Transmembrane receptor turnover during neuronal plasticity

PhD thesis

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Introduction

Synaptic plasticity is regulated in an activity-dependent manner and is known to underlie memory formation and learning. One key element of synaptic strength modulation is alteration of the actin network within dendritic spines, leading to the modification of the size, number and shape of dendritic spines. Regulation of neurotransmitter receptor recycling in the postsynaptic membrane is also involved in the modulation of neuronal plasticity (Kennedy and Ehlers, 2006).

It is known that protein kinase D (PKD) controls the extent of the dendritic tree, the integrity of the Golgi apparatus (Czöndör et al., 2009), and the somatodendritic distribution of transferrin receptor (Bisbal et al., 2008). Our previous results show that endogenous PKD is active within dendritic spines and stabilizes actin cytoskeleton during synaptic plasticity (Bencsik et al., 2015). Since PKD participates in neuronal transport mechanisms and controls endocytosis in non-neuronal cells, PKD may have additional role in regulating AMPA receptor turnover, which is important in the modulation of synaptic plasticity.

Ras and Rab interactor 1 (RIN1) protein, a known substrate of PKD, has been already indicated in controlling neuronal endocytosis (Deininger et al., 2008). It has been reported that RIN1 has the ability to modulate two downstream pathways. First of all, RIN1 directly activates Abl kinases, which have a role in actin cytoskeleton remodelling (Hu et al., 2005). Secondly, RIN1 can also act as a Rab5 GEF protein, thereby regulating Rab5-dependent clathrin-mediated endocytosis (Tall et al., 2001). Protein interactions and cellular functions of RIN1 are regulated by the phosphorylation of distinct amino acid residues. Phosphorylation of serine 351 enhances the interaction with 14-3-3 adaptor proteins, which sequester active RIN1 from the cytoplasm, thus modulating its Rab5 GEF and Abl kinase activity (Wang et al., 2002). Consequently, mutation of 351 serine to alanine (RIN1-S351A) prevents the association between RIN1 and 14-3-3 proteins, therefore RIN1 is constitutively present in the cytoplasm (Balaji et al., 2012). RIN1 directly interacts with Abl kinases, leading to the subsequent phosphorylation of RIN1 at tyrosine 36 (Y36). This process enhances Abl kinase activity followed by increased phosphorylation of its substrates (Hu et al., 2005). Of note, mutation of tyrosine 36 together with three secondary tyrosine residues within RIN1 (RIN1-QM; Hu et al., 2008) blocks the activation of Abl kinases (Balaji et al., 2012). RIN1-mediated activation of Abl kinases is further modulated by PKD-mediated phosphorylation of serine 292, therefore PKD regulates Abl kinase activity through RIN1 (Ziegler et al., 2011).
Mutation of glutamate 574 in RIN1 results a Rab5 GEF deficient RIN1-E574A mutant, which disrupts RIN1-mediated Rab5 GTP-ase actions (Galvis et al., 2009).

**Main goals**
1. Our aim was to provide more evidence regarding the effect of PKD on actin cytoskeleton remodelling within dendritic spines. Our goal was to complement previous studies from our laboratories which indicated that PKD regulates actin dynamics via increasing phospho-cofilin levels in dendritic spines.
2. We wished to investigate the role of RIN1 in dendritic filopodial motility, and whether RIN1 controls this process in a PKD-dependent manner.
3. We investigated whether PKD participates in transmembrane receptor turnover, particularly focusing on the surface expression of AMPA receptors, and whether PKD regulates this process through RIN1.
4. In order to obtain data on RIN1-dependent actions during neuronal plasticity, we aimed to investigate the role of RIN1 in AMPA receptor turnover during long-term depression and whether it is dependent on PKD activity.

**Applied methodology**
- preparation and maintenance of primary hippocampal neuronal cultures
- transfection of neurons with fluorescently-tagged PKD and RIN1 constructs
- chemically induced long-term potentiation (cLTP) and long-term depression (cLTD) in hippocampal neurons
- actin barbed end assay to analyse actin incorporation in dendritic spines
- fluorescent live cell imaging of transfected neurons to investigate the motility of filopodial dendritic protrusions
- transferrin uptake to study regular endocytosis by pulse labelling the neurons with fluorescently labelled transferrin conjugate, and antibody feeding assay to analyse the cellular localisation of AMPA receptor GluA1 subunit level by introducing anti-GluA1 antibody to living neurons
- surface biotinylation and Western blot assay to investigate the PKD and RIN1-dependent changes in plasma membrane localised GluA1 amount
- immunocytochemistry in fixed cells and confocal microscopy
Results

1. The effect of PKD and RIN1 on actin cytoskeleton remodelling in dendritic spines

1.1. PKD activity inhibits actin incorporation within dendritic spines

Actin barbed end assay was carried out to investigate actin dynamics within dendritic spines in relation to PKD activity. Application of a selective PKD inhibitor kbNB 142-70 led to increased actin-rhodamine incorporation within the head of the dendritic spines indicating enhanced rate of F-actin turnover. Following chemically induced LTP (cLTP), when the expanded heads of dendritic spines contain stabilized F-actin network, the extent of actin incorporation was decreased. Inhibition of endogenous PKD activity during this stage also increased actin dynamics. Consequently, PKD activity stabilizes F-actin within dendritic spines under normal conditions as well as during LTP.

1.2. RIN1 accelerates filopodial motility depending on the activation of Abl kinase pathway

According to our laboratory’s previous result, PKD activity decreases filopodial motility, which is in line with our findings that PKD stabilizes actin network within dendritic spines. It is known that phosphorylation of RIN1 at serine 292 (S292) by PKD stimulates the activity of Abl kinases (Ziegler et al., 2011). Since RIN1 participates in actin cytoskeleton remodelling through Abl kinase pathway (Hu et al., 2005), we investigated the influence of RIN1-dependent Abl kinase activity on filopodial motility in a relation to the upstream phosphorylation of S292.

Transfection with fluorescently-tagged RIN1 constructs was carried out in RIN1 knockout (RIN1-KO) neurons to directly compare the effect of wild type and RIN1 point mutants. Expression of wild type RIN1 (RIN1-WT) as well as the RIN1 mutant form (RIN1-S351A), which can not be sequestered from the cytoplasm, significantly increased the motility of protrusions compared to the EGFP transfected, control RIN1-KO neurons. This effect was completely reverted by the application of the Abl kinase inhibitor imatinib. Introduction of RIN1 mutants defective in Abl kinase activation (RIN1-QM) or lacking upstream phosphorylation of the S292 site (RIN1-S292A) prevented the onset of increased filopodial motility observed upon wild type RIN1 expression. On the contrary, the Rab5 GEF activity of RIN1 did not influence RIN1-evoked increase in filopodial motility, as the RIN1-E574A point mutant also increased the motility of protrusions. These data suggest that RIN1-mediated Abl kinase activation increases the motility of filopodial protrusions probably via increased actin cytoskeleton remodelling, which effect might be stimulated by PKD.
2. The role of PKD and RIN1 in regulation of cell surface receptor endocytosis in neurons

2.1. Investigation of neuronal transferrin endocytosis

2.1.1. PKD and RIN1 activity enhances the endocytosis of transferrin in neurons

The endocytosis of transferrin receptor was analysed in relation to PKD and RIN1 activity. Our results show that overexpression of the constitutively active PKD mutant accelerated transferrin uptake, which effect was completely abolished by the application of a selective PKD inhibitor. Consequently, PKD activity contributes to increased transferrin endocytosis in hippocampal neurons.

Expression of RIN1-WT in RIN1-KO neurons significantly enhanced the endocytosis of transferrin, similarly to RIN1-S351A construct. Our results clearly show that the regulation of transferrin endocytosis was dependent on the Rab5 GEF activity of RIN1, as the Rab5 GEF deficient RIN1-E574A mutant completely blocked enhanced endocytosis. We additionally proved that the Abl kinase pathway is not involved in this process.

2.1.2. PKD activity enhances the early and recycling endosomal functions

Constitutively active PKD increased the colocalization between transferrin and the early endosomal marker protein Rab5 positive vesicles, and enhanced the association with Rab11 positive recycling endosomes at the early steps of endocytosis. These effects were reverted by the application of a PKD-specific inhibitor. Thus, PKD activity plays a role in the regulation of transferrin receptor endocytosis via early endosomal system close to the plasma membrane or via interacting with recycling endosomal compartments.

2.2. Regulation of AMPA receptor turnover in relation to PKD and RIN1 activity

2.2.1. Short or long-term modulation of PKD activity has an influence on cell surface distribution of AMPA receptor

We analysed PKD-mediated effects on the surface expression of AMPA receptors by cell surface biotinylation and antibody feeding. According to our results, long-term inhibition of endogenous PKD activity decreased the surface GluA1 level, probably via interacting with post-Golgi secretory pathway. Short-term inhibition of PKD activity increased the postsynaptic GluA1 amount localised on the surface of dendritic branches.

2.2.2. Rab5 GEF activity of RIN1 is responsible for the AMPA receptor turnover

Introducing RIN1-WT construct into RIN1-KO neurons significantly decreased the GluA1 signal within the postsynaptic region. This effect was even more prominent in the presence of RIN1-S351A. The postsynaptic GluA1 level was clearly dependent on the Rab5 GEF activity of RIN1, as the expression of the Rab5 GEF deficient RIN1-E574A point
mutant did not induce changes in the relative GluA1 values compared to the control, EGFP-expressing neurons. On the other hand, expression of the Abl-deficient RIN1 mutant (RIN1-QM), and the lack of phosphorylation at the S292 RIN1 site led to a similar drop in surface GluA1 intensities as observed upon wild-type RIN1 expression. These data suggest that RIN1 regulates the amount of GluA1 subunits within the postsynaptic sites via its Rab5 GEF activity and independently from the Abl kinase pathway.

2.2.3. The Rab5 GEF activity of RIN1 is responsible for the cLTD-induced endocytosis of AMPA receptor

To examine whether RIN1 plays a role in endocytosis of surface AMPA receptors during LTD, chemically induced LTD (cLTD) was carried out in CD1 wild type and in RIN1-KO neuronal cultures. According to the cell surface biotinylation assay, cLTD treatment evoked a significant loss in the surface GluA1 subunit level in CD1 wild type neurons. In the absence of RIN1, on the other hand, neurons were not able to downregulate surface AMPA receptors after cLTD induction. These results were confirmed with antibody feeding assay, as well. Importantly, cLTD-mediated effects on surface GluA1 localisation were restored only when RIN1-KO neurons were transfected with RIN1 constructs possessing intact Rab5 GEF activity (RIN1-WT and RIN1-QM). On the other hand, the Rab5 GEF deficient RIN1-E574A was not able to downregulate the surface GluA1 level upon cLTD treatment. Taken together, our data indicate that cLTD-evoked loss of surface AMPA receptors depends on the Rab5-GEF activity of RIN1.

Conclusions

1. PKD stabilizes actin cytoskeleton independently of RIN1 activity in neurons

1.1. PKD stabilizes dendritic spines via regulating F-actin network

We have shown that PKD promotes plasticity induced F-actin stabilization within dendritic spines via decreasing the effect of cofilin phosphatase (Bencsik et al., 2015). This is in agreement with previous findings obtained in non neuronal cells, that PKD stabilizes the actin cytoskeleton system (Olayioye et al., 2013).

1.2. RIN1 may act against synaptic stabilization via enhancing F-actin remodelling

According to our results, RIN1 enhances filopodial motility through its downstream effectors, Abl kinases. Consequently, RIN1 can act against synapse stabilization via increasing filopodial motility. Abolished upstream phosphorylation at the S292 site (RIN1-S292A) prevents RIN1-mediated increase in filopodial motility. As phosphorylation of the serine 292 site of RIN1 enhances the interaction between RIN1 and Abl kinase (Ziegler et
PKD activity probably increases Abl kinase activity through RIN1. This observation is contradictory, as PKD activity decreases filopodial motility. Nevertheless, we can not exclude that other kinases are involved in the serine 292 site phosphorylation of RIN1. Additionally, RIN1-dependent pathways can compensate for the effect of PKD on filopodial motility.

2. PKD and RIN1 regulate neuronal transmembrane receptor turnover independently

2.1. PKD controls receptor turnover during endocytosis or by modulating the secretory pathway

According to our results, PKD can control transferrin receptor endocytosis at the plasma membrane via regulating the early or recycling endosomal systems. Based upon the different outcome of short or long-term inhibition of PKD activity, PKD seems to regulate receptor turnover by dual mechanisms. Firstly, PKD can regulate receptor turnover by controlling the post-Golgi secretory pathway, and secondly, PKD can direct endocytosis close to the plasma membrane, as well.

It has been reported that the kinase dead mutant form of PKD led to the fragmentation of neuronal Golgi apparatus (Czöndör et al., 2009), and the axonal mistargeting of transferrin receptor (Bisbal et al., 2008). Thus, long-term disturbance in PKD activity may effect the secretory pathway of AMPA receptors.

PKD can also regulate transmembrane receptor turnover close to the plasma membrane via interacting with clathrin-coated vesicles or early/recycling endosomes. Early endosomes can be regulated by the substrates of PKD, as RIN1 or Rabaptin5 are involved in Rab5-dependent endocytosis (Deininger et al., 2008; Christoforides et al., 2012). According to our results, PKD controls endocytosis independently from RIN1 activity, as mutation of serine at 292 to alanine in RIN1 (RIN1-S292A), which disrupts the PKD-mediated phosphorylation site did not effect postsynaptic AMPA receptor distribution. Further studies are needed to clarify the downstream effectors of PKD in endocytosis.

We have proved that PKD plays a role in regulating AMPA receptor turnover, however additional experiments should be done regarding the effect of PKD activity on AMPA receptor turnover in relation to synaptic plasticity.

2.2. RIN1 is required for the endocytosis of AMPA receptors during cLTD

We have shown by cell surface biotinylation and antibody feeding assay that RIN1 regulates surface expression of AMPA receptor. As introduction of the RIN1-S292A mutant into RIN1-KO neurons decreased postsynaptic GluA1 level similarly to the wild type RIN1
construct, and that surface GluA1 level was not affected by the phosphorylation of serine 292, we assume that PKD regulates AMPA receptor turnover independently of RIN1 activity.

We have proved that in CD1 wild type neuronal cultures, chemically induced LTD led to AMPA receptor internalization, which is in agreement with previous findings (Collingridge et al., 2010). On the other hand, RIN1-KO neurons were not able to downregulate surface AMPA receptor after cLTD induction. Expression of wild-type RIN1, on the other hand, restored the endocytosis of AMPA receptor during LTD. Our results also indicate that internalization of surface AMPA receptor occurs only if the Rab5 GEF activity of RIN1 is intact.

Further studies are required to clarify the detailed role of PKD and RIN1 in actin remodelling and AMPA receptor turnover during long-term potentiation and long-term depression. These results would highlight important informations about the role of PKD and RIN1 in synaptic plasticity and psychiatric diseases.

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Publications based on the presented work


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