observations, wtPKD1-EGFP was transported in the axon bidirectionally and in a stop-and-go manner. In cotransfection studies using dual

colour fast live cell imaging, wtPKD1-mCherry was transported independently from the fast component proteins synaptophysin and synaptotagmin, but was partially cotransported with synapsin-EGFP or myosin Vb-EGFP signals, which are known markers of Slow Component b (SCb) type of axonal transport. wtPKD1-EGFP was also colocalised with synuclein or synapsin I in stationary deposits localised at branching points or at axonal terminal.

Observations of fixed hippocampal neuronal cultures indicated that in contrast to wtPKD1-EGFP, cytoplasmic kdPKD1-EGFP signal gradually disappeared from the transfected axons. In live cell imaging recordings kdPKD1-EGFP associated structures were occasionally detected in the axons showing random and short-term movements. Upon cotransfection with axonal transport markers kdPKD1-EGFP did not colocalise with any of the investigated constructs.

Conclusion

According to our studies, endogenous PKD activity is polarised in mature neurons being present in the somatodendritic regions and excluded from the axons. However, the polarised activity of PKD developed gradually during neuronal maturation as phosphorylation of the PKD activity reporter was detected in every compartment of young, non-polarised neurons. The ubiquitous distribution of endogenous PKD activity in non-polarised neurons indicates a role of PKD during early neuronal development. Indeed, recent publications have shown that PKD can contribute to neuronal polarisation processes during early developmental stages (Watkins et al., 2008; Yin et al., 2008) and to dendritic growth (Horton et al., 2005). According to our preliminary observations, PKD can also influence developmental processes by providing the necessary membrane supply for neurite elongation or via controlling the required cytoskeletal changes. However, further experiments are needed in order to explore the versatile function of PKD during neuronal development.

Despite the lack of endogenous PKD activity in axons of mature neurons, immunocytochemical stainings revealed the presence of endogenous PKD in the axon indicating the involvement of PKD in axonal processes, as well. Additionally, live cell imaging experiments have shown that overexpressed wild type PKD is clearly transported within the axons. Characterisation of the movement of wtPKD1-EGFP associated structures as well as its cotransport with known SCb marker proteins indicates that PKD is transported in the SCb type of axonal transport. In contrast to wtPKD1 construct, kdPKD1-EGFP was

excluded from the axon in parallel with increasing posttransfection time, suggesting that the axonal transport of PKD depends on its kinase activity. As endogenous PKD is inactive in the axon, kinase activity can be needed for connecting PKD with motor proteins either directly or via scaffolding/adaptor proteins. We assume that kinase activity can be required for the (auto)phosphorylation of a serine located at the C-terminus of PKD, which is responsible for protein-protein interactions via PDZ domains (Sanchez-Ruiloba et al., 2006). As several PDZ proteins are involved in transport processes by coupling to kinesins as well as to transport carriers (Kim and Sheng, 2004), it is a feasible idea that wtPKD is transported along microtubules by binding to kinesins via PDZ domain containing adaptor proteins.

In our mature hippocampal cultures, PKD1 constructs were enriched at the TGN and Golgi targeted PKD reporter revealed high endogenous PKD activity at the neuronal Golgi complex. These observations already suggest a role for PKD in neuronal Golgi function, similarly to non-neuronal cells (Liljedahl et al., 2001). Importantly, kdPKD1 expression led to the dispersal of the neuronal Golgi apparatus into smaller fragments. This effect might evolve due to the disturbed balance of Golgi membrane dynamics or as a consequence of altered cytoskeletal organisation occurring upon altered PKD activity. Fragmentation of the Golgi apparatus is at variance with the well-described tubulation of the TGN in kdPKD expressing non-neuronal cells, which is widely accepted as a consequence of impaired vesicle fission (Bard and Malhotra, 2006). However, the seemingly opposite effect of kdPKD on Golgi morphology might be explained by the basic differences concerning Golgi organisation and function described in neurons compared to non-neuronal cells. Therefore, despite differences in phenotypical changes, our results are consistent with observations from earlier studies carried out in non-neuronal cells in the following aspects: *i*) PKD activity is required for the maintenance of the cell type specific Golgi complex architecture and *ii*) interfering with PKD activity rapidly changes Golgi complex integrity.

Besides the effect on Golgi organisation, kdPKD1 expression led to dramatic shrinkage of the dendritic tree within 24 hours in mature neurons. The strong correlation between the onset of Golgi apparatus disruption and the extent of impaired dendritic arborisation in kdPKD1 transfected neurons indicate that the reduction of dendritic arborisation can be a consequence of impaired secretory function(s) and decreased membrane supply. This is in accordance with recent publications showing the importance of Golgi organisation in the maintenance of the dendritic arbour (Horton et al., 2005;Ye et al., 2007). Besides acting on the secretory pathway, PKD can influence dendritic structure also via local effects, as high level of PKD activity was observed not only around the neuronal Golgi, but also in the

cytoplasm of dendrites. On one side, kdPKD1 was shown to exert an effect on endosomal dynamics as kinase inactive PKD resulted in increased endocytosis of dendritic membrane proteins (Bisbal et al., 2008). On the other, direct or indirect cytoskeletal effects of PKD can also participate in the observed dendritic rearrangements in transfected neurons as it is known that PKD activity regulates cytoskeletal organisation during cell migration and invasion in non-neuronal cells (De Kimpe et al., 2009; Scholz et al., 2009).

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