Title
Protein Kinase D1 In Neuronal Signaling

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Publication Date
2011

Peer reviewed|Thesis/dissertation

Protein Kinase D1 In Neuronal Signaling

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Protein Kinase D1 and Neuronal Signaling

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ABSTRACT

Long-term changes in synaptic strength are thought to underlie many forms of learning and memory. To achieve these changes in synaptic strength, neurons must be able to regulate the trafficking of neurotransmitter receptors to and from the synapse locally within dendrites. The dendritic trafficking of AMPAR-type glutamate receptors critically modulates synaptic strength, though the regulatory mechanisms are not fully understood. Here I describe a novel protein kinase D1 (PKD1)-dependent pathway that mediates the postsynaptic trafficking of AMPARs. Without functional PKD1, GluA2-containing AMPARs accumulated in endosomes, decreasing the number of extrasynaptic AMPARs. This is due to decreased GluA2 exocytosis, as knockdown of PKD1 reduced GluA2 recycling, but not uptake, in response to glutamate. PKD1 is in turn dually regulated by glutamate. We found that mGluRs control PKD1 activity, whereas NMDARs control PKD1 localization. Ca^{2+} influx through NMDARs caused movement of PKD1 from the dendritic cytoplasm to AMPAR-containing endosomes. PKD1 translocation is a multistep process requiring both its membrane-binding and kinase domains. DAG generated downstream of NMDARs recruited PKD1 to membranes, and PKD1 subsequently translocated to endosomes via its kinase domain. Mutating PKD1's activation loop serines to alanines (SSAA) disrupted endosomal localization, despite the fact that NMDARs do not induce PKD1 phosphorylation. I generated an ATP analog inhibitor–sensitive allele of PKD1 to determine if phosphoryl transfer activity is required for

endosomal localization, but found inhibitor binding did not block PKD1 translocation. On the contrary, I found inhibitor binding rescued endosomal localization of the SSAA mutant, suggesting inhibitor binding induces the closed or "active" kinase conformation, and this conformation may be required for endosomal targeting. My results underscore the complexity with which PKD1 is regulated in neurons, and opens up the possibility that PKD1 localization to endosomes with and without concomitant kinase activation have different effects on AMPAR trafficking. Thus, PKD1 may be a critical node in a plasticity pathway that receives distinct synaptic signals from NMDARs and mGluRs and mediates changes in the availability of extrasynaptic AMPARs.

DEDICATION AND ACKNOWLEDGMENTS

I'd like to thank my friends and family for supporting me during my graduate career. I would especially like to thank my husband, to whom this work is dedicated.

All my lab mates have my undying gratitude for their camaraderie over the years. I'd especially like to thank my mates Sarah R. Carter and Hong Joo Kim, for their essential contributions to my project: Sarah for initiating the NMDAR-PKD1 signaling experiments and performing the initial pharmacology experiments; Hong Joo for shRNA constructs, antibodies, and advice.

Thanks to my collaborators for contributing to the following projects: Aryn H. Gittis and Anatol Kreitzer (electrophysiology, Chapter 2), Kristina Micheva, Brad L. Busse and Stephen J Smith (array tomography, Chapter 2), Chao Zhang and Kevan M. Shokat (ATP-analog sensitive kinase, Chapter 3).

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PREFACE

How do we remember what happened yesterday or how to ride a bike? What kicks off the forgetfulness of an early Alzheimer's patient? What starts a seizure? Why do some drug users become drug addicts, and why are some unable to stay clean? How does the development of a normal child's brain differ from one with autism?

The common theme running through all of these questions is the remarkable phenomenon of plasticity. Every day, our brains sort through a complex mix of chemical and electrical signals. Some of these signals may initiate a change, such as the formation of a memory within the circuits of our brain. For this circuitry to change and store information, the individual cells that make up those circuits must adjust their connections to one another. My dissertation focuses on this aspect of learning and memory: the plasticity of individual brain cells or neurons.

Neurons talk to each other by connections called synapses. Information passes from one cell to another as the presynaptic cell releases a chemical message, neurotransmitter, onto a postsynaptic cell, and the postsynaptic cell translates it into a new chemical or electrical signal. Neurons adjust their connectivity by altering their synapses. They can change the number of synapses they have, making new connections or dismantling unneeded ones. On a shorter timescale, neurons can alter the properties of existing synapses by fine tuning either the presynaptic neurotransmitter release machinery or the postsynaptic neurotransmitter receptors.

Although all of the mechanisms I mentioned do occur in the brain, neuroscientists have found that plasticity at the postsynaptic density (i.e., changing the number, type, chemistry, and molecular interactions of neurotransmitter receptors) is a widely used and

fundamental basis for plasticity throughout the brain. The most common neurotransmitter in the brain is glutamate, and so the plasticity of glutamate receptors is the basis of much of our learning.

We still do not fully understand how plasticity at the glutamatergic synapse works, and so I centered my dissertation research on this problem. I asked how the neurotransmitter glutamate initiates plastic changes to the synapse, focusing on a signaling molecule called protein kinase D1 (PKD1). I found that glutamate regulates the activity and movement of PKD1 in neurons, and in turn, PKD1 regulates the movement of glutamate receptors. Below, I describe these two pathways, and how they might impact learning and memory.

CHAPTER 1. INTRODUCTION.

SYNAPTIC PLASTICITY

Activity-dependent adaptive changes in synaptic function, collectively called synaptic plasticity, are critical to learning and memory. Two of the best studied forms of synaptic plasticity are long-term potentiation (LTP), which strengthens synaptic responses, and long-term depression (LTD), which weakens them. The early phases of LTP and LTD occur within a few minutes after the stimulus and are characterized by the modulation of receptors at the synapse, particularly the phosphorylation and insertion or removal of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (AMPARs) from the synapse[1]. In contrast, the late phase of LTP or LTD occurs 30 minutes or more after the stimulus and involves cytoskeletal rearrangements in dendrites, gene transcription, and protein synthesis[2]. These changes modulate the sensitivity of existing synapses and remove or add additional synapses.

LTP and LTD require different types of input for initiation. In the case of LTP, high-frequency or theta burst stimulation leads to synaptic strengthening, while LTD occurs after low-frequency stimulation. The type of input a dendrite receives restricts LTP and LTD spatially and temporally to regions of the dendrite that have received the appropriate synaptic input. The synapse specificities of LTP and LTP are a remarkable phenomenon, especially considering the number of synapses a neuron has (tens of thousands) spread out over a tremendous distance (hundreds of microns) and surface area (more than 10^5 μm^2). To address the challenges associated with the extensive size of dendritic branching,

and the need for specific targeting to certain synapses, neurons have developed a unique arrangement of the protein synthesis and trafficking machinery.

Dendritic protein trafficking and plasticity. The canonical view of organelle organization within cells held that the endoplasmic reticulum (ER) extends throughout the cell, while the Golgi apparatus is exclusively perinuclear. However, the finding that mRNAs encoding transmembrane proteins traffic to dendrites[3] foreshadowed the discovery of Golgi elements in more distal locations. Indeed, *cis*, medial, and *trans*-Golgi markers are found in dendrites[4-6]. Dendrites isolated from the soma add AMPARs translated from exogenous mRNA to their surface[7,8], indicating that dendrites have functional rough ER and Golgi network.

Additionally, dendrites and spines have an elaborate endosomal system that regulates trafficking of receptors and secreted factors[9]. After retrieval from the plasma membrane, small endocytic vesicles join early endosomes. From early endosomes, proteins are sorted for either recycling or degradation. In the case of degradation, vesicles bud off the early endosome to join late endosomes and finally lysosomes. Proteins destined to return to the plasma membrane may cycle straight from early endosomes to the plasma membrane in so-called "short loop" recycling. A longer route requires proteins to go from early endosomes to recycling endosomes and finally to the plasma membrane.

Protein trafficking through the dendritic secretory pathway may be regulated by neuronal activity. Neuronal activity can alter the intended cargoes directly, for example, by changing the phosphorylation state of an ion channel's cytoplasmic domain[10]. Such physical changes to a cargo change the cargo's binding partners, which can, in turn, result

in recruitment of trafficking machinery. Indeed, synaptic activity also regulates a wide variety of trafficking effectors, including small GTPases, lipid-modifying enzymes, and exocyst complex proteins[11].

As I alluded to above, glutamate receptors are among the synaptic molecules whose trafficking is most highly regulated in plasticity. While glutamate is the predominant excitatory neurotransmitter in the brain, there are several types of glutamate receptors, which can mediate different downstream responses.

GLUTAMATE SIGNALING IN DENDRITES

The typical glutamatergic synapse is illustrated below. The postsynaptic density (PSD) generally contains three types of glutamate receptors: metabotropic glutamate receptors (mGluRs), *N*-methyl-D-aspartate receptors (NMDARs), and 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid receptors (AMPARs). The latter two are ion channels named after their specific pharmacologic agonists, but *in vivo* all these receptors are activated by glutamate.

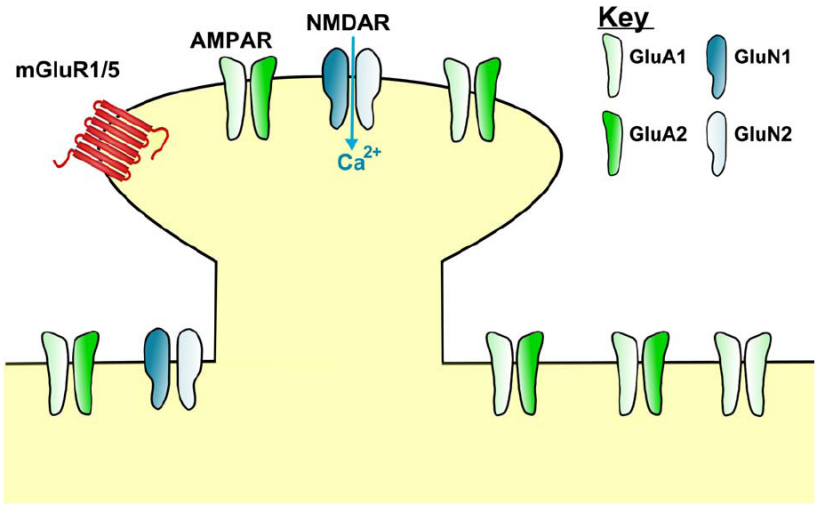

Figure 1. The three major types of glutamate receptors: mGluRs, NMDARs, and AMPARs

AMPA Receptors

***AMPAR properties*.** AMPARs are tetrameric cation channels, with four possible subunits (GluA1–4)[1,12]. GluA2 is the most abundant subunit and often forms heteromeric channels with either GluA1 or GluA3[13]. The ion permeability of AMPARs is determined by the GluA2 subunit. AMPARs that lack GluA2 are Ca^{2+} permeable, and the number of Ca^{2+}-permable AMPARs at a synapse is tightly regulated[13,14]. In most primary neurons in the cortex and hippocampus, the majority (>95%) of AMPARs are GluA1/2 heteromers and thus calcium impermeable[15]. My thesis focuses on these cortical and hippocampal neurons exclusively.

AMPARs are protein complexes with regulatory machinery bound to the cytoplasmic side of the channel[1,12]. Each AMPAR subunit has a unique cytoplasmic C-terminal domain, which contains regulatory phosphorylation sites as well as protein-protein interaction domains[12]. An AMPAR's phosphorylation state and binding partners determine the receptor's localization with respect to both the cell surface and the post synaptic density[16].

AMPAR trafficking in synaptic plasticity. AMPARs mediate the majority of fast, excitatory neurotransmission in the central nervous system, and their number and subunit composition are major determinants of synaptic strength. One critical way that NMDARs and mGluRs effect rapid change in the synapse is by regulating the removal and insertion of AMPARs[12]. How the endo- and exocytosis of AMPARs at the plasma membrane is regulated depends on the subunit composition of the AMPAR.

Internalization and insertion of GluA1-containing AMPARs are regulated, in part, by the phosphorylation state of GluA1 C-terminus. During LTP, NMDAR signaling activates PKA. PKA then phosphorylates GluA1 at S845, resulting in increased AMPAR exocytosis[17]. For synaptic retention of these AMPARs, an additional phosphorylation event at S831 by CamKII or PKC may be required[18]. During NMDAR-LTD, dephosphorylation of S845 by calcineurin causes AMPARs to be internalized[19].

The phosphorylation state of the GluA2 C-terminus also affects its localization. GluA2 phosphorylation changes its affinity for the binding partners GRIP, which promotes stability at the plasma membrane, and PICK1, which promotes internalization of AMPARs. Phosphorylation of GluA2 at Ser880 by PKC causes GluA2 to dissociate from GRIP and bind to PICK1, leading to internalization of the AMPAR[20,21]. GluA2 can also interact with the clathrin adaptor protein AP2 as a part of endocytosis[22].

After internalization from the plasma membrane, some AMPARs are shuttled to lysosomes and degraded, but most are reinserted in the plasma membrane[23,24]. The GluA2-associated protein NEEP21 plays a role at endosomes to promote AMPAR recycling[25,26]. Different stimuli can affect the fate of internalized AMPARs; however, it remains unclear how the switch in trafficking destinations is determined or regulated.

Metabotropic Glutamate Receptors

mGluR properties. The metabotropic glutamate receptors (mGluRs) are seven-pass transmembrane G-protein coupled receptors found at both pre and post synaptic terminals. There are eight known mGluRs which fall into three categories: Group I mGluRs are Gαq coupled and are found only at the post synaptic density; Group II and

III mGluRs are Gi/o coupled and are predominantly presynaptic[27,28]. As my studies were focused on postsynaptic plasticity, I will describe only the Group I mGluRs, although both Group II and III receptors are important for presynaptic mechanisms of plasticity[27].

Type I mGluRs activate PLC through a direct association of the heterotrimeric G protein subunits and PLC[27,28]. Activation of PLC leads to generation of diacylglycerol (DAG) and IP_3, which can lead to rises in intracellular calcium via opening of IP3 receptors gating smooth ER calcium stores[29]. mGluRs 1 and 5 also couple to intracellular Ca^{2+} stores via the scaffolding molecule Homer[30,31]. Thus, mGluRs 1 and 5 mediate rises in intracellular calcium from intracellular stores via two distinct mechanisms. The generation of DAG, in conjunction with calcium release, activates protein phosphatases as well as protein kinases C (PKC), both of which critically regulate AMPAR trafficking[32].

mGluR signaling and AMPAR trafficking. Type I mGluRs mediate early and late-phase LTD at some regions of the hippocampus and cerebellum by regulating AMPAR localization and translation of pre-existing mRNA[2]. mGluR-mediated activation of PKC leads to phosphorylation and internalization of the GluA2 subunit[33,34], discussed in greater detail above. Another mechanism by which mGluRs initiate removal of synaptic AMPARs is through the rapid, local translation of Arc[35,36]. Arc mRNA is present in dendrites at low levels constitutively, and mGluR activation leads to its rapid translation. Newly synthesized Arc protein causes AMPAR internalization through its association with the endocytic proteins endophilin and dynamin[37].

NMDA Receptors

NMDAR properties. The *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) are ion channels, mediating their effects on the post synaptic cell through a combination of depolarization and second messenger signaling via calcium. NMDARs are heteromers of primarily GluN1 and GluN2 subunits[38]. The GluN1 subunit is obligatory for NMDAR channel function, while four different GluN2 subunits (GluN2A-D) are differentially expressed throughout development and confer distinct channel properties and intracellular binding partners[39-42].

NMDARs have the unique property of being both voltage and ligand gated. Binding glutamate is not sufficient to open NMDARs as they are blocked by Mg^{2+} at resting membrane potentials. When the postsynaptic membrane is depolarized, the Mg^{2+} block is alleviated and NMDARs can pass cations, including calcium. NMDARs thus serve as coincidence detectors for the postsynaptic cell: enough synaptic activity must occur for both NMDARs to bind glutamate and for the postsynaptic cell to be depolarized by other channels, such as AMPARs.

NMDAR signaling and AMPAR trafficking. Ca^{2+} influx through NMDARs activates a broad range of signaling pathways that lead to insertion or removal of surface receptors[1,12], as well as gene transcription and protein synthesis[2,43]. During the early phase of LTP, NMDARs activate protein kinases whose activity leads to AMPAR exocytosis and synaptic retention. Protein kinase A (PKA) is activated by cAMP produced in response to NMDARs by a Ca^{2+}-dependent adenylyl cyclase[44]. PKA phosphorylates many synaptic proteins, including the AMPAR subunit GluA1[45,46], which

leads to AMPAR insertion into the plasma membrane. The calmodulin-dependent kinase CamKII is also activated by NMDARs in LTP. CamKII phosphorylates the AMPAR subunit GluA1, as well as the auxiliary subunit stargazin, causing retention of AMPARs at the PSD[47]. In late phase LTP, NMDAR signaling leads to de novo synthesis of the atypical protein kinase C, PKMζ. Once translated, PKMζ is constitutively active and promotes GluA2 exocytosis[48,49]. In contrast to LTP, Ca^{2+} signaling from NMDARs in LTD leads to loss of synaptic AMPARs. This is due in part to the activation of Ca^{2+}-dependent phosphatases[50], which dephosphorylate GluA1 and thus reverse AMPAR synaptic retention.

Although synaptic protein trafficking is essential for plasticity, our understanding of how AMPARs travel through the dendritic secretory/endosomal pathway is nowhere near complete. To find new and important regulators of postsynaptic receptor trafficking, I turned to a novel kinase, protein kinase D, whose functions in non-neuronal cells made it a promising candidate.

PROTEIN KINASE D

Protein kinase D structure and properties

PKD domain organization. Protein kinase D1 (PKD1) was first cloned from mouse in 1994 by Eric Rozengurt's group[51], who identified it as a kinase regulated by diacylglycerol and phorbol-ester binding. They noted that the N-terminal domain of PKD contains regulatory regions similar to protein kinases c (PKC), including two cysteine rich, diacylglycerol binding domains and a pleckstrin homology domain (Figure 2). Despite similarities in the regulatory regions, PKD1 kinase domain is quite divergent

from PKCs. PKD1 kinase is more closely related to the calmodulin-dependent kinase superfamily. Its closest homolog is the kinase domain of Checkpoint 2 (Chk2), with which it shares 37% identity. The positions of key amino acids and motifs within PKD1 motifs are listed in Table 1.

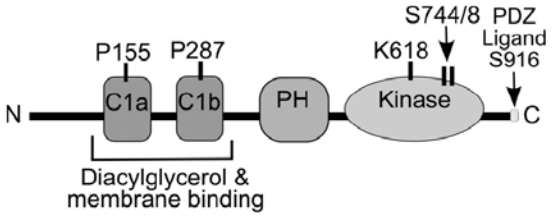

Figure 2. PKD1 Kinase Domains. Key- C1a, cysteine rich domain 1a; C1b, cysteine rich domain 1b; PH, pleckstrin homology domain.

Table 1. **Key Residues and Motifs in PKD1**

Domain or Motif	Residues	Function	Notable Residues and Mutations
Cysteine-rich domain 1a (C1a)	H145-S223	Binds DAG, usually at internal membranes, such as TGN	P155G blocks DAG binding to C1a
Cysteine-rich domain 1b (C1b)	H277-D353	Binds DAG, usually at plasma membrane	P287G blocks DAG binding to C1b
Pleckstrin Homology	V429-G557	Protein-protein interactions, including P14K and Rap1	Deletion of this domain results in constitutively active PKD1
Kinase Domain	I589-L845	Serine/threonine protein kinase	
Glyine Loop	G596-V603	ATP binding (phosphates, adenine)	
VAIK	615-618	ATP binding (phosphates)	K618N is dominant-negative allele for protein secretion from TGN
Gatekeeper residue	Met665	Restricts size of ATP binding pocket	M665A generates ATP-analog inhibitor sensitive PKD1 allele; see Ch.3
Catalytic Loop	H-Z	Mg2+ binding; catalysis	PKD1 has unusual catalytic loop motif: HCD instead of HRD (see Ch.3)
DFG Triplet	733-735	Mg2+ binding; catalysis	
Activation Loop	SFRRS 744-748	Phosphorylation at Ser744/8 induces PKD1 kinase activity	SSAA is kinase inactive but is not dominant negative at TGN
PDZ Ligand	VSIL 915-918	Binding to PDZ domains, autophosphorylation site at Ser916	

PKD1 regulation by phosphorylation. As PKD1 contains DAG binding domains, it was assumed PKD1 kinase activity would be regulated by PLC signaling and DAG mimetics

such as phorbol esters. Like PKC, PKD1 is indeed activated by DAG or phorbol esters, but in the case of PKD1, the activation is not direct. DAG is very important in controlling PKD1 localization. PKD1 translocation to subcellular domains is essential for its ability to regulate diverse cellular functions. Its two cysteine-rich domains, C1a and C1b, cause PKD1 to translocate to the plasma membrane or to internal membranous structures, such as the TGN, upon DAG production[52]. DAG co-recruits PKC to membranes, and it is PKC that activates PKD1 by phosphorylating its "activation loop" serines (S744 and S748)[53]. Activation loop phosphorylation alleviates autoinhibition of PKD1 by its pleckstrin homology domain[54]. The now active PKD1 can autophosphorylate its C-terminus[55] within a type I PDZ ligand (VSIL). Phosphorylation on this site may determine the duration of PKD1 activity[56] and PKD1's interactions with PDZ domain–containing proteins[55,57]. In addition to the phospho-serine sites, PKD1 is subject to tyrosine phosphorylation. Phosphorylation by Src at Tyr469 and Tyr93[58] allows PKD1 to interact with the NF-κB stress response pathway[59].

PKD phosphorylation consensus site. To date, relatively few PKD substrates have been identified. PKD is a basophilic kinase with a preference for arginine or lysine at the -3 position[60]. PKD also demonstrates a strong preference for a leucine at the -5 position; isoleucine and valine are acceptable substitutions[60]. Specific substrates are discussed below as they relate to cellular signaling pathways.

PKD Cellular Functions

PKD1 and two closely related isoforms, PKDs 2 and 3, are widely expressed throughout the body. In these different tissues, PKD1 has been linked to four major signaling pathways: 1) prosurvival responses to oxidative stress, 2) cell motility via the actin cytoskeleton, 3) G-protein coupled receptor elicited gene transcription, and 4) secretory protein trafficking. The Finkbeiner laboratory has found evidence that neurons use the latter two PKD1 pathways.

PKD1, Gαq signaling, histone deacetylases and gene transcription. In numerous tissue types, PKD1 mediates changes in gene expression[61]. The best-characterized pathway by which this occurs requires Gαq signaling, leading to PLCβ activity and DAG formation. DAG co-recruits PKC and PKD1, resulting in phosphorylation of PKD1 by PKC. Typically, calcium-independent PKCs are implicated in this response, as novel PKCs (PKCδ, ε, η, and θ) have the highest affinity for the PKD1 activation loop[62,63]. Activated PKD1 can translocate to the nucleus or perinuclear cytosol, where it phosphorylates type IIa histone deacetylases (HDACs). HDACs are transcriptional repressors when bound to promoter complexes at their target genes, but they also constitutively cycle in and out of the nucleus[64]. Once phosphorylated by PKD1, phospho-HDACs are excluded from the nucleus, relieving transcriptional repression[65,66].

A striking example of PKD1's importance in activity-related gene transcription can be seen in the heart[66]. In cardiac muscle, stresses, such as chronic exposure to angiotensin II or β-adrenergic agonsists, lead to decreased cardiac performance and compensatory hypertrophy. PKD1 regulates the induction of key genes under the control of MEF2 and CAMTA2–Nkx2-5, which are required for this cardiac stress response[67]. In the absence

of PKD1, stress-induced hypertrophy is abolished[68]. HDACIIa-associated transcription factors, particularly MEF2, are also important in immune system function[69] and synaptic plasticity[70,71]. PKD1 regulates transcription in immune tolerance[72], but its potential role in neuronal gene expression has been largely unexplored.

PKD1 and protein secretion. In several mammalian cell types, PKD1 regulates Golgi function. Kinase-dead PKD disrupts normal TGN morphology[73]; kinase-dead PKD1 or transient knockdown of PKD1 impairs secretion of proteins or delivery of certain proteins from the TGN to the plasma membrane[7,74]. Our understanding of the signaling that leads to PKD1-mediated trafficking is limited. However, the DAGl-sensitive protein kinase C–like regulatory N-terminal domain is required to recruit PKD1 to the TGN[75]. After the N-terminal cysteine-rich C1a domain binds DAG on the TGN, PKD1 is activated by phosphorylation on the activation loop by co-recruited PKC[76]. Once active, PKD1 phosphorylates at least one critical downstream target, phosphotidylinositol-4-phosphate kinase (PI4K), to initiate budding of vesicles from the TGN bound for the plasma membrane[59].

Kinase-dead PKD1 or PKD1 knockdown is sufficient to hamper trafficking of its cargoes, indicating PKD1 is important for constitutive trafficking. However, PKD1 is also essential for secretion and recycling of proteins in response to extracellular signals. PKD1 facilitates the recycling of β3 integrins to focal adhesions in epithelia in wound-healing[77], mediates Ca^{2+}-dependent insulin secretion in pancreatic β cells[78], and is required for hormone-induced secretion of neurotensin from the gut[79].

PKD1 in the Nervous System

Little is known about the function of PKD1 in the nervous system. Several studies have established PKD1 as important for secretory trafficking in neuronal development[80,81]. However, what functions PKD1 might serve in mature neurons has not been addressed. For my dissertation, I sought to answer two questions. First, does PKD1 regulate the dendritic trafficking of important synaptic cargoes? In Chapter 2, I show that PKD1 controls AMPAR trafficking in dendrites via the GluA2 subunit. Second, I asked whether neuronal activity regulates PKD1. In Chapter 2, I show that PKD1 is dually regulated by the neurotransmitter glutamate. mGluRs induce PKD1 kinase activity, whereas NMDARs direct PKD1 to AMPAR-containing endosomes. In Chapter 3, I seek to better understand the mechanism by which NMDARs direct PKD1 localization. I show that PKD1 translocation requires both DAG-binding by PKD1's C1b domain and PKD1's kinase domain.

CHAPTER 2. THE ROLE OF PKD1 IN AMPA RECEPTOR TRAFFICKING.

ABSTRACT

Long-term changes in synaptic strength are thought to underlie many forms of learning and memory. Changes in the localization of AMPAR-type glutamate receptors critically modulate synaptic strength through mechanisms that are not fully understood. Here we describe a novel protein kinase D1 (PKD1)-dependent pathway that mediates the postsynaptic trafficking of AMPARs. Without functional PKD1, GluA2-containing AMPARs accumulated in endosomes, decreasing the number of extrasynaptic AMPARs. This is due to decreased GluA2 exocytosis, as knockdown of PKD1 reduced GluA2 recycling, but not uptake, in response to glutamate. PKD1 is in turn dually regulated by glutamate. We found that mGluRs control PKD1 activity, whereas NMDARs control PKD1 localization. Ca^{2+} influx through NMDARs caused movement of PKD1 from the dendritic cytoplasm to AMPAR-containing endosomes. Thus, PKD1 may be a critical node in a plasticity pathway that receives distinct synaptic signals from NMDARs and mGluRs and mediates changes in the availability of extrasynaptic AMPARs.

INTRODUCTION

AMPA-type glutamate receptors (AMPARs) mediate most fast, excitatory neurotransmission in the central nervous system, and their number and subunit composition are major determinants of synaptic strength. When changes in AMPARs at the postsynaptic density (PSD) are required, two sources of dendritic AMPARs are available. Extrasynaptic AMPARs are already in the plasma membrane but not tethered to the PSD, and diffuse freely within spine necks and dendritic shafts[82,83]. These are important for rapid changes at the PSD, such as in early long-term potentiation[84]. An internal receptor pool is found in endosomes throughout the dendritic arbor. These AMPARs, from cell surface receptors, are recruited to synapses after synaptic activity[85]. Understanding how different AMPARs traffic to endosomes and back to the plasma membrane is critical to understand synaptic plasticity.

In non-neuronal cells, extracellular signals regulate endosomal trafficking through protein kinase D1 (PKD1). PKD1 facilitates recycling of β3 integrins to focal adhesions in epithelia in wound-healing[77] and mediates Ca^{2+}-dependent insulin secretion in pancreatic β cells[78]. PKD1 is expressed in neurons, but its function is mostly unknown. It regulates transport of the neurotrophin receptor subunit ARMS[57] and may help in somato-dendritic protein trafficking during development[80,81].

Does PKD1 function in the mature brain and mediate activity-dependent changes in protein composition and at synapses? We examined PKD1 localization and function in neurons and found PKD1 in dendrites *in vitro* and *in vivo*. Distinct glutamate receptors dually regulate PKD1: mGluRs activate PKD1 while NMDARs trigger its translocation within dendrites. Ca^{2+} influx through NMDARs and phospholipase C (PLC) activity

induces PKD1 to associate with endosomes that have GluA2-containing AMPARs. Disrupting PKD1 function caused intracellular retention of GluA2-containing AMPARs and increased the proportion of GluA2-lacking AMPARs at the cell surface, indicating PKD1 regulates AMPAR trafficking and subunit composition. Thus, we elucidated a new pathway mediated by PKD1 in neurons that integrates distinct synaptic signals from NMDARs and mGluRs to rapidly alter the number and composition of GluA2-containing AMPARs.

RESULTS

Endogenous PKD1 has a different distribution in neurons than PKD2 and 3

To define the role of PKD in the nervous system, we determined where it is found. Transcripts encoding the three known PKD isoforms were widely expressed in adult mouse brain, including in cortex, hippocampus, striatum, and cerebellum (Fig. 3a). PKD1, the most abundant isoform, was highly expressed in primary neuronal cultures of mouse cortex and hippocampus, which are well-established model systems for mechanistic studies of synaptic plasticity[86] and protein trafficking[4]. PKD1 levels were high and relatively constant at 2, 7, and 14 days in vitro (DIV) (Fig. 3b), when synapses form and mature.

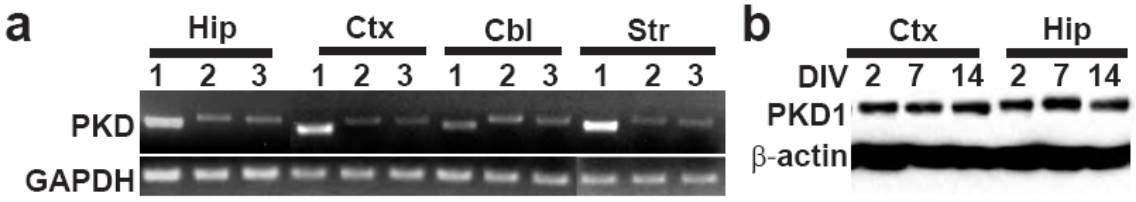

Figure 3. PKD1 is widely expressed in mouse brain and neurons. (**a**) RT-PCR showing expression of PKD1, 2, and 3 in hippocampus (Hip), cortex (Ctx), cerebellum (Cbl), and striatum (Str). (**b**) Western blot showing PKD1 expression in primary mouse cortical (Ctx) and hippocampal (Hip) neuronal cultures.

To identify the types and subcellular locations of PKDs, we analyzed cortical cultures with isoform-specific polyclonal antibodies to PKD1, PKD2, and PKD3. All three occurred in MAP2-positive (Fig. 4a, b) and -negative cells (not shown), indicating expression in neurons and glia. In neurons, PKD2 and PKD3 were mostly localized to the cell body. PKD2's distribution overlapped that of the *cis*-Golgi marker GM130. PKD3 localized to the *cis*-Golgi in some neurons but in others was diffusely distributed

throughout the soma, as in other cell types[87,88]. Their association with the Golgi is consistent with a role in protein trafficking.

Unlike the other isoforms, PKD1 was present throughout the soma and dendrites in diffuse and punctate distributions (Fig. 4b). In dendrites, puncta were seen in the shaft and some spines, suggesting unique roles for PKD1 in neuronal function and that PKD1 might regulate synaptic function.

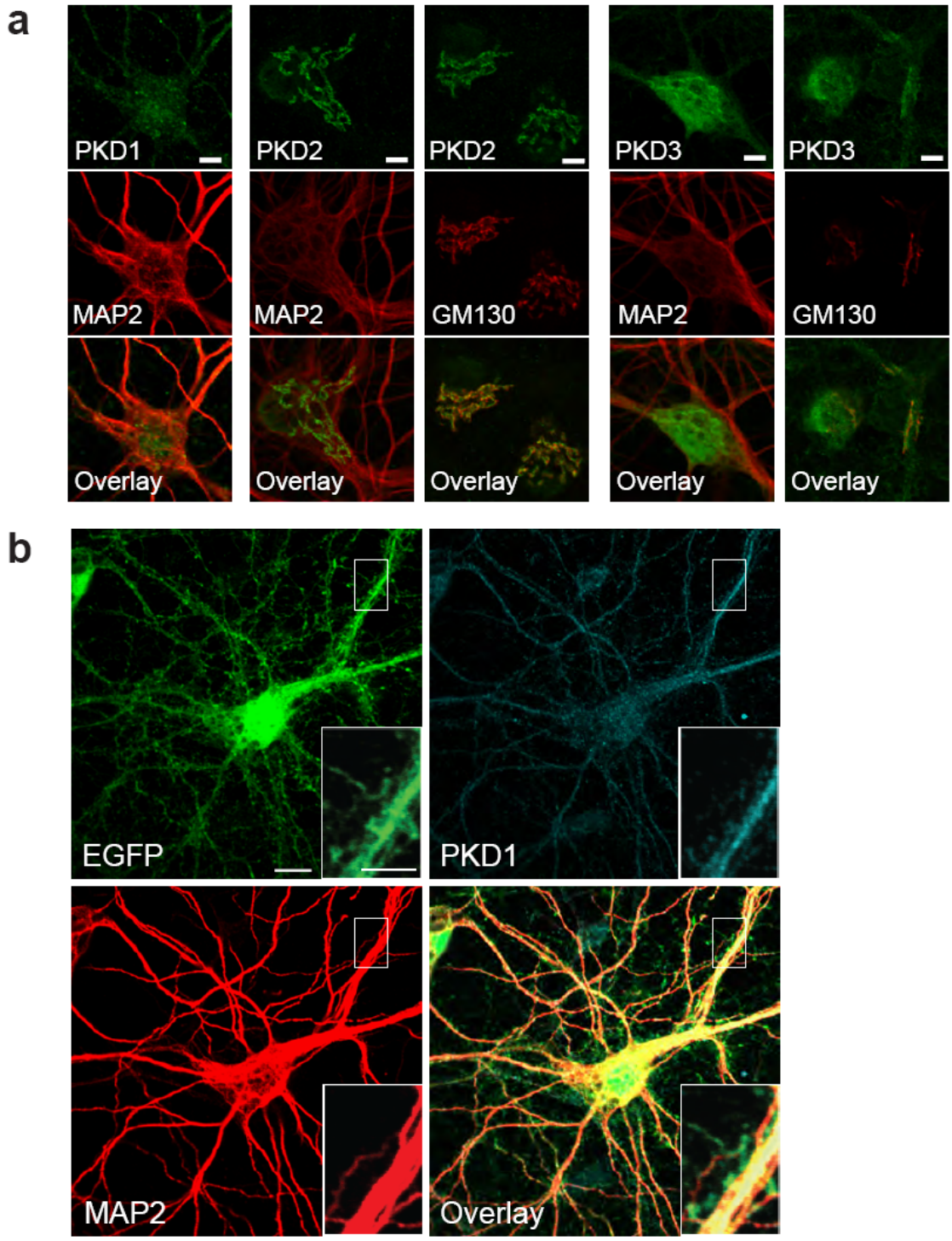

Figure 4. PKD1 has a unique dendritic distribution among PKD isoforms. (**a**) Immunocytochemical staining of PKD1, 2, and 3 in neuronal cell bodies. MAP2 serves as a neuronal marker, and GM130 labels *cis*-Golgi apparatus. Scale bar, 5 μm. (**b**) Immunocytochemical staining of a primary cortical neuron showing distribution of endogenous PKD1 in the cell body and dendrites. The neuron was infected with lentivirus encoding EGFP, which serves as a morphology marker. MAP2 specifically labels neuronal cell bodies and dendrites. Scale bars, 10 μm (main image) and 2 μm (inset).

Interfering with PKD1 function disrupts GluA2 trafficking

The unique distribution of PKD1 within dendrites led us to ask what function it might be performing there. Two of the best-characterized pathways that PKD1 regulates include gene transcription and secretory protein trafficking[89]. As neurons have a uniquely elaborate system of protein secretion machinery throughout their dendritic arbor[9], we thought PKD1 might regulate protein trafficking in dendrites. To test this, we first took advantage of a kinase-dead PKD1 mutant. PKD1-K618N lacks the lysine of the VAIK motif critical to catalysis and acts as a dominant negative on protein secretion. Kinase-dead PKD1 constitutively localizes to the *trans*-Golgi network (TGN) and TGN-derived vesicles[57,74] and prevents certain proteins from trafficking to the plasma membrane, trapping them in endosomes where PKD1 localizes[57,74].

To find dendritically localized PKD1 cargoes, we co-expressed Venus-tagged PKD1 and PKD1-K618N with transmembrane and secreted proteins important for synaptic transmission and plasticity. To assess puncta formation of different potential PKD1 cargoes, we developed a puncta index based on standard deviations of pixel intensities throughout the image[86]. Venus-PKD1 K618N altered the subcellular localization of coexpressed HA-GluA2: HA-GluA2 formed puncta that colocalized with Venus-PKD1 K618N (Fig. 5a, b). Unlike exogenously expressed HA-GluA2, Venus-PKD1 K618N did not colocalize with HA-GluA1 or affect HA-GluA1 localization, indicating kinase-dead PKD1 specifically disrupts GluA2 trafficking without impairing localization of other transmembrane proteins. This is consistent with other observations that kinase-dead PKD isoforms dominantly interfere with their specific cargoes but allow other proteins to be secreted normally[74].

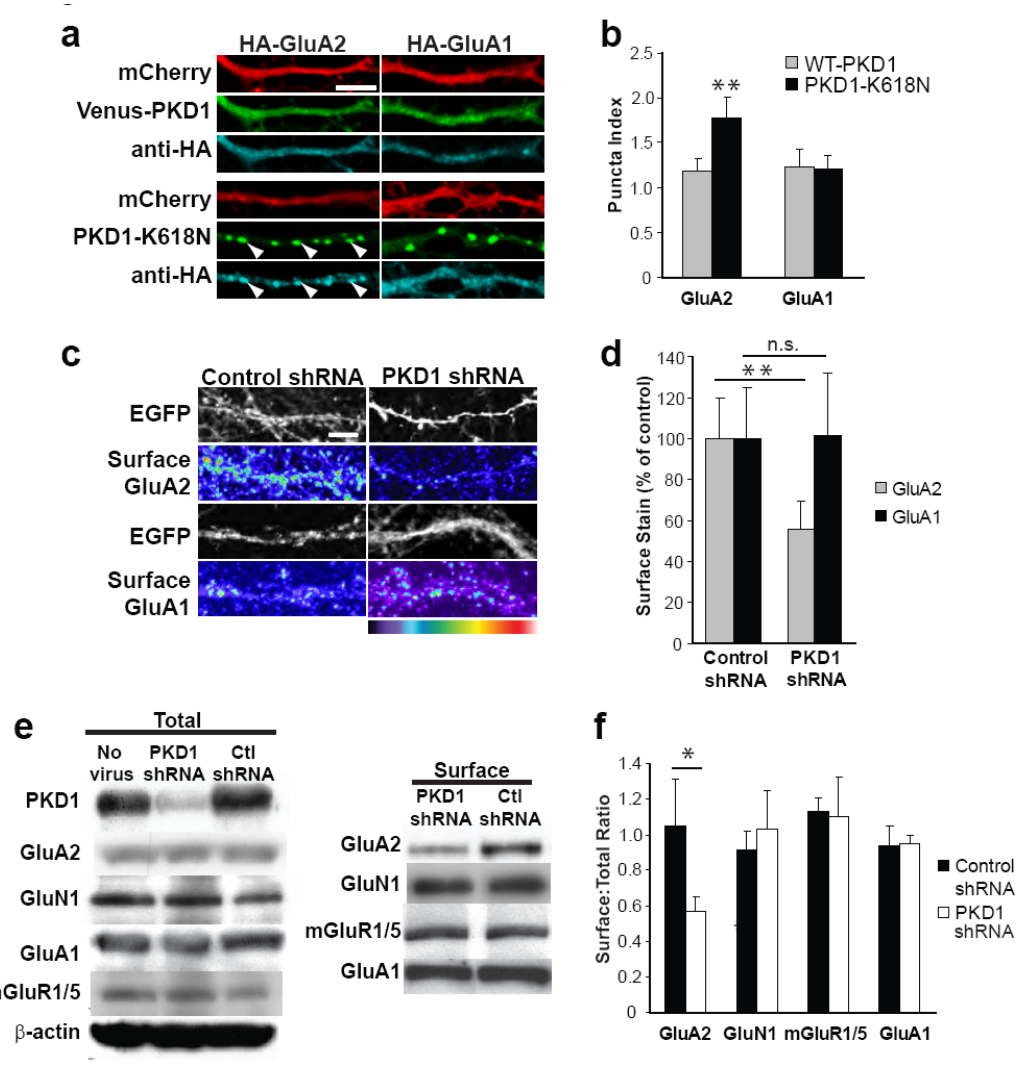

Figure 5. Interfering with PKD1 specifically disrupts GluA2 trafficking. (**a**) Neurons were co-transfected with Venus-PKD1 or Venus-PKD1 K618N and HA-GluA2 or HA-GluA1. One day after transfection, neurons were fixed and stained with anti-HA antibody. Arrowheads show colocalizing puncta. Scale bar, 5 µm. Images are representative (n>9 neurons, 4 experiments). (**b**) Quantification of GluA puncta in the presence of wild-type or K618N PKD1. (n=45 dendrites from n=9 neurons, 4 experiments). ** p<0.01, unpaired t test. (**c**) Neurons were infected with lentivirus encoding GFP and a control shRNA or shRNA against PKD1. Surface staining for endogenous GluA2 or GluA1 is shown in lower panels; blue pixels indicate lower intensity stain than red pixels. Scale bar, 5 µm. (**d**) Quantification of surface GluA2 and GluA1 staining shown in **c**. Values are mean±SD. n>80 dendrites, >15 cells per condition over three experiments. **p<0.001, n.s. not significant by unpaired t test. (**e**) Surface biotinylation assay showing total protein (left) and surface receptor levels (right) after PKD1 knockdown. (**f**) Quantification of biotinylation assay in **e**. Bars show mean±SD. n=3 experiments per receptor. *p<0.05, unpaired *t* test.

To further establish the specificity and physiological relevance of this finding, we used a complementary knockdown approach to determine if loss of PKD1 led to corresponding reductions in endogenous GluA2 subunits at the plasma membrane. At 7–8 DIV, neurons were infected with a lentivirus encoding EGFP and a short hairpin RNA (shRNA) against PKD1 or a control mutated shRNA. Spiny proximal dendrites of pyramidal and stellate neurons were stained for surface GluA1 or GluA2 at 12–14 DIV. PKD1 knockdown reduced surface GluA2 levels to ~60% of that in control cells, while surface GluA1 expression remained unaffected (Fig. 5c, d). Total GluA1 and GluA2 protein levels were unaffected by the knockdown. Thus, PKD1 regulates trafficking, rather than expression, of GluA2.

That GluA2, but not GluA1 trafficking, is altered by disrupting PKD1 function, suggests GluA2 is a specific cargo for PKD1. To investigate whether trafficking of other glutamate receptors is unaffected by PKD1 knockdown, we examined trafficking of NMDARs and Group I mGluRs. Surface biotinylation assays after PKD1 knockdown by lentiviral infection, as above, showed decreased surface expression of GluA2, but not of the obligatory NMDAR subunit GluN1 or mGluRs or of GluA1 (Fig. 5e, f). This result suggests two conclusions. First, the fact that group I mGluR and NMDAR surface localization remains intact after PKD1 knockdown suggests that surface loss of GluA2 is not an indirect effect of PKD1 on mGluRs and/or NMDARs, which also regulate AMPAR trafficking. Second, GluA2 is a specific cargo of PKD1, and global protein trafficking is likely unaffected by PKD1 knockdown.

PKD1 co-localizes with GluA2 *in vivo*

If PKD1 constitutively regulates GluA2 trafficking *in vivo*, we might expect the two molecules to colocalize. We used array tomography[90] to obtain high-resolution immunofluorescence images of 48 70-nm serial sections from a transgenic mouse expressing YFP in pyramidal cells of cortical layer 5[91]. Thus, YFP was a morphology marker to assess PKD1's subcellular localization. PKD1 was distributed in puncta throughout neurons of layers 4 and 5 of adult mouse cortex (Fig. 6). Puncta were in the cytosol and adjacent to the plasma membrane in the neuropil and cell bodies. As these tissue arrays can be probed multiple times[90], we tested PKD1 co-localization with several synaptic markers in the same sample. To quantify this colocalization, we developed a van Steensel-like correlation coefficient[92]. Few PKD1 puncta were at synapses; there was little co-localization with PSD95 or synapsin. However, PKD1 showed strong correlation with GluA2, suggesting PKD1 is associated with a nonsynaptic fraction of GluA2. This is consistent with a role for PKD1 in trafficking GluA2 through the dendritic secretory pathway.

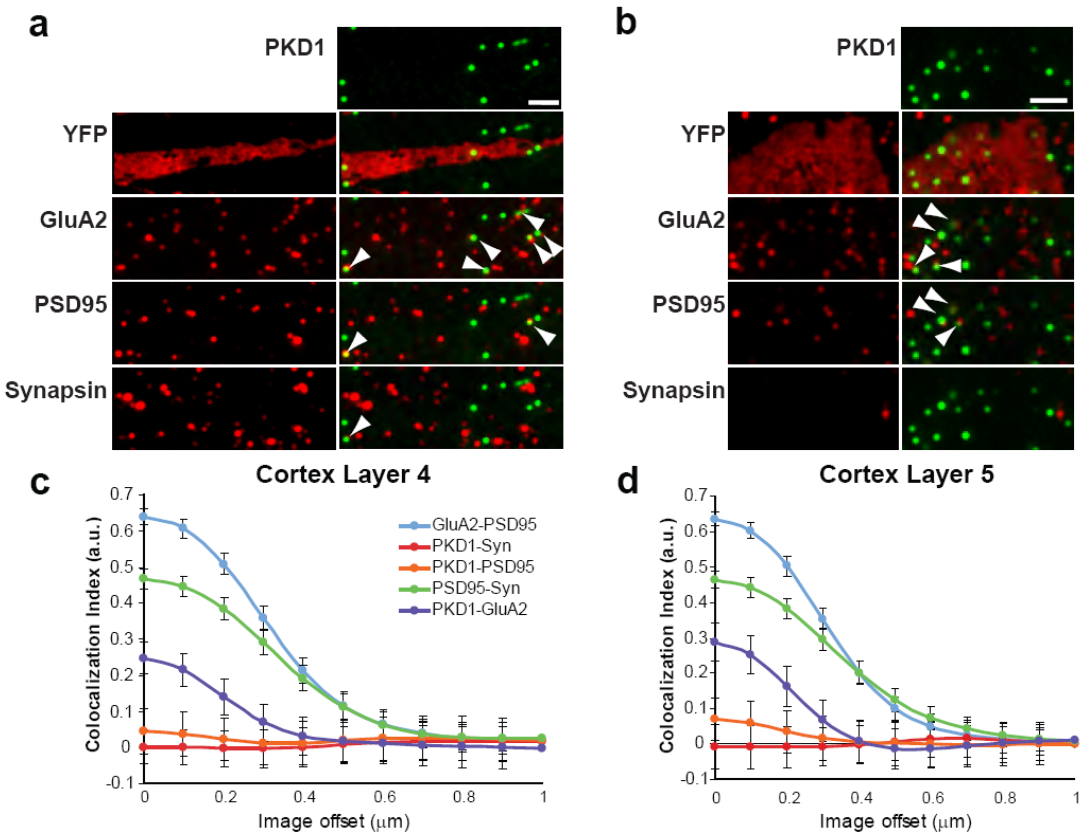

Figure 6. PKD1 primarily co-localizes with a nonsynaptic fraction of GluA2 *in vivo* at steady state. (**a**) Array tomography images of immunoreactivity of endogenous PKD1, GluA2, PSD95, and synapsin in dendrites of cortical layer 4 of mouse brain transgenically expressing YFP as a morphology marker. Arrowheads point to colocalizing puncta. Scale bar = 2 μm. (**b**) Array tomography images of immunoreactivity of endogenous PKD1, GluA2, PSD95, and synapsin in the cell body layer, cortical layer 5 of mouse brain transgenically expressing YFP, which serves as a morphology marker. Arrowheads point to colocalizing puncta. Scale bar = 2 μm. (**c**) Quantification of colocalization of PKD1 and synaptic markers in layer 4 of mouse cortex. Points along the line are mean correlation coefficients based on analysis of 48 70-nm sections; error bars show SD. (**d**) Extent of colocalization of PKD1 and synaptic markers in layer 5 of mouse cortex. Points along the line are mean correlation coefficients based on analysis of 48 70-nm sections; error bars show SD.

Interfering with PKD1 decreases availability of extrasynaptic AMPARs

If PKD1 regulates GluA2 surface expression, it might determine AMPAR subunit composition or availability. GluA2 governs Ca^{2+} permeability and other biophysical properties of AMPARs[13], and specific AMPAR antagonists exist, which block only receptors lacking the GluA2 subunit. Thus, the relative proportion of GluA2-lacking AMPARs at the synapse can be assessed electrophysiologically and pharmacologically.

We used IEM-1460, an open-channel blocker with high affinity for GluA2-lacking AMPARs[93]. We measured EPSCs in cells expressing shRNA against PKD1 or mutated control shRNA before and after IEM-1460 application. Controls had no change in EPSC size, consistent with basal AMPARs comprising GluA1/2 or GluA2/3 subunits. However, cells lacking PKD1 had smaller EPSCs after IEM-1460, indicating these cells had a synaptic population of GluA2-lacking AMPARs (Fig. 7a,b). Disrupting PKD1 function altered AMPAR composition and led to a relative increase in functional AMPARs lacking GluA2.

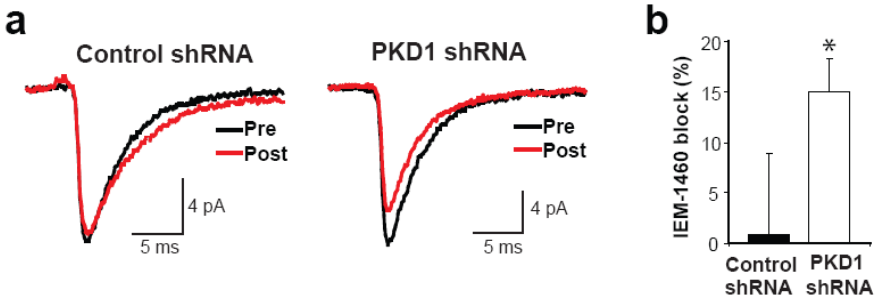

Figure 7. PKD1 knockdown alters synaptic AMPAR composition. (**a**) Primary hippocampal neurons were infected with lentivirus encoding EGFP and a control shRNA or shRNA against PKD1. Neurons were patched and held at -80 mV while EPSCs were recorded for 3 minutes to assess baseline EPSC amplitude. IEM-1460 (50 μM), an antagonist of GluR2 lacking AMPARs, was then applied, and changes to EPSCs were monitored for 10 minutes. Black traces show average EPSCs before drug application, red traces show average EPSCs after IEM-1460. Representative traces are from single neurons. (**b**) Quantification of average percent blocks of EPSCs by IEM-1460 in control and PKD1 shRNA expressing neurons. n=3 cells per condition. *$p<0.05$, t test.

Although PKD1 knockdown increased functional synaptic AMPARs lacking GluA2, the magnitude of change (15% ± 3.2%) was smaller than expected. Our cell-biology data indicate a 30–40% loss of surface GluA2 (Fig. 5). Were extrasynaptic AMPARs affected by loss of PKD1? A differential effect on synaptic and extrasynaptic AMPARs is possible: PSD may retain a set number of AMPARs via extracellular and cytoplasmic protein-protein interactions even when their net availability in the plasma membrane is

altered[94]. To test whether extrasynaptic AMPARs had altered subunit composition, we measured whole-cell currents from cells expressing shRNA against PKD1 or a control shRNA. Although whole-cell currents include responses mediated by synaptic AMPARs, they are dominated by those mediated by extrasynaptic receptors. AMPARs lacking GluA2 are inwardly rectifying, owing to a voltage-dependent block by endogenous polyamines, and rectification can be detected from whole-cell currents by measuring I/V curves. Disrupting PKD1 function did not significantly change rectification at the whole-cell level ($p=0.1$, Fig. 8a). The failure to demonstrate significant rectification at the whole-cell level, despite the appearance of some synaptic currents mediated by GluA2-lacking AMPARs suggests PKD1 disruption did not significantly affect the subunit composition of extrasynaptic AMPARs or extrasynaptic GluA2-lacking AMPARs are not robustly rectifying[95].

We hypothesized most of the 30-40% loss of surface GluA2 and the intracellular GluA2 retention that follows PKD1 disruption (Fig. 5) resulted from reducing total available extrasynaptic AMPARs without changing rectification. We "puffed" glutamate onto neurons to elicit extrasynaptic AMPAR current and performed a dose-response curve (Fig.8b). In neurons where PKD1 was knocked-down, peak AMPAR current was decreased ($p=0.004$, Wilcoxon rank test), as expected for a loss of extrasynaptic AMPARs. At maximum glutamate dose, PKD1 knockdown reduced maximal AMPAR current by over 30%, consistent with significantly decreased surface GluA2 as measured by surface staining and biotinylation (Fig. 5).

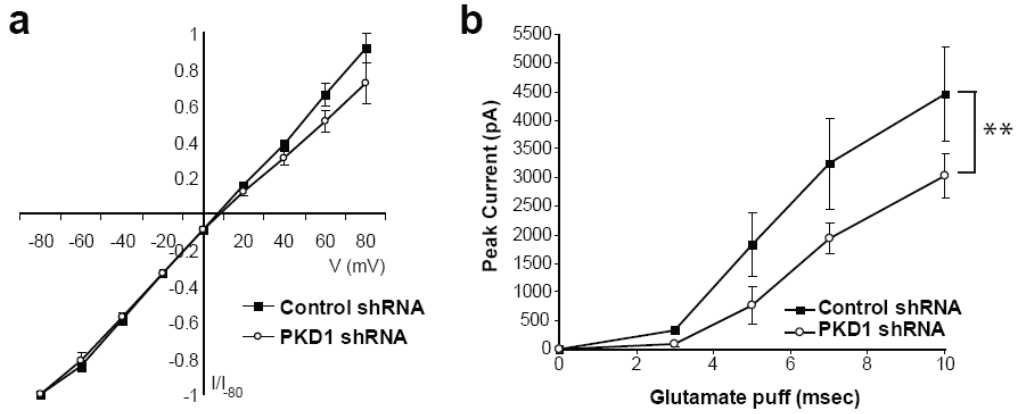

Figure 8. PKD1 knockdown reduces the extrasynaptic AMPAR pool. (**a**) Average I/V curves from cells expressing shRNA against PKD1 or control mutant shRNA, n=7 cells per condition. Rectification indices (current at +40 divided by current at -60) not significant, p=0.1 by student's t test. (**b**) Primary hippocampal neurons were infected with lentivirus encoding EGFP and a control shRNA or shRNA against PKD1. Neurons were patched and held at -80 mV while glutamate (1 mM) was puffed onto the cell for varying durations. **p=0.004, Wilcoxon rank test. n=5 cells per condition.

PKD1 regulates GluA2 reinsertion, not internalization, during glutamate-induced recycling

Dominant-negative PKD1 and PKD1 knockdown led to intracellular retention of GluA2, which could result if PKD1 decreases the endocytosis of GluA2 or promotes its exocytosis. To test the first possibility, we measured surface GluA2 levels after glutamate-induced endocytosis (Fig. 9a–c). If PKD1 decreases GluA2 endocytosis, the relative decrease in surface GluA2 should be greater after PKD1 knockdown than in control neurons. However, we found no difference in surface GluA2 reduction after glutamate between control and PKD1 knockdown cells.

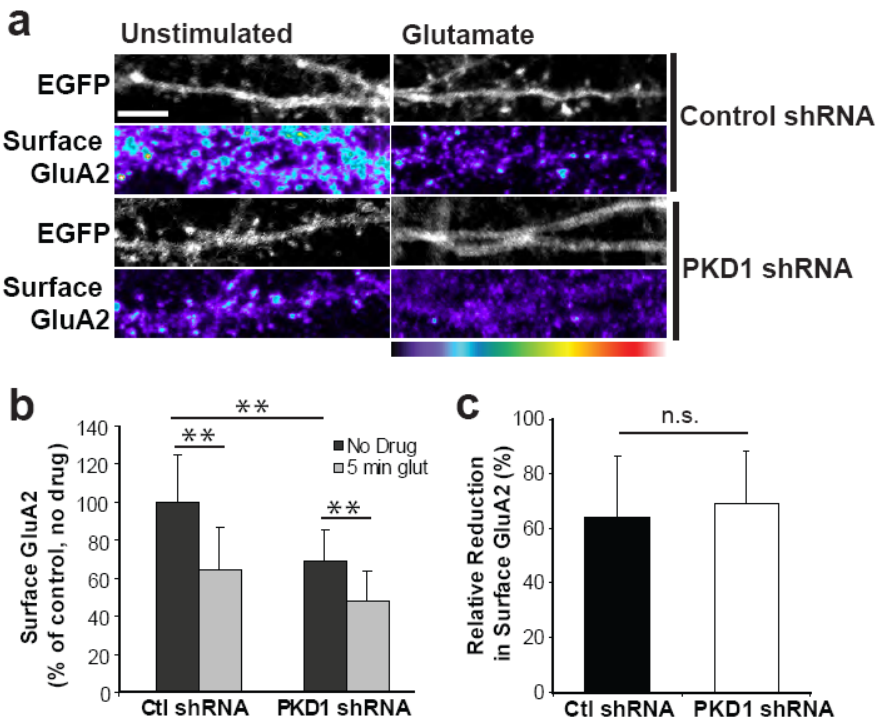

Figure 9. PKD1 does not regulate GluA2 uptake in response to glutamate. (**a**) Neurons were infected with lentivirus encoding GFP and a control shRNA or shRNA against PKD1. Surface staining for endogenous GluA2 is shown in lower panels; blue pixels indicate lower intensity staining than red pixels. Glutamate (30 μM with 10 μM glycine) was applied for 5 minutes when indicated. Scale bar, 5 μm. (**b**) Quantification of surface staining in A. Values are mean±SD. n=13–15 cells, >65 dendrites/condition. **p<0.01 (unpaired *t* test with Bonferroni correction). (**c**) Comparison of relative reduction in surface GluA2 after glutamate application in control and PKD1 knockdown cells in **a**. The difference was not significant (n.s.; p>0.1, unpaired *t* test).

To test whether PKD1 promotes GluA2 exocytosis, we measured exocytosis using GluA2 tagged on its N-terminus with superecliptic pHluorin (pH-GluA2). pHluorin fluorescence is reversibly quenched by the acidic endosome environment, so its fluorescence intensity correlates with its extracellular localization[96]. Cells were transfected with pH-GluA2 and an shRNA against PKD1 or a mutated control. Immediately after 5 min of glutamate stimulation, pH-GluA2 endocytosis is maximal, as indicated by reduced pH-GluA2 fluorescence intensity of 20% of baseline. The magnitude of normalized peak endocytosis was similar with endogenous PKD1 or after

PKD1 knockdown. At 55 min after stimulation, pH-GluA2 fluorescence recovered to >90% of baseline in control transfected neurons. However, in neurons lacking PKD1, fluorescence recovery was significantly delayed (Fig. 10). Parallel experiments performed with pHluorin-tagged GluA1 revealed no deficit in GluA1 recycling after glutamate-induced endocytosis (Fig. 11), confirming again PKD1 specificity for GluA2 trafficking. Thus, PKD1 seems to be important in regulating the rate of GluA2 exocytosis in response to glutamatergic activity.

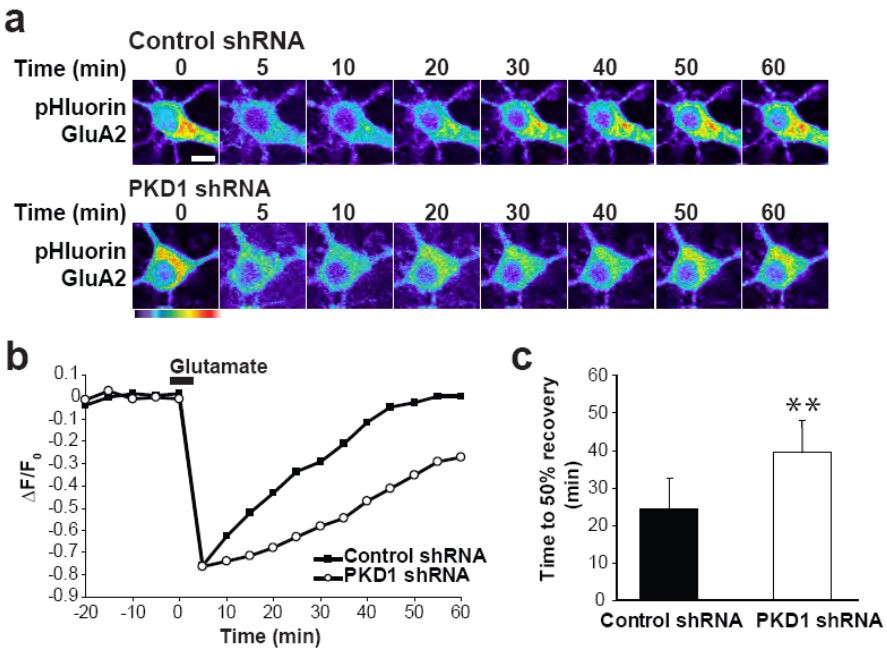

Figure 10. PKD1 regulates GluA2 recycling in response to glutamate. (**a**) Representative images of neurons expressing pH-GluA2 and PKD1 shRNA or mutated control. At t=0 min, glutamate was applied for 5 minutes and washed out. Surface expression of pH-GluA2 correlates with fluorescence intensity, which is color-coded in the images and ranges from low levels (indigo) to high levels (red). Scale bar, 10 μm. (**b**) Quantification of pH-GluA2 intensity of images shown in D. (**c**) Quantification of recovery of pH-GluA2 fluorescence intensity expressed as the time required for fluorescence to recover halfway back to baseline from the time point of maximal internalization. **p<0.01 (unpaired *t* test). n=7 cells for control, 8 cells for PKD1 shRNA.

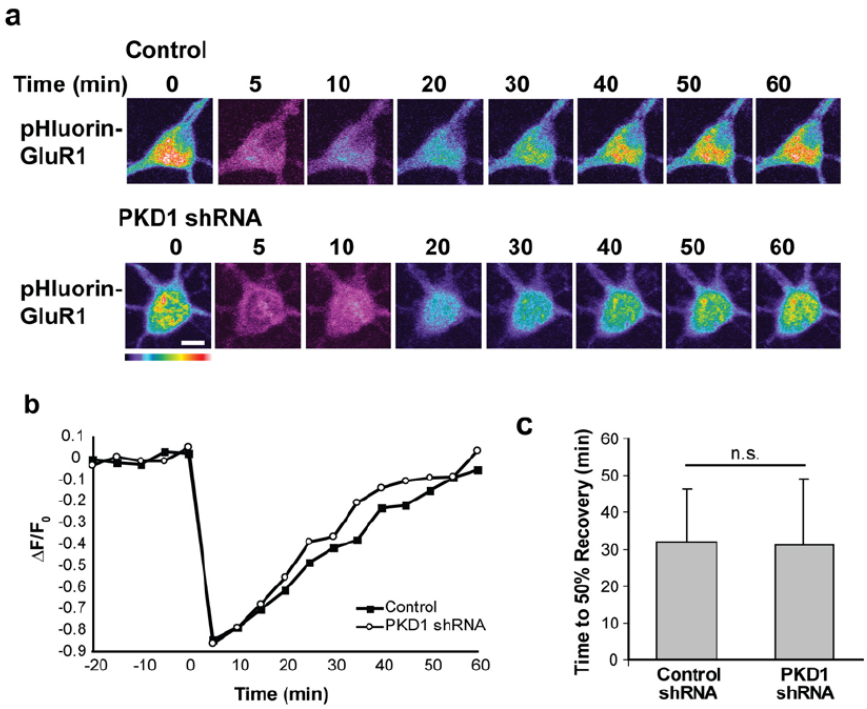

Figure 11. PKD1 knockdown does not affect GluA1 recycling. (**a**) Representative images of neurons expressing pH-GluA1 and PKD1 shRNA or mutated control shRNA. At t=0 min, glutamate was applied for 5 minutes and washed out. Blue pixels indicate lower intensity pH-GluA1 signal than red pixels. Scale bar, 10 μm.(**b**) Quantification of pH-GluA1 intensity of images shown in **a**.(**c**) Quantification of recovery of pH-GluA1 fluorescence intensity halfway back to baseline. n=6 cells for control, 5 cells for PKD1 shRNA. n.s., p>0.1 by unpaired t test.

PKD1 activation is downstream of mGluRs

How does glutamate regulate PKD1 activity? We stimulated mouse cortical cultures with bath-applied glutamate (30 μM) and prepared extracts for western analysis. After 5 min of stimulation, PKD1 was phosphorylated at the activation loop (S744/8) and autophosphorylation (S916) sites (Fig. 12), indicating it was activated[55]. Phosphorylation persisted for up to 30 min after stimulation but returned to near control levels after an hour of washout. By contrast, activation of voltage-gated Ca^{2+} channels with K^{+} depolarization[86] or TrkB receptors with brain-derived neurotophic factor (BDNF) did not activate PKD1 (Fig.12). Thus, PKD1 activity was rapidly but selectively controlled by the major excitatory neurotransmitter glutamate.

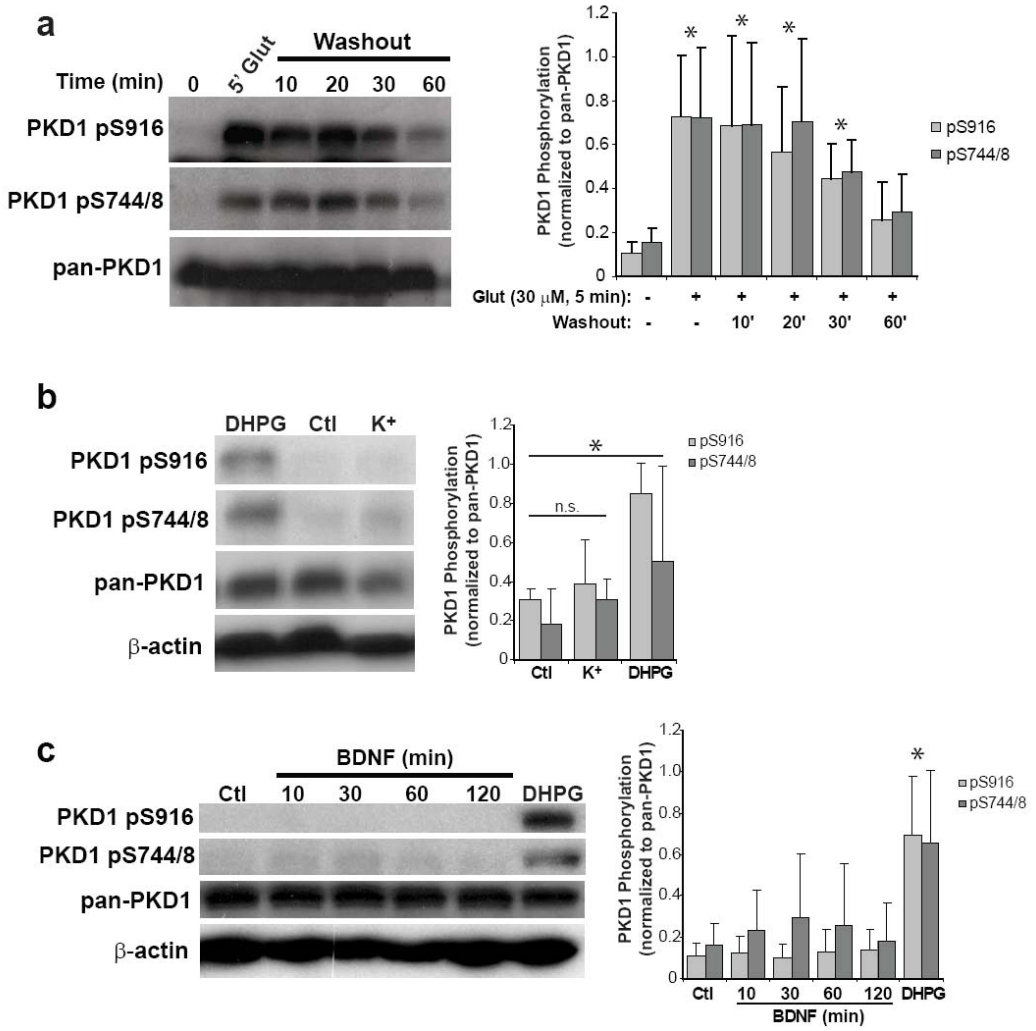

Figure 12. PKD1 kinase activity is elicited by glutamate, but not voltage gated Ca^{2+} channels or BDNF. (**a**) Mouse cortical neurons were stimulated with 30 μM glutamate for the indicated times (n=4). Phospho band intensities were quantified by densitometry and normalized to total PKD1. Bars show mean±SD. * $p < 0.05$ for both phospho-sites per condition by unpaired t test. (**b**) Mouse cortical neurons were depolarized with K^+ (55 mM + 50 μM D-APV) or DHPG (50 μM) for 5 minutes before lysis and analysis by western blot. Representative blot on left; quantification from n=3 experiments on right. * $p<0.05$ for both phospho-PKD1-specific antibodies, unpaired t-test. (**c**) Neurons were stimulated with the Group I mGluR agonist DHPG (50 μM) for 5 minutes or BDNF (100 ng/ml) for the lengths of time indicated before lysis and analysis by western blot. Representative blot on left; quantification from n=3 experiments on right. * $p<0.05$ for both phospho antibodies; n.s., $p>0.1$ for both phospho antibodies, unpaired t-test.

In non-neuronal cells, PKD1 is commonly activated by Gαq-coupled receptors and isoforms of protein kinase C (PKC) or by an influx of extracellular Ca^{2+} [97]. Since glutamate activates Gαq-coupled mGluRs and Ca^{2+}-permeable NMDARs, we determined

which glutamate receptor type activates PKD1 in neurons. A Group I mGluR antagonist (MCPG, Fig. 13) or a Group I mGluR antagonist cocktail (10 μM CPCCOEt and 100 nM MPEP, Fig. 12) reduced glutamate-induced PKD1 phosphorylation, and mGluR-specific agonist DHPG induced phosphorylation (Fig. 13). The NMDAR antagonist 2-amino-5-phosphonovaleric acid (APV) did not block phosphorylation of PKD1 by glutamate, and NMDA application did not induce phosphorylation. Thus, mGluR activation seems to be necessary and sufficient to mediate PKD1 activation in response to glutamate, whereas NMDARs are dispensable.

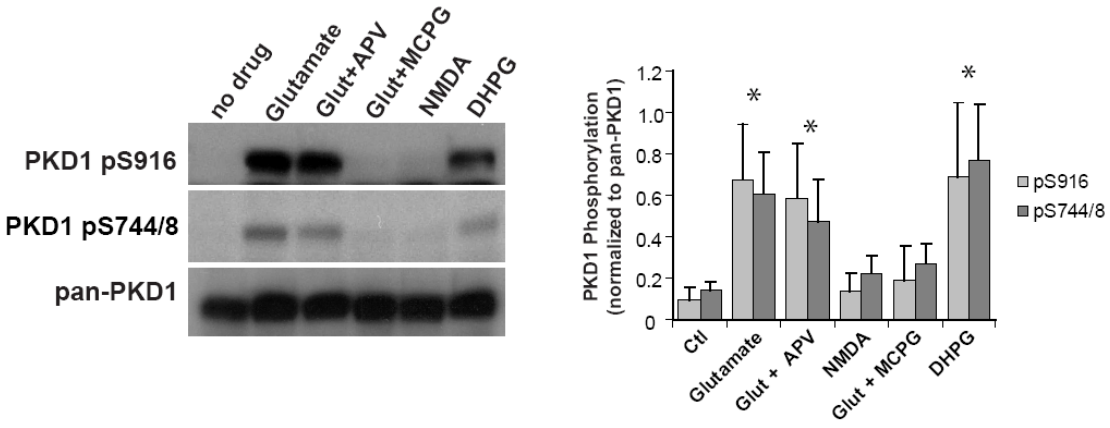

Figure 13. PKD1 is activated by mGluRs. Neurons were stimulated as indicated, harvested, and examined by western blot (n=4). Phospho band intensities were quantified by densitometry and normalized to total PKD1. Bars show mean±SD. * $p < 0.05$ for both phospho-sites per condition by unpaired t test.

PKD1 translocates to endosomes in response to Ca^{2+} influx through NMDARs

PKD1 subcellular localization after stimulation of non-neuronal cells provided clues about its function. Certain stimuli cause PKD1 to move from the cytosol to the plasma membrane[61], nucleus[98], mitochondria[59], and *trans*-Golgi network (TGN). We assessed PKD1 movement in neurons in response to glutamate. To facilitate our studies, we made a plasmid encoding PKD1 with the fluorescent protein Venus fused to its N-terminus (Venus-PKD1). Transfected Venus-PKD1 was localized diffusely throughout the

cytoplasm in neurons under basal conditions but translocated to discrete puncta throughout the dendrites in response to a brief application of glutamate (2 min; Fig. 14a, b). Neuronal morphology, demarcated by co-transfected fluorescent mCherry protein, appeared healthy and remained unchanged during puncta formation.

Next, we discovered the receptor responsible for glutamate-induced translocation of Venus-PKD1. Puncta formation after glutamate stimulation was unaffected by the metabotropic glutamate inhibitors CPCCoET and MPEP, and DHPG did not induce translocation, even at a dose (100 μM) above that required for PKD1 activation (50 μM; Fig. 6b). In contrast, puncta formation was induced by NMDA alone and blocked by the NMDAR antagonist APV or the absence of extracellular Ca^{2+}. To determine if the source of Ca^{2+} influx is important, we depolarized neurons with high K^{+} (30 mM) with APV, which should selectively activate voltage-sensitive Ca^{2+} channels[86]. No puncta formed (Fig. 7b). Thus, although mGluRs activate PKD1 after glutamatergic stimulation, PKD1 translocation to dendritic puncta is mediated by Ca^{2+} influx through NMDARs.

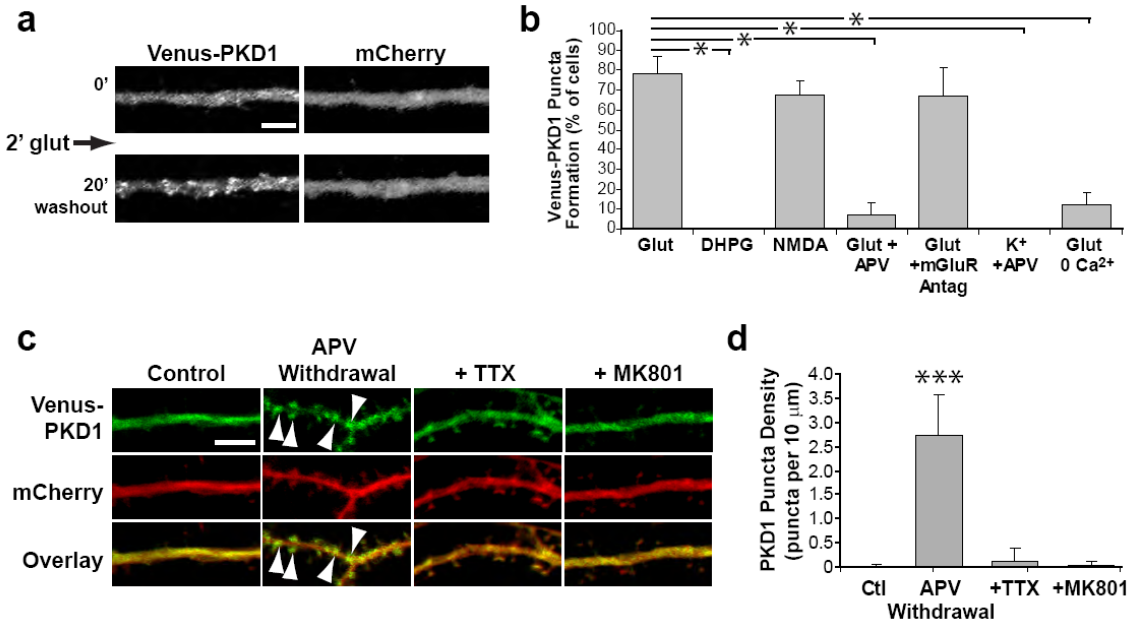

Figure 14. PKD1 translocates to puncta in response to NMDARs. (**a**) Live-cell confocal images of Venus-PKD1 and mCherry before and after stimulation with 30 μM glutamate. Scale bar, 5 μm. Images are representative (n>30 neurons, >3 experiments). (**b**) Puncta formation of Venus-PKD1 after glutamate and other pharmacologic stimuli. *p<0.05, different from glutamate treatment. n=24-40 neurons from n=2-5 experiments per condition. (**c**) Confocal microscopy images of Venus PKD1 and mCherry with or without APV withdrawal (30 minutes). Arrowheads show Venus-PKD1 puncta. Third and fourth columns show APV withdrawal with the addition of TTX (1 μM) or MK-801 (5 μM). Scale bar, 5 μm. Images are representative (n>30 neurons, n=3 experiments). (**d**) Quantification of puncta formation shown in **c**. Density is calculated as the number of PKD1 puncta per 10μm of dendrite. Bars show mean±SD. *** $p < 0.001$ by unpaired t test with Bonferroni correction.

Is synaptic activation of NMDARs sufficient to induce PKD1 puncta? To induce predominantly NMDAR-mediated synaptic activity, we used APV withdrawal. Culturing neurons chronically with APV yields specific upregulation of NMDARs without altering synapse number or other aspects of synaptic physiology[99]. Subsequent withdrawal of APV results in a bout of synaptic NMDAR stimulation via spontaneous activity of the culture, and this paradigm has been used extensively to study synaptic NMDAR-mediated changes in signaling and receptor trafficking[100-102]. APV withdrawal for 30 min caused robust PKD1 translocation (Fig. 14c). If this translocation was mediated by synaptically evoked NMDARs, it should be blocked by preventing action potentials with

tetrodotoxin or by the use-dependent NMDAR antagonist MK-801. These treatments prevented PKD1 translocation in response to APV withdrawal, suggesting synaptic NMDAR activity causes PKD1 puncta formation (Fig. 14c-d).

If NMDAR-dependent PKD1 puncta formation is a physiologically relevant process, PKD1 puncta should form spontaneously in culture. By examining neurons transfected with Venus-PKD1 in normal medium, we found ~19% of cells had spontaneous PKD1 puncta at DIV15; only ~3% of cells grown in medium supplemented with APV (Fig. 15).

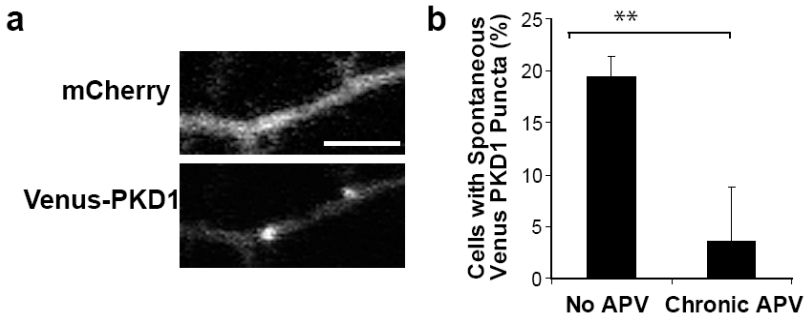

Figure 15. Venus-PKD1 puncta form spontaneously in culture. (**a**) Spontaneous Venus PKD1 puncta found in neurons were transfected with Venus-PKD1 and mCherry and imaged live at 15 days *in vitro* (DIV). (Left) A neuron with Venus-PKD1 puncta induced by low-frequency stimulation with a field stimulator. (Right) The same neuron 24 h later. Puncta have disappeared, indicating that puncta formation is not associated with cell death. Scale bar, 5 μm. (**b**) Summary data of the number of cells observed to form spontaneous Venus PKD1 puncta in normal media at 15 DIV versus those grown in the NMDAR antagonist APV until 18 DIV. **$p<0.01$ by student's t test.

PKD1 colocalizes with early endosomes and AMPARs in response to NMDARs

As PKD1 regulates AMPAR recycling in response to glutamate, and glutamate causes PKD1 to translocate to dendritic puncta, we hypothesized that glutamate-induced PKD1 puncta were endosomes involved in AMPAR recycling. To characterize Venus-PKD1 puncta, we examined colocalization of Venus-PKD1 and GluA2 or Rab5 and Rab11 Venus-PKD1 and HA-tagged GluA2, Rab5 were strongly colocalized, and Rab11 less so

(Fig. 16). As a negative control, we coexpressed Venus-PKD1 with Lamp1a-HA, a marker of lysosomes. Venus PKD1 puncta did not colocalize with Lamp1, consistent with PKD1 playing a role in AMPAR recycling but not degradation. Co-localization of PKD1 with endosomes but not lysosomes agrees with our data showing PKD1 knockdown regulates GluA2 trafficking but not does not affect total GluA2 levels.

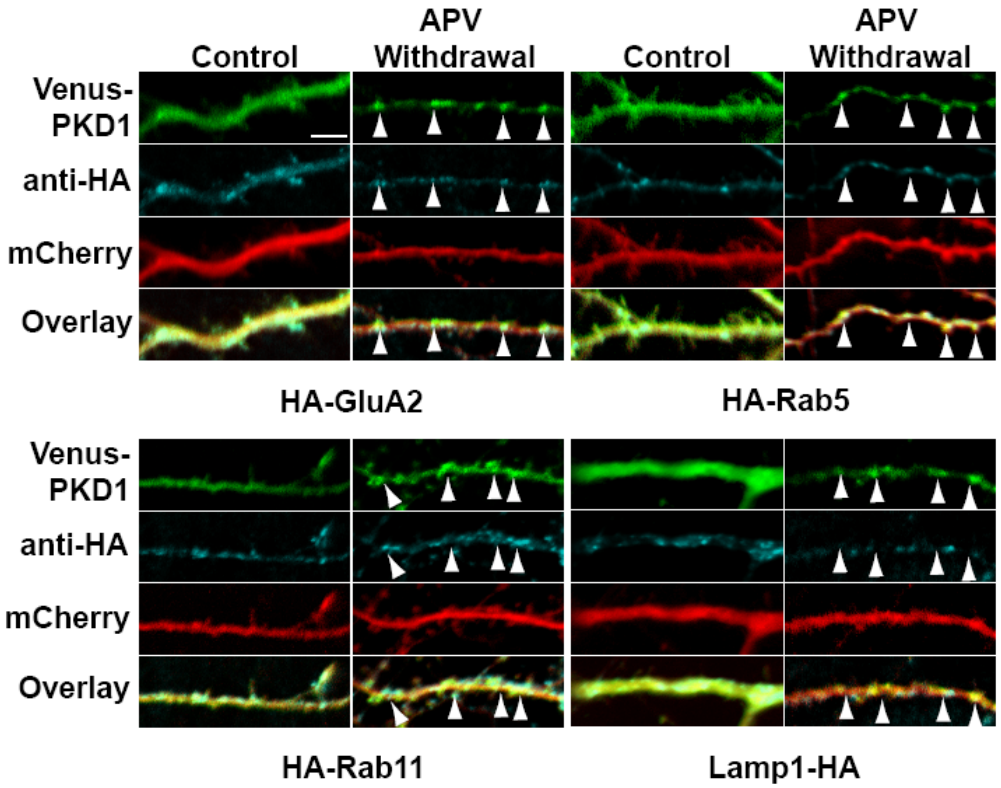

Figure 16. PKD1 puncta are AMPAR-containing endosomes. Neurons were transfected mCherry, Venus-PKD1 and HA-GluA2 or other HA-tagged endosome markers. Neurons were subjected to APV withdrawal and were stained with anti-HA antibodies. Arrows show location of Venus-PKD1 puncta. Images are representative (n>30 neurons, n=3 experiments). Scale bar = 5 μm.

DISCUSSION

Here we elucidated a new pathway mediated by PKD1, dually controlled by mGluRs and NMDARs, that regulates the composition and trafficking of AMPARs. PKD1 is expressed throughout dendrites, and it regulates trafficking of GluA2-containing

AMPARs. GluA2 surface expression was reduced after PKD1 knockdown, and GluA2 exocytosis was delayed after glutamate stimulation. Cells lacking PKD1 showed a large decrease in extrasynaptic AMPAR current, but only modest increases in GluA2-lacking synaptic AMPARs. We conclude that PKD1 primarily functions to promote trafficking of GluA2-containing AMPARs from internal compartments to the cell surface, thereby regulating the size of the extrasynaptic AMPAR pool. PKD1 is dually regulated by glutamate: its kinase activity is downstream of mGluR signaling, and its translocation to early endosomes containing AMPARs requires NMDAR signaling. Our findings suggest PKD1 is a target of neuronal activity and regulates AMPAR surface expression through dendritic GluA2 trafficking (Fig. 17).

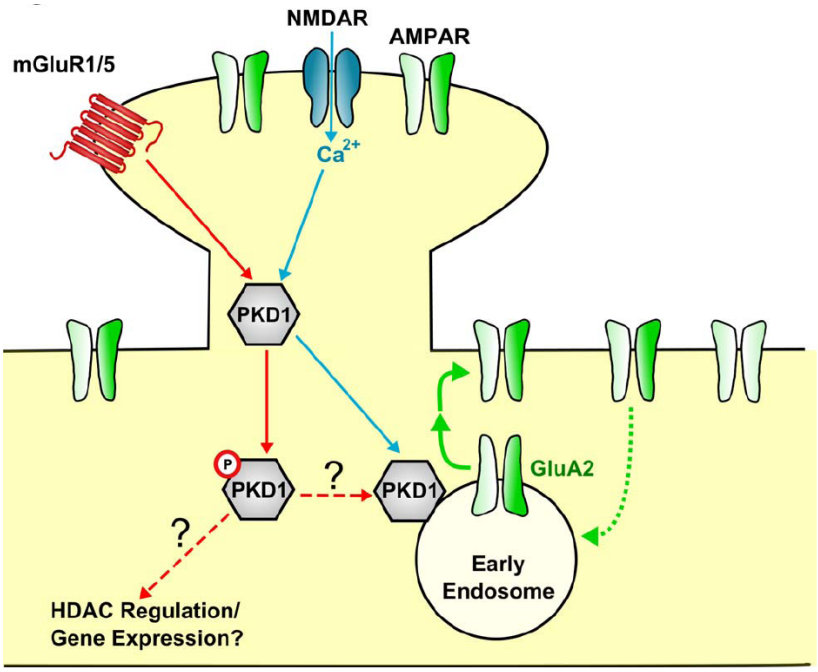

Figure 17. Model for PKD1's role in glutamate signaling and receptor trafficking. Red pathway, Group I mGluRs lead to PKD1 phosphorylation and kinase activation. Blue pathway, calcium influx through NMDARs induces PKD1 translocation to AMPAR-containing endosomes. PKD1 in turn promotes the recycling/insertion of AMPAR containing GluA2 into the extrasynaptic membrane. Whether PKD1 kinase activity is required for GluA2 recycling, or serves another function such as mediating gene transcription, remains to be seen.

When PKD was first cloned from mouse in 1994 by Eric Rozengurt and colleagues[51], they noted PKD1 mRNA was strongly expressed in brain. However, only recently PKD1 emerged as an important player in neuronal secretory trafficking. In other cell types, PKD1 regulates basolateral protein trafficking in non-neuronal polarized cells[74]. As neurons employ the basolateral sorting pathway to send transmembrane and secreted protein to dendrites[9], it is not wholly surprising that PKD1 has been implicated in neuronal development and in establishing polarized dendrites[80,81]. Indeed, disrupting PKD1 function early *in vitro* (1–3 days in culture) results in a truncated dendritic arbor and excess axonal sprouting[80,81]. This may be due, in part, to PKD1's regulation of proteins important for neuronal development, such as the neurotrophin receptor subunit ARMS/Kidins220[103]. However, none of these studies addressed the function of PKD1 in more adult neurons. Here, we show that PKD1 expression persists as neurons mature (Fig. 1b) and regulates the trafficking of an important synaptic cargo, AMPARs.

We showed PKD1 regulates AMPAR surface localization through the GluA2 subunit. Manipulating PKD1 function by shRNA knockdown altered GluA2 surface expression without affecting the obligatory NMDAR subunit GluN1, Group I mGluRs, or another AMPAR subunit, GluA1. How might PKD1 regulate GluA2? PKD1 promotes secretion of cargoes from the TGN[73,74], as well as recycling from the so-called short-loop pathway in early endosomes[77]. Dendrites contain TGN outposts[104], and new GluA2 is synthesized there[7], so PKD1 could alter trafficking of newly synthesized AMPARs. In the present study, we show an activity-dependent translocation of PKD1 to Rab5-positive early endosomes (Fig. 7). This implies PKD1 promotes short-loop recycling of GluA2 or the sorting and transition of GluA2 into recycling endosomes. As the mechanisms by which

AMPARs are sorted through endosomes remain murky, it will be important to determine what PKD1's function in endosomal sorting might be.

We were surprised to find that although in many assays PKD1 specifically regulated GluA2, PKD1 knockdown had only a modest effect on AMPAR rectification. However, there was a large reduction in the size of the extrasynaptic AMPAR pool. How might we reconcile a large loss of GluA2 without a proportional change in rectification? One possibility is that when PKD1 is lost, there is either a concomitant or compensatory decrease in polyamine sensitivity in the remaining GluA2 lacking channels. Such changes are seen in cases of increased TARP association[105]. Elucidating the composition of extrasynaptic AMPARs in the absence of PKD1 will be important for understanding the extent and the mechanisms by which PKD1 regulates AMPARs.

Despite the burgeoning evidence for PKD1's importance in neurobiology, how neuronal activity controls PKD1 function has been largely unexplored. Our results show PKD1 is dually regulated by glutamate. NMDAR signaling caused translocation of PKD1 to GluA2-containing endosomes but did not activate the kinase. Translocation of Venus-PKD1 was Ca^{2+}-dependent and specific to Ca^{2+} influx through NMDARs; Ca^{2+} influx through voltage-gated Ca^{2+} channels failed to induce translocation of Venus-PKD1 (Fig. 7b). Thus, for PKD1 signaling, Ca^{2+}-sensitive signaling proteins close to the channel mouth may be more important than bulk Ca^{2+} flow[86], underscoring the critical importance of NMDARs in this signaling pathway.

What function might PKD1 translocation serve? Some kinases translocate in response to activity, promoting synaptic plasticity. For example, activity-dependent Ca^{2+} influx activates calcium-calmodulin kinase II (CaMKII) and induces its rapid redistribution to

synapses. Sustained and concentrated CaMKII activity at synapses is essential for changes in AMPAR phosphorylation and localization and thereby turns a transient signal into long-term potentiation of the synapse[106]. Neuron-specific PKCγ also undergoes Ca^{2+}-dependent translocation. PKCγ is activated by binding to the plasma membrane in neurons in response to Ca^{2+} and PLC activity[107]. We speculate PKD1 translocation propagates information from NMDARs, and that whether the endosome-localized kinase is active provides an additional level of regulation.

In contrast to NMDAR activity, stimulating mGluRs activated PKD1 but did not affect translocation to endosomes. What role mGluR-mediated PKD1 kinase activity has in AMPAR trafficking or other neuronal processes is unclear. At the TGN, PKD1 induces vesicle budding by phosphorylating PI4K[59]. PKD1 also influences trafficking by phosphorylating its cargoes or by autophosphorylation[57]. Thus, one reason mGluRs and NMDARs signal distinctly to PKD1 might be that PKD1 serves as a coincidence detector. While translocation of CaMKII and PKCγ in response to NMDAR activity concomitantly activates these kinases, PKD1 translocation to GluA2-containing endosomes occurs independently of PKD1 activation. Thus, coactivation of mGluRs and NMDARs may be required to put active PKD1 in the same subcellular compartment as AMPARs and regulate their trafficking. In agreement with this, kinase-dead PKD1 altered GluA2 localization in dendrites. Separating the regulation of PKD1 localization from its activation could allow combinatorial control of PKD1 function during complex processes, such as synaptic plasticity.

How might PKD1 affect synaptic transmission? Under basal conditions in culture, PKD1 appears to be critical for building the extrasynaptic AMPAR pool. This could be

due to PKD1's regulation of GluA2 trafficking, as GluA2 constitutively cycles to and from the plasma membrane[108-110]. Nearly half of cell-surface AMPARs are in a mobile, extrasynaptic pool[82,83]. Although extrasynaptic AMPARs are not directly activated by synaptic glutamate release, they are important for shaping postsynaptic currents. In the short term (milliseconds), desensitized synaptic receptors can be exchanged for extrasynaptic AMPARs to alleviate EPSC depression[111]. Quickly overcoming desensitization is important for fidelity of the EPSC between low- and high-frequency activity. Without PKD1, the dearth of extrasynaptic AMPARs could limit exchange of desensitized receptors and result in frequency-dependent rundown of the EPSC.

In addition to its constitutive effect on extrasynaptic AMPARs, PKD1 regulates the recycling rate of GluA2 in response to glutamate (Fig. 5). The cycling of AMPARs to and from the cell surface has been the subject of much scrutiny, as the trafficking of these receptors is essential for plasticity. Briefly, AMPARs are removed from the synapse first by diffusing into the extrasynaptic space where they may enter endocytic hot zones and be endocytosed via clathrin coated pits/vesicles. These vesicles fuse with early endosomes, from whence AMPARs may be sorted for degradation or recycling[9]. Recycled AMPARs are inserted into the extrasynaptic space and incorporated into the synapse by interactions with the PSD[47]. Thus, both surface localization and exchange between synaptic and extrasynaptic AMPARs are critically regulated in long-term plasticity. Glutamate signaling through NMDARs and mGluRs regulates each of the above steps, putting PKD1 in an ideal position to contribute to glutamate-induced plasticity.

As PKD1 promotes AMPAR exocytosis, PKD1 could contribute to LTP. NMDAR-dependent LTP requires coincident activation of signals to both insert and retain AMPARs to increase synaptic strength, as extrasynaptic AMPAR insertion precedes synaptic incorporation[112]. As PKD1 may be important for plasma membrane insertion of AMPARs, in the context of synaptic activity PKD1 may be important in long-term synaptic strengthening. On a shorter timescale, the early phase of LTP requires synaptic incorporation of pre-existing extrasynaptic AMPARs[84,113]. Without PKD1 to fuel the extrasynaptic AMPAR pool, inadequate reserves might hinder early LTP.

PKD1 might also regulate LTD. At some synapses, LTD requires an exchange of GluA2-lacking AMPARs for GluA2-containing ones[114,115]. In the context of homomeric, overexpressed AMPAR subunits, PKD1 preferentially regulates GluA2-containing receptors over GluA1 homomers. If this preference holds true *in vivo*, PKD1 may help regulate AMPAR composition during long-term changes in synaptic efficacy.

In the present study, we uncovered new functions for PKD1 in AMPAR localization and glutamatergic signaling, two crucial processes in plasticity. Given its strategic distribution throughout dendrites and its unique dual regulation by NMDARs and mGluRs, PKD1 likely serves other roles in neuronal signaling besides protein trafficking. Notably, activation of NMDARs and mGluRs is required to trigger different forms of plasticity, and these two receptor types control discrete aspects of PKD1. This brings up a possibility of another model for PKD1 in neurobiology, where NMDARs cause PKD1 translocation to regulate AMPAR trafficking, and mGluRs activate PKD1 for another purpose (Fig. 8). One possible pathway is activity-dependent gene expression. In cardiac muscle, PKD1 is essential for the program of gene transcription required for adaptive

stress-induced changes[68]. PKD1 activation by Gαq-coupled receptors leads to its phosphorylation of class IIa histone deacetylases, alleviating transcriptional repression[89]. Though mGluRs are not well-known regulators of transcription, Group I mGluRs can induce transcription of plasticity genes, such as Arc[116]. What role PKD1 plays in mGluR signaling is unknown; however, PKD1's ability to regulate protein trafficking and gene expression suggests it is uniquely positioned to contribute to short- and long-term synaptic plasticity.

METHODS

Cortical cultures

Embryonic mouse primary cortical neurons were cultured as described[86] from wild-type C57/BL6 mice (Charles River). Neurons from embryonic day (E)18–19 embryos were plated at a density of 0.6×10^6 cells/cm^2 on 12-mm glass coverslips coated with poly D-lysine and maintained in Neurobasal medium supplemented with B27 (Invitrogen). Experiments were done at 8–14 DIV. Neurons were transfected with the calcium phosphate method[86].

Reverse transcription PCR

Total RNA was extracted from C57/BL6 mouse brain with RNEasy kits (Qiagen). cDNA was generated with SuperScript II reverse transcriptase. PKDs 1, 2, and 3 were amplified with isoform specific primers as described[117].

Western blots

Neurons were lysed with ice-cold RIPA buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, and protease inhibitor cocktail; Roche). Samples were centrifuged, and supernatants were loaded onto gels, separated by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against pan-PKD1 (1:1000, Cell Signaling or 1:5000, HJK), phospho-S744/8 PKD1 (1:1000, Cell Signaling), phospho-S916 PKD1 (1:1000, Cell Signaling), β-actin (1:4000, Sigma) or anti-tubulin (1:500,000, Sigma). Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase were used on all blots and imaged by enhanced chemiluminescence (Amersham Biosciences) or with SuperSignal West Femto substrate (Pierce). Band intensities were quantified with ImageJ Gel Analyzer tool[118].

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde with 4% sucrose for 10 min and blocked in PBS with 3% bovine serum albumin, 2% goat or donkey serum, and 0.1% Triton-X100. For surface staining, primary antibody was applied to live cells for 45 min before fixation, and detergent was omitted from the blocking step. The primary antibody concentrations were: PKD1 (1:5000, HJK), PKD2 (1:2000, Bethyl Laboratories) PKD3 (1:5000, HJK) MAP2 (1:400, Chemicon), HA (1:1000, Cell Signaling), GluA2 (1:300, Millipore). All fluorescently tagged secondary antibodies were used at 1:250 (Invitrogen).

PKD1 Knockdown

PKD1 shRNA was constructed as a short hairpin RNA in mammalian expression vector pSilencer 2.0 using the target PKD1 sequence AAAGAGTGTTTGTTGTTATGG, followed by a 9-bp linker and the inverse 21-bp sequence. The control m-shRNA used the same target sequence with four mutations (underlined): AAAGTGTGATTGTTGTTTAGG. To generate lentiviral constructs of shRNAs, the U6 promoter and short hairpin sequences from the pSilencer constructs were amplified by PCR, subcloned into viral vector FUGW2, and expressed in HEK293FT cells (Invitrogen) along with viral packaging proteins Δ8.9 and VSVG. Viruses were harvested, titered, and used for infection as described[119]. Neurons were harvested and analyzed 5–7 days after infection.

Pharmacology

Stimulations were performed at room temperature in HEPES-buffered saline (HBS: 119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 30 mM glucose, 1 μM tetrodotoxin, 10 μM NBQX, pH 7.4). Neurons were incubated in HBS for 10–20 min before stimulation. Doses used (unless otherwise indicated): PMA (100 nM), glutamate (30 μM with 10 μM glycine), APV (100 μM), NMDA (60 μM with 10 μM glycine), DHPG (50 μM), MCPG (1 mM), mGluR antagonist cocktail of CPCCOEt (10 μM) and MPEP (100 nM), and K^+ (55 mM).

APV withdrawal

Cells were cultured in neurobasal medium as described until 9 DIV, when they were transfected using Lipofectamine2000 (Invitrogen). After transfection, the cells were

placed back in their conditioned medium supplemented 1:1 with fresh neurobasal medium and 50 μM D-APV (final concentration). The medium was refreshed 1:1 with fresh neurobasal and 50 μM D-APV every 4 days after transfection. At 18 DIV, APV withdrawal was performed in HEPES-buffered saline (HBS: 119 mM NaCl, 2.5 mM KCl, 25 mM HEPES, 30 mM glucose, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 μM glycine, 5 μM strychnine, pH 7.4) for 30 min at 37 °C before fixation with 4% PFA, 4% sucrose in PBS pH 7.4, followed by analysis with confocal microscopy. Control cells were placed in the same HBS lacking glycine and strychnine but containing 50 μM D-APV. For TTX and MK-801 experiments, cells were incubated for 5 min in their conditioned, APV-containing medium with TTX (1 μM) or MK-801 (5 μM) before washout with APV-lacking HBS (as above) containing TTX or MK-801.

Imaging and analysis

For live-cell measurements of Venus PKD1 puncta formation, images were taken with an upright LSM 510 scanning laser confocal microscope with a 40× water immersion dipping objective. Venus-PKD1 and mCherry images of the same field were taken before and after stimulation. For PKD1 K618N, APV withdrawal, and endosome colocalization studies, images were obtained with a Zeiss LSM 510 laser scanning confocal microscope. Puncta formation for HA-GluA1 or 2 coexpressed with Venus PKD K618N were calculated based on a puncta index (PI) developed previously[86]. PIs were calculated with MetaMorph software (Molecular Devices) as the standard deviation (SD) of anti-HA immunofluorescence along a region of dendrite divided by the SD of mCherry fluorescence along the same region. Puncta formation was determined by calculating PIs

in the presence of wild-type or K618N PKD1. To quantify PKD1 puncta formation after APV withdrawal, dendrite length was measured and puncta were counted in ImageJ. For colocalization with endosome markers, thresholds for puncta were set to fivefold mean background intensity for each channel. The soma was excluded from the analysis. Thresholding and overlap quantification were performed in MetaMorph software.

Electrophysiology

Recordings from cultured neurons were performed in voltage-clamp mode (V_{hold} = -80 mV) in Tyrode's solution (in mM): 150 NaCl, 3.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4, 305 mOsm) at room temperature. GABAergic signaling was blocked with 50 μM picrotoxin, For experiments measuring IPSC sensitivity to IEM-1460 (50 μM, Tocris), recordings were made from 17 DIV hippocampal neurons. For experiments measuring whole-cell AMPAR current sensitivities, recordings were made from 12–13 DIV cortical neurons. During measurement of whole-cell AMPAR currents, 50 μM cyclothiazide (Tocris) and 10 μM SYM 2081 (Tocris) were added to the external solution to prevent AMPAR desensitization and to desensitize kainate receptors, respectively.

Data were collected with a MultiClamp 700B amplifier (Molecular Devices) and ITC-18 A/D board (HEKA) with Igor Pro software (Wavemetrics). Voltage-clamp recordings were filtered at 2 kHz and digitized at 10 kHz. Electrodes were made from borosilicate glass (pipette resistance, 2–4 MΩ). For all recordings, the internal solution contained (in mM): 120 CsMeSO3, 15 CsCl, 8 NaCl, 0.5 EGTA, 10 Hepes, 2 MgATP, 0.3 NaGTP; pH to 7.5 and Osm 290. For experiments measuring whole-cell AMPA

currents, 0.1 mM spermine and 10 mM BAPTA were also included in the internal solution.

To measure whole-cell AMPAR currents, 1 mM glutamate dissolved in Tyrode's solution was puffed onto neurons using a Picospritzer III (Parker Instrumentation). Puffer pipettes were positioned 15–20 μm from the soma and air pressure was set to 0.2 PSI.

Constructs

GW1-Venus-PKD1 was created from a Clontech vector containing GFP-PKD1 as described[61]. A 46–amino acid stretch links Venus to the N-terminus of mouse PKD1. The K618N point mutation in Venus-PKD1 was generated with a site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. HA-Rab11, HA-Rab5, and HA-Lamp1 were cloned into pGW1. HA-GluA1 and HA-GluA2 were described[108]. For details on PKD1 shRNA and control constructs for knockdown, see Supplementary Methods.

Array tomography

Arrays of brain tissue from YFP-H transgenic mice[91] were prepared with 70-nm slices as described[90]. Primary antibodies were incubated on arrays overnight at 4°C as follows: PKD1 (1:300, HJK), PSD95 (1:50, NeuroMabs) synapsin (1:100, Millipore), GluR2 (1:30, Chemicon), Rab5 (1:100, BD Biosciences), and GM130 (1:50, BD Biosciences). To examine the spatial relationship of the channels of interest, we used a cross-correlation analysis similar to that of van Steensel and colleagues [92]. For each pair of channels, patches of neuropil were convoluted to find the raw colocalization score, Sr, for a range of lateral offsets. To correct for differences in mean brightness in different

channels, the analysis was repeated with one channel transposed (and therefore uncorrelated) to get a baseline score, St. The colocalization index (Ci) of two channels was determined by normalizing by difference-over-sum as Sr-St/Sr+St = Ci. Ci = 1 indicates ideal colocalization, Ci = 0 no colocalization above chance, and Ci <0 anti-localization. With this method, colocalization of different labels can be objectively compared in a channel-independent manner.

Surface biotinylation

Primary cortical neurons (7 DIV) were infected with lentivirus encoding either shRNA against PKD1 or control shRNA. Five days after infection, cells were placed on ice and rinsed twice with ice-cold phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4). Sulfo-NHS-LC-biotin (2 mg/ml, Pierce) was added, and cells were incubated at 4°C with gentle rocking. After 30 min, cells were rinsed three times with ice-cold PBS and harvested in PBS containing 1% Triton X-100, 0.1% SDS, and protease cocktail inhibitor with EDTA (Roche). Lysates were sonicated briefly and centrifuged at 13,000 rpm in a tabletop centrifuge at 4°C. Protein concentration was measured by Bradford assay, and lysates were diluted to 200 μg/ml in PBS + 1% NP-40. Avidin Sepharose beads (50 μl slurry, Pierce) were added to diluted lysate (500 μl) and incubated overnight at 4°C. Beads were washed three times in ice-cold PBS, and biotinylated protein was eluted by boiling in 2× Laemmli buffer. Surface proteins were analyzed by western blot with anti-GluR2 (1:1000, NeuroMabs), anti-GluR1 (1:200, Millipore), anti-NR1 (1:1000, Upstate Biotechnology), or anti-mGluR1/5 (1:1000, NeuroMAbs). Total protein (40 μg per sample) was reserved and run on parallel western

blots to control for expression between samples. Band intensities were quantified with ImageJ Gel Analyzer tool[118].

Electrophysiology

Recordings from 17 DIV hippocampal cultures were performed in voltage-clamp mode (V_{hold} = -80 mV) in Tyrode's solution (in mM): 150 NaCl, 3.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4, 305 mOsm at room temperature. Data were collected with a MultiClamp 700B amplifier (Molecular Devices) and ITC-18 A/D board (HEKA) using Igor Pro software (Wavemetrics). Voltage-clamp recordings were filtered at 2 kHz and digitized at 10 kHz. Electrodes were made from borosilicate glass (pipette resistance, 2–4 MΩ). The intracellular recording solution contained (in mM): 120 $CsMeSO_3$, 15 CsCl, 8 NaCl, 0.5 EGTA, 10 Hepes, 2 MgATP, 0.3 NaGTP; pH to 7.5 and Osm 290.

pHluorin GluA1 and 2 recycling assay

Rat GluA2 tagged with superecliptic pHluorin was cloned into pGW1 mammalian expression vector. Rat GluA1 tagged with superecliptic pHluorin was cloned into pCAGGS mammalian expression vector. mCherry was subcloned to replace EGFP in FUGW PKD1 shRNA and control vectors. mCherry served as a morphology marker to monitor the overall health of the cell. pHluorin-GluA constructs and and PKD1 shRNA or mutant control were transfected into mouse cortical neurons with Lipofectamine2000 (Invitrogen). At 12 DIV, neurons were imaged on a Zeiss LSM 510 confocal microscope with a 40× water immersion lens in HBS, only nimodipine was excluded. After 20 minutes of baseline imaging, 30 μM glutamate and 10 μM glycine were washed on to

cells by gravity flow perfusion for 5 minutes, followed by washout with control HBS solution. Images were collected every 5 minutes. Average pixel intensity of pH-GluA1 or 2 in the cell body was calculated in MetaMorph, and background was measured in a cell-free region of the image and subtracted from fluorescence intensities before further calculations. Fractional change in fluorescent intensity ($\Delta F/F_0$) was calculated as F_t-F_0/F_0, where F_t is pH-GluA intensity at time t and F_0 is the average pH-GluA fluorescence intensity at four points before glutamate application.

CHAPTER 3. PKD1 LOCALIZATION TO ENDOSOMES

ABSTRACT

To preserve temporal and spatial information of biochemical signals within neurons, protein kinases translocate in response to synaptic activity. In response to synaptic NMDAR activation, PKD1 localizes to endosomes and regulates exocytic AMPAR trafficking. Here, I show that PKD1 translocation is a multistep process requiring both its membrane-binding and kinase domains. DAG generated downstream of NMDARs recruited PKD1 to membranes, and PKD1 subsequently translocated to endosomes via its kinase domain. Mutating PKD1's activation loop serines to alanines (SSAA) disrupted endosomal localization, despite the fact that NMDARs do not induce PKD1 phosphorylation. I generated an ATP analog inhibitor–sensitive allele of PKD1 to determine if phosphoryl transfer activity is required for endosomal localization, but found inhibitor binding did not block PKD1 translocation. On the contrary, I found inhibitor binding rescued endosomal localization of the SSAA mutant, suggesting inhibitor binding induces the closed or "active" kinase conformation, and this conformation may be required for endosomal targeting. My results underscore the complexity with which PKD1 is regulated in neurons, and opens up the possibility that PKD1 localization to endosomes with and without concomitant kinase activation have different effects on AMPAR trafficking.

INTRODUCTION

Protein phosphorylation is a fundamental post-translational modification in signal transduction pathways. The enzymes that catalyze phosphorylation, protein kinases, comprise a large family with overlapping expression profiles, upstream regulators, and substrate preferences. Even so, in the vast dendritic arbor of a neuron, synapse-specific changes can take place through kinase signaling. How are these biochemical messages propagated with such precision? The spatial organization of signaling machinery provides one level of specificity. By gathering multiple members of a signal transduction pathway at one subcellular location or on a single scaffolding molecule, phosphorylation is restricted to substrates of interest at the correct site.

As seen in the previous chapter, PKD1 regulates AMPAR trafficking by promoting receptor exocytosis after glutamate stimulation. Presumably, PKD1's site of action is early endosomes, as glutamate induces PKD1 translocation to Rab5-positive puncta. Interestingly, PKD1 localization and enzymatic activity are controlled by distinct signaling pathways. While mGluRs elicit PKD1 kinase activity, NMDAR signaling results in PKD1 translocation. This raises the question of how PKD1 translocation is regulated: PKD1 translocation without concomitant kinase activation has not been documented.

To better understand how NMDARs regulate PKD1 localization, I set out to characterize the signaling pathway leading from NMDARs to PKD1 translocation.

RESULTS

PKD1 Translocation Requires PLC Activity

NMDAR activity causes PKD1 to translocate from the cytosol to AMPAR-containing endosomes. Intriguingly, in our APV withdrawal paradigm, PKD1 puncta formed within the shaft of dendrites and spines (Ch. 2, Figs. 14,16). We wondered if this was due to a primary signal generated by NMDARs at the plasma membrane or in spines. In non-neuronal cells, PKD1 is redistributed after it binds its N-terminal cysteine-rich domains to newly generated diacylglycerol (DAG) in plasma and intracellular membranes[120-122]. To test whether NMDAR-dependent PKD1 translocation in neurons requires phospholipase C (PLC)-dependent DAG production, we used the PLC inhibitor U73122 and found it blocked glutamate-induced puncta formation (Fig. 18). PLC can also recruit protein kinase C (PKC) to membranes, resulting in PKD1 phosphorylation and kinase activation. However, PKC antagonists GF 109203X or Gö6983 did not prevent PKD1 translocation, consistent with our finding that NMDARs do not induce PKD1 kinase activity.

Next, we determined if PKD1 translocation requires direct binding to DAG. Binding of DAG to the C1a and C1b domains of PKD1 is disrupted by two single amino acid substitutions, P155G and P287G, respectively[75,120,123]. In Venus-PKD, only the P287G mutation prevented glutamate-induced puncta formation (Fig. 18). Thus, DAG produced downstream of NMDARs binds PKD1 and induces its translocation. Mutating two other regulatory domains of PKD1, the pleckstrin homology domain and the PDZ ligand, did not affect translocation, emphasizing the specificity of the response to DAG.

PKD1 Binds Plasma Membrane via C1b Domain before Forming Puncta

The C1b domain of PKD1 mediates association with the plasma membrane[123], and the C1a domain mediates binding to internal membranes, such as TGN[75,120]. Thus, the requirement of C1b for puncta formation was surprising and suggested another intermediate translocation step.

In other cell types, PKD1 first translocates to the plasma membrane in response to PLC activation[124,125], so we determined if PKD1 associates with membranes before forming puncta. Confocal imaging revealed that puncta formation is the result of a two-step translocation. After 10 min of stimulation, Venus-PKD1 localized to the plasma membrane in the cell body and dendrites in many neurons and was relatively absent from the cytoplasm (Fig. 18). After 30 min, Venus-PKD1 formed puncta in the cytoplasm, which persisted with continued bath application of glutamate. In contrast, the P287G mutant did not translocate to the plasma membrane (Fig. 18) and did not form puncta. Thus, prior plasma membrane association is required for localization of PKD1 to puncta.

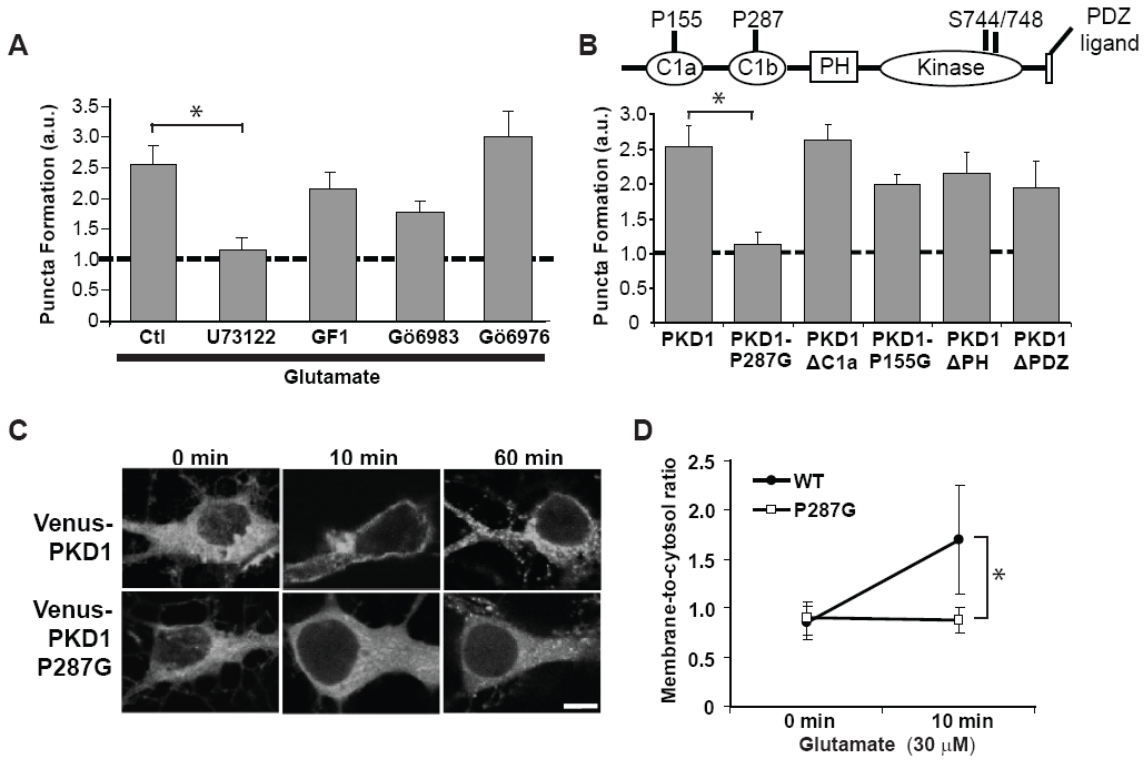

Figure 18. PKD1 translocation is a two-step process requiring PLC**. A**) Neurons transfected with Venus-PKD1 and mCherry were stimulated with glutamate (30 μM, 60 min, plus indicated drugs), and their respective puncta formations were calculated (see Methods). Only U73122, the PLC inhibitor, blocks puncta formation. PKC inhibitors GF109203X, Gö6976, and Gö6983 had no effect. Bars show mean + SD. *p<0.05, before vs. after stimulation (two-tailed, paired *t* test). n>4 dendrites from at least three neurons in at least two experiments. **B**) (Top) Primary structure of PKD1 with critical amino acids highlighted. Bottom shows puncta formation (see Methods for calculation) of each construct in response to 30 μM glutamate. Bars show mean + SD. *p<0.05, before vs. after stimulation (two-tailed, paired *t* test). n>4 dendrites from at least three neurons in at least two experiments. **C**) Neurons were stimulated, fixed, and imaged by confocal microscopy. Neurons transfected with Venus-PKD1 P287G do not show plasma membrane association after 10 min or puncta formation at 60 min. Scale bar, 5 μm. Membrane localization after glutamate stimulation was observed in 44 of 63 cells expressing Venus-PKD1, compared to 8 of 55 cells expressing Venus-PKD1 P287G (n=3 experiments). Experimenter was blinded to conditions for cell counting. Scale bar, 5 μm. **D**) Quantification of membrane localization 10 minutes after glutamate stimulation (30 μM plus 10 μM glycine; for quantification details, see Methods). * p<0.05 by unpaired t test. Error bars show mean ± SD. Quantification from n>9 cells per condition from three experiments

PKD1 Kinase Domain Is Required for Translocation to Endosomes

We performed further mutational analysis to try to block the second step of PKD1 translocation (i.e., from the plasma membrane to endosomes). Having already made a

series of deletion or inactivating mutations in all the major domains or motifs of PKD1 except the kinase domain, I began to make mutations there. We found that mutating PKD1 activation loop serines to alanines (Venus-PKD1 SSAA) blocked PKD1 puncta formation (Fig. 19)

We were very surprised at this finding. We previously showed that NMDARs do not cause PKD1 activation loop phosphorylation (Ch.2, Fig. 13). We wondered whether we were missing a phosphorylation event as the anti-phosphoSS744/8 antibody recognizes a dually phosphorylated epitope. Perhaps only one phospho-residue in the activation loop is required for translocation, and we are unable to detect this with our antibody. Another possibility was that the serine-to-alanine mutation alters the secondary structure or hydrophobicity of the activation loop, and a more serine-like, polar residue would be sufficient for translocation, even without the negative charge conferred by a phospho group.

To address these hypotheses, I mutated the activation loop serines to glutamines (Venus-PKD1 SSQQ). Glutamines cannot be phosphorylated but are approximately the same size and charge as serines and may be a more conservative substitution than alanine. Venus-PKD1 SSQQ translocated in response to glutamate equally well as wildtype PKD1 and Venus-PKD1 SSEE (Fig. 19). Thus, negative charge at the activation loop is not required for translocation, and either charged or polarized residues in the activation loop are permissive for endosomal localization.

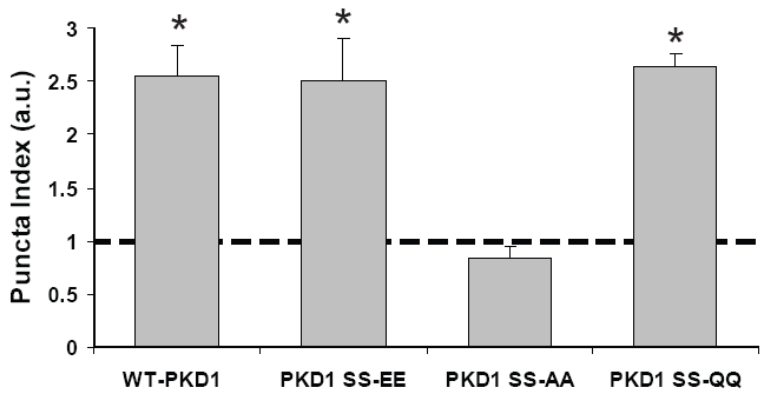

Figure 19. PKD1 translocation does not require activation loop phosphorylation. Neurons were transfected with various Venus-PKD1 constructs and mCherry. Neurons were stimulated with glutamate (30 μM, 60 min), and their respective puncta formation were calculated (see Methods for calculation). Bars show mean + SD. *p<0.05, before vs. after stimulation (two-tailed, paired *t* test). n>15 dendrites from at least five neurons in three experiments.

Generating an ATP Analog-Sensitive PKD1 Allele

As phosphorylation of the PKD1 activation loop is not required for translocation, as seen in the SSQQ mutant, we wanted to know whether phosphoryl transfer activity of PKD1, independent of activation loop phosphorylation, is required for localization to endosomes. The obvious experiment would be to use another mutant of PKD1 without kinase activity. Unfortunately, the most commonly used kinase-dead PKD1 mutant, PKD1 K618N, is constitutively punctate[57,74] (see Ch.2 Fig. 5) and cannot be used to assess translocation. I thus decided to collaborate with the Shokat lab to generate a form of PKD1 that can be inhibited and that is suitable for my translocation studies.

To block PKD1 activity more acutely and transiently, I developed an allele of PKD1 that is specifically sensitive to an ATP analog inhibitor that does not affect wild-type (WT) kinase. The Shokat lab[126] developed an approach to subtly mutate a kinase of interest so that it can accommodate large ATP analogs in its active site. The analog-sensitive allele can then be reversibly inhibited by drugs that do not affect the WT allele.

With advice from the Shokat lab, I made an analogous mutation in the ATP binding pocket of PKD1 (M665A) to create an allele that is specifically inhibited by the ATP analog 1NA-PP1 (Fig. 20). In HEK cells, inhibition of PKD1 autophosphorylation is seen within 10 min. In neurons, application (1 h) of 1NA-PP1 causes Venus-PKD1 M665A, but not WT PKD1, to form puncta reminiscent of the kinase-dead allele (Fig. 20, compare to Ch.2, Fig. 5). These data suggest that, after prolonged inhibitor exposure, the analog-sensitive mutant behaves similar to kinase dead PKD1.

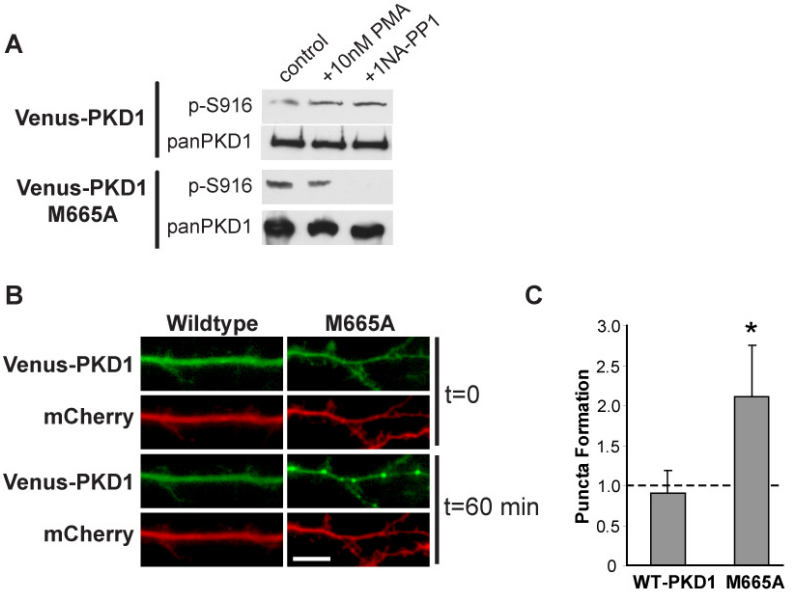

Figure 20. Generation of analog-sensitive protein kinase D1 allele. **A**) HEK cells were transfected with Venus-PKD1 constucts, stimulated as indicated, lysed, and analyzed by western blot. **B**) Live-cell imaging of Venus-tagged PKD1 before and after addition of 1 μM 1NA-PP1. n=10 cells, n>3 experiments per condition. **C**) Quantification of imaging shown in B.

PKD1 Phosphoryl Transfer Activity Is Not Required for Translocation

To determine if PKD1 kinase activity is required for translocation, I stimulated neurons with glutamate with or without co-application of 1NA-PP1. Both Venus-PKD1 and Venus-PKD1 M665A translocated equally well in the presence or absence of 1NA-PP1 (Fig. 21), indicating kinase activity was not required for PKD1 puncta formation.

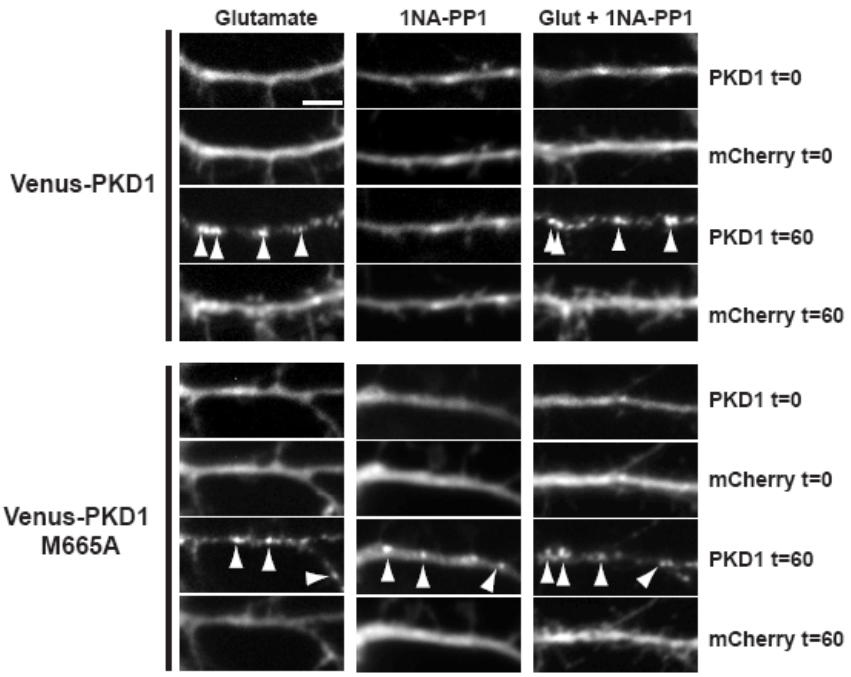

Figure 21. PKD1 kinase activity is not required for translocation. Neurons were transfected with Venus-PKD1 and mCherry and imaged before and after stimulation with glutamate (30 μM, 60 min) 1NA-PP1 (1 μM), or both. Images are representative (n>9 cells from three experiments). Scale bar = 5 μm.

If phosphoryl transfer activity is not necessary for PKD1 translocation, why is the PKD1 SSAA mutant non-responsive to glutamate stimulation? The activation loop phosphorylation state of protein kinases acts as a molecular switch, inducing the active or "on" conformation of a kinase. Inhibitor binding to kinases in the ATP binding site can cause the protein to adopt a conformation normally associated with the active kinase[127]. We hypothesized that inhibitor binding to PKD1 might rescue the SSAA translocation phenotype. I made the M665A mutation in the background of the Venus-PKD1 SSAA construct and tested translocation in response to glutamate with and without 1NA-PP1 (Fig. 22). Indeed, the M665A mutation in the presence of 1NA-PP1 rescued Venus-PKD1 SSAA translocation. Importantly, translocation of Venus PKD1 M665A SSAA did not occur on inhibitor binding alone; concomitant glutamate stimulation was required.

This is consistent with a two-step translocation model whereby DAG must be generated at the plasma membrane before PKD1 binds endosomes.

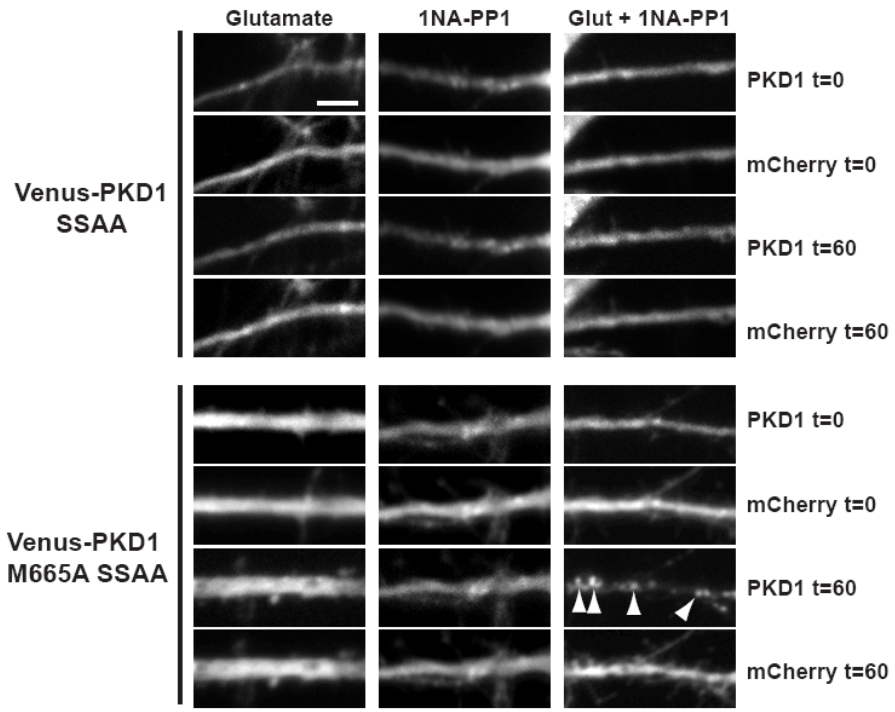

Figure 22. 1NA-PP1 binding rescues PKD1-SSAA translocation in response to glutamate. Neurons were transfected with Venus-PKD1 and mCherry and imaged before and after stimulation with glutamate (30 μM, 60 min) 1NA-PP1 (1 μM), or both. Images are representative (n>9 cells from three experiments). Scale bar = 5 μm.

DISCUSSION

In the previous chapter, I demonstrated that PKD1 is a target of NMDAR signaling, and this pathway regulates AMPAR recycling through endosomes. Here, I expand upon this signaling pathway and show that the regulation of PKD1 endosomal localization is complex and multi-step. NMDARs must activate PLC, which generates DAG at the plasma membrane and recruits PKD1 through its C1b domain. PKD1's kinase domain is required for its subsequent association with endosomes, even though its kinase activity is

dispensable. While the potential role of PKD1 kinase activity at endosomes remains unclear, these results indicate PKD1's activity-dependent regulation of exocytosis in neurons, as well as other cell types, may be governed by sophisticated mechanisms.

NMDAR to PKD1 Signaling

Understanding how neurons alter the strength of some synapses, and not others, remains an important problem in neurobiology. Experiments using highly focused stimuli, such as glutamate uncaging, have shown single stimulated spines, and not neighboring ones, will change the number of AMPARs at the spine head[128]. AMPAR insertion and recruitment require phosphorylation of the AMPAR cytoplasmic tail. Indeed, kinases involved in this process, such as CamKII, translocate in response to activity[129], and this may be restricted to stimulated spines[130]. It will be important to determine if PKD1 is also recruited to endosomes near sites of local NMDAR activation. If so, PKD1 is poised to participate in synapse-specific changes in efficacy, such as LTP and LTD.

Interestingly, PKD1 kinase activation downstream of Gαq receptors[131], such as mGluRs, and its endosomal localization downstream of NMDARs require PLC activity. Even so, these processes can occur independently. A Ca^{2+} signaling microdomain at the channel pore might also explain the pattern of NMDA-induced PKD1 translocation. NMDAR-mediated PLC activation causes PKD1 to bind the plasma membrane via one of its cysteine-rich DAG binding domains. PKD1 has two DAG binding domains, C1a and C1b[52]. C1a associates with DAG in intracellular membranes, such as the TGN[120], and C1b has greater specificity for DAG at the plasma membrane[123]. We found that the C1b domain mediates PKD1's association with the plasma membrane downstream of

NMDARs. The specific translocation of PKD1 to the plasma membrane suggests that DAG generation in response to NMDARs is compartmentalized and restricted to the plasma membrane.

While the role of mGluR-PLC signaling has been fairly well characterized in mGluR-LTD[32], NMDAR-PLC signaling is less well understood in the context of plasticity. For example, PLC is required for NMDAR-dependent LTD *in vitro*[132], but the precise effector has not been identified. That PKD1 is responsive to NMDAR-PLC signaling suggests it participates in the same biological processes and may, thus, also regulate NMDAR-LTD.

PKD1 Activation Loop in Signal Transduction

NMDARs do not elicit PKD1 kinase activity, so we were very surprised that mutating the activation loop disrupted NMDAR-dependent PKD1 translocation. How does activation loop mutation disrupt PKD1 translocation? To address this, one must first consider what function the PKD1 activation loop serves within the kinase. Protein kinases are tightly regulated and have inactive and active states. To switch between the two, kinase domains are highly dynamic, undergoing large conformational changes upon activation. Activation loop phosphorylation contributes to this conformational shift.

Most kinases contain an HRD motif in the catalytic loop, and phosphorylation of a residue within the activation loop creates a salt bridge with the catalytic loop arginine, thus orienting the DFG motif for catalysis[132]. Interestingly, PKD1 is not an RD kinase: its catalytic loop contains HCD. In some non-RD kinases, an arginine in the P+1 loop just N terminal of the APE is used for a salt bridge instead[133], but PKD1 lacks this arginine as

well. In fact, a number other CamK family members are non-RD kinases without P+1 loop arginines, and their activation by phosphorylation is not fully understood. Exploring the mechanism by which the PKD1 activation loop regulates the kinase may shed light on PKD biology and other closely related kinases.

Regardless of the precise mechanism, as activation loop phosphorylation induces a dynamic rearrangement of the kinase, I hypothesized the serine to alanine mutations within the kinase domain disrupt the kinase conformation so that it no longer forms multimers or interacts with another binding partner. The rescue of translocation by M665A in the presence of 1NA-PP1 supports the idea that only some conformations of PKD1 are competent to interact with endosomes.

If kinase activity is not required for PKD1 localization to endosomes, what is PKD1's function there? Is it to phosphorylate a substrate that impinges on AMPAR trafficking, or does PKD1 have kinase-independent effects? Some active kinases have non-enzymatic roles; however, these are usually mediated through domains other than the kinase domain. For example, polo-like kinase 2 (Plk2) regulates GluA2's interaction with the exocytic protein N-ethylmaleimide-sensitive factor (NSF), but this is through a motif C-terminal to its kinase domain[134].

On the other hand, the conformation of the kinase domain can be adapted for non-catalytic functions. These kinase inactive "pseudokinases" are important protein-protein interaction domains and allosteric modulators[135]. Few active kinases have been ascribed pseudokinase functionality, but some examples have emerged recently. Kinase suppressor of Ras (KSR) is an active kinase but also acts as a scaffold to stabilize the interaction between the small GTPase Raf and mitogen-activated protein kinase

kinase[132,136]. The ER stress sensor Ire1 autophosphorylates in trans, and this mulitmerization through the activation loop activates the protein's RNase domain. However, the phosphorylation event is not actually required for RNase activity: binding of an ATP analog inhibitor produces the same conformational change and oligomerization as ATP binding[137,138]. This is reminiscent of PKD1, in which inhibitor binding is permissive for at least part of its function (endosomal localization).

Our data thus support three possible models for PKD1's endosomal localization. As kinase activity is not required for endosomal association; PKD1's regulation of GluA2 trafficking may be non-enzymatic. This could occur if PKD1 allosterically regulates trafficking proteins, or physically couples AMPARs with trafficking machinery. Alternatively, as the phospho-mimetic mutant, PKD1 SSEE, can localize to endosomes, perhaps active PKD1 is what drives GluA2 trafficking, and endosomal localization is required but not sufficient. In this case, PKD1 would be a is a coincidence detector between NMDAR signaling (localization) and mGluR signaling (activation). Finally, these two possibilities are not mutually exclusive: PKD1 could regulate AMPARs differentially depending on whether it is active. This third possibility is particularly appealing in light of the PKD1 SSQQ mutant translocating to endosomes. One could imagine the non-phosphorylated PKD1 has a different action than the phosphorylated kinase or phospho-mimetic mutant.

PKD1's regulation of AMPAR trafficking indicates its importance in neurobiology. By delving into the mechanism by which NMDARs regulate PKD1's localization, I showed PKD1 kinase localization is not determined simply by its kinase activity. I believe further investigation will reveal PKD1 is among the protein kinases we now

understand also serve non-enzymatic functions. I believe future work will confirm PKD1 is an essential player in synaptic plasticity, and understanding its regulation in dendrites will have broader implications in kinase biology.

FUTURE DIRECTIONS

Finding PKD1's Protein-Protein Interactions at Endosomes

Determining PKD1's binding partners at endosomes remains a challenge. Two promising candidates are Rap1 and PICK1. Rap1 is a small GTPase that regulates AMPAR trafficking in neurons[139]. Interestingly, Rap1 directly binds PKD1 in T cells, and this interaction promotes β integrin trafficking to the plasma membrane[140]. I hypothesized that PKD1 might bind Rap1 in neurons and regulate AMPAR trafficking by a parallel mechanism. I performed co-immunoprecipitation experiments with an anti-PKD1 antibody and probed for Rap1 by a protocol based on the one used to originally find the PKD1-Rap1 interaction[140]. However, under control conditions and after APV withdrawal, I could not detect PKD1-Rap1 binding in neurons (Fig. 23).

PICK1 is a GluA2-binding protein that participates in AMPAR trafficking through dendritic endosomes[141]. As PICK1 regulates GluA2-specific trafficking, localizes to AMPAR containing endosomes, and binds PKC, we conjectured PKD1 might participate in PICK1-PKC signaling. However, I was unable to pull down PICK1 when I immunoprecipitated PKD1 (Fig. 23), indicating the two proteins were not interacting.

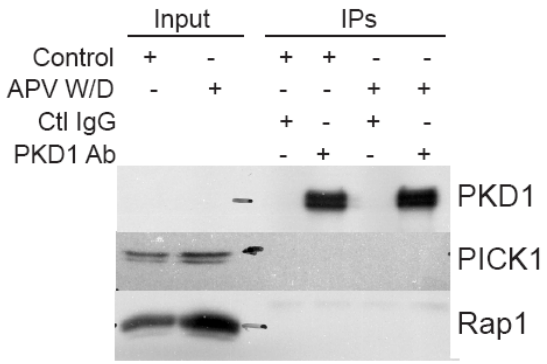

Figure 23. PKD1 does not co-IP with either PICK1 or Rap1. Lysates from 18 DIV in neurons with either subjected to APV withdrawal (APV W/D) or control medium, lysed, and PKD1 was immunoprecipitated. Normal rabbit IgG was used as a control (Ctl IgG). Right-hand labels indicate the antibody used for western blotting. Images are representative of n=3 experiments.

Co-immunoprecipitation experiments are fraught with technical challenges, so while the preliminary experiments I performed could have yielded false negatives, I believe Rap1 and PICK1 are less appealing as possible neuronal PKD1 interactors. As the candidate approach has not proved fruitful, perhaps a screen-based approach will be needed to find PKD1's neuronal effectors.

Measuring the Effect of Activation Loop Mutation on PKD1 Conformation and Dynamics

How the activation loop of PKD1 contributes to its endosomal localization remains unclear. However, our preliminary "rescue" data indicating that 1NA-PP1 binding permits PKD1 SSAA translocation (Fig. 21) suggest the conformation of the kinase is altered by activation loop mutation. I hypothesize that the activation loop serine to alanine mutations change PKD1 kinase conformation and thus disrupt its multimerization, or interaction with another protein.

To test whether the PKD1 kinase domain conformation is altered by mutation of the activation loop serines to alanines, I will take advantage of an assay designed by the Rauh

laboratory to distinguish active and inactive kinase conformations in solution. As I alluded to above, the transition from the inactive to active conformation of a kinase requires significant conformational changes. By labeling a residue in either the glycine loop[142] or activation loop[143] with the environmentally sensitive fluor, acrylodan, shifts in the acrylodan emission can be correlated to conformational changes in a kinase.

I generated constructs to express PKD1 kinase recombinantly in bacteria. To determine what portion of PKD1 kinase domain would express robustly and remain soluble, I aligned the PKD1 kinase domain sequence with that of Checkpoint2 (Chk2). PKD1 and Chk2 share 37% identity, and Chk2 is the closest homolog of PKD1 whose structure has been solved[144].

I tested expression of PKD1 tagged with an N terminal His6, N terminal GST, or the cysteine protease domain of *Vibrio cholera* toxin[145] at the C terminus. Of these three tags, I found GST yielded the best PKD1 expression. The kinase appeared to be active, as the purified protein was strongly phosphorylated on the activation loop (Fig. 24). While this usefully indicated recombinant PKD1 folds properly, I wanted to generate PKD1 where the activation loop serines are consistently non-phosphorylated and compare this to kinase with phosphorylation or phospho-mimetic mutations. To accomplish this, I transformed Rosetta2 cells with a plasmid encoding phage λ phosphatase and generated a line of competent cells. GST-mPKD1 kinase expressed in the presence of λ phosphatase was no longer recognized by a phospho-specific antibody against the PKD1 activation loop (Fig. 24).

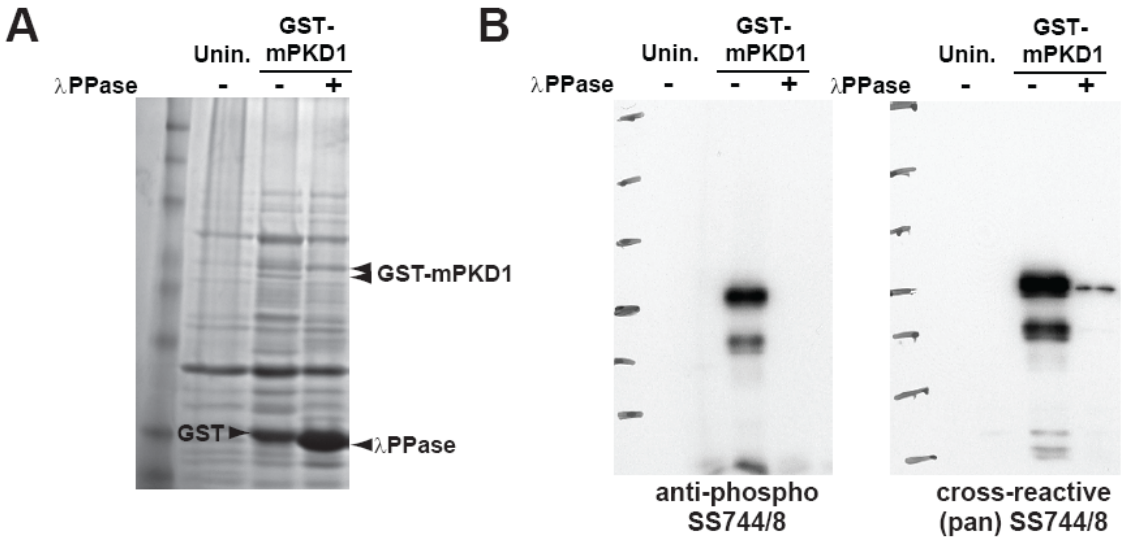

Figure 24. Recombinant GST-mPKD1 is soluble and phosphorylated. **A**) Coomassie-stained gel showing GST-mPKD1 576-902 expressed in *E. coli* with and without phage λ phosphatase (λPPase). Note the loss of the lower mPKD1 band in the presence of λPPase, consistent with dephosphorylation. **B**) Western blot on the same samples, using antibodies generated against the PKD1 activation loop. The phospho specific antibody recognizes a band only in lysate without λPPase, whereas the cross-reactive antibody recognizes PKD1 in both conditions.

I next generated a GST-mPKD1 construct lacking cysteines by creating substitutions by site-directed mutagenesis (i.e., C653S, C711S, C732S, C859S), hereafter referred to as mPKD1 4CS. Within the mPKD1 4CS construct, I chose three positions to label to examine PKD1 kinase conformation: F600C in the glycine loop, and A736C and F737C by the activation loop. With these tools in hand, I will compare the fluorescence of labeled activation and/or glycine loop in PKD1 with and without SSAA and SSEE mutations in the activation loop. I will also examine changes in both the activation and glycine loop conformation upon 1NA-PP1 binding in PKD1 M665A. I predict that PKD1 SSAA adopts distinct glycine and activation loop orientations from the wildtype kinase, and 1NA-PP1 binding corrects this.

METHODS

Constructs

GW1-Venus-PKD1 was created from a Clontech vector containing GFP-PKD1 described previously[61]. A 46–amino acid stretch links Venus to the N-terminus of mouse PKD1. For recombinant GST-PKD1 kinase domain expression, the kinase domain was amplified by PCR from GW1-Venus-PKD1 and inserted into a modified pGEX-4T (GE Healthcare). Modified pGEX-4T is identical to the original vector, except the multiple cloning site has been replaced with that of pET-22 (XXX Company). Point mutations in PKD1 were generated with a site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing.

HEK293 Cell Culture

HEK293 FT cells (ATCC) were cultured in 6-well dishes coated with poly D-lysine. At 70% confluency, cells were transfected by the calcium phosphate method (ref), with the modification that the DNA precipitate was added directly to HEK growth medium and washed off 15 hours later.

Pharmacology

Drug application for both HEK cells and neurons was performed at room temperature in HEPES-buffered saline (HBS: 119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, pH 7.4).

Western Blots

HEK cells were lysed with ice-cold RIPA buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, and protease inhibitor cocktail;

Roche). Samples were centrifuged, and supernatants were loaded onto gels, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against pan-PKD1 (1:1000, Cell Signaling or 1:5000, HJK), phospho-S744/8 PKD1 (1:1000, Cell Signaling), phospho-S916 PKD1 (1:1000, Cell Signaling), or β-actin (1:30,000, Sigma). Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase were used on all blots and imaged by enhanced chemiluminescence (GE Healthcare). Band intensities were quantified with ImageJ Gel Analyzer tool[118].

Cortical Cultures

Embryonic mouse primary cortical neurons were as described [86] using C57/BL6 wildtype mice (Charles River). Neurons from E18–19 embryos were plated at a density of 0.6×10^6 cells/cm^2 on 12-mm glass coverslips coated with poly D-lysine and maintained in Neurobasal medium supplemented with B27 (Invitrogen). Experiments were done at 8–14 DIV. Neurons were transfected by the calcium phosphate method [86].

Imaging and Analysis

For puncta analysis, images were taken with an inverted epifluorescence microscope (Nikon, Japan) and a cooled CCD digital camera (Hamamatsu Orca II) [146]. Venus-PKD1 and mCherry images of the same field were taken before and after stimulation. Calculations of puncta indices (PIs) were based on a previous study [86]. PIs were calculated with MetaMorph software (Molecular Devices) as the standard deviation (SD) of Venus fluorescence along a region of dendrite divided by the SD of mCherry fluorescence along the same region. Puncta formation was determined by calculating PIs

before and after a 60-min application of 1NA-PP1 and expressed as the ratio of PI (time 60)/PI (time 0).

Co-Immunoprecipitations

Neuronal cultures were grown and maintained for APV withdrawal as described in Chapter 2. At 18 DIV, cells either underwent APV withdrawal or received a control medium change (Ch. 2). Thirty min later, cells were lysed in RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Trition X-100, 0.1% SDS) containing protease (Thermo Scientific) and protease (Roche) cocktails and incubated with either anti-PKD1 antibody or control rabbit IgG (Santa Cruz) overnight at 4º C, tumbling end over end. The next day, magnetic protein A–conjugated beads (Thermo Scientific) were added to the immunoprecipitation reactions and incubated for 15 min at 4 º C, tumbling end over end. The reactions were washed three times in 50 mM Tris pH 8, 150 mM NaCl, and 0.5% Triton X-100 before elution in 2X Laemelli sample buffer. After SDS-PAGE and transfer to PVDF membrane, samples were blotted with anti-PKD1 (1:5000), anti-Rap1 (1:1000, Chemicon), or anti-PICK1 (1:1000, AbCam).

Recombinant PKD1 Expression

Rosetta2 DE3 cells (EMD Chemicals) were transformed with phage l phosphatase in pCDF-Duet1 vector and used to make chemically competent cells. GST-mPKD1 plasmids were transformed into this cell line and grown in 2XYT medium at 37 º C, shaking at 225 rpm until they reached OD_{600} = 0.8. Cultures were induced with 2 mM IPTG, supplemented with 1 g of glucose, 2 g of Na_2HPO_4, and 1 g of KH_2PO_4 per liter

culture, and incubated overnight at 20 º C. Cultures were harvested the next day and lysed in PBS with one cycle of freeze-thawing, followed by sonication.

CHAPTER 4. SUMMARY OF FINDINGS AND CONCLUSIONS.

The means by which a neuron may regulate and adapt its synaptic strength is perhaps its most vital set of tools. Synaptic plasticity is critical for the neurobiology of learning and memory, as well as neurological diseases ranging from epilepsy to Alzheimer's. While many of the molecular mechanisms underlying plasticity have been elucidated, our understanding is far from complete.

In this dissertation, we uncovered a new pathway mediated by PKD1, dually controlled by mGluRs and NMDARs, that regulates the composition and trafficking of AMPARs. We found PKD1 kinase regulates the size of the extrasynaptic AMPAR pool by promoting the exocytosis of GluA2-containing receptors. While GluA2 trafficking is altered by PKD1 constitutively, we also found PKD1 regulates glutamate-induced recycling of AMPARs. PKD1 is in turn is dually regulated by glutamate: its kinase activity is downstream of mGluR signaling, and its translocation to early endosomes containing AMPARs requires NMDAR signaling.

As the distinct regulation of enzymatic activity and subcellular localization is unusual, we went on to more fully characterize the signaling from NMDARs to PKD1. The regulation of PKD1 endosomal localization is complex and multi-step. NMDARs must activate PLC, which generates DAG at the plasma membrane and recruits PKD1 through its C1b domain. PKD1's kinase domain is required for its subsequent association with endosomes, even though its kinase activity is dispensable. While the potential role of PKD1 kinase activity at endosomes remains unclear, these results indicate PKD1's

activity-dependent regulation of exocytosis in neurons, as well as other cell types, may be governed by sophisticated mechanisms.

Looking forward, I believe future work on the role of PKD1 in neurobiology will uncover its fundamental role in plasticity and learning. Activity-dependent changes in AMPAR trafficking are fundamental to synaptic plasticity. While disruption of PKD1 is not sufficient to alter synaptic physiology, PKD1 is likely to regulate key trafficking processes in the context of plasticity-inducing stimuli requiring NMDAR or mGluR signaling. Furthermore, PKD1 may regulate activity dependent gene-expression in neurons, as it regulates transcription in other cell types. PKD1's ability to regulate protein trafficking and gene expression suggests it is uniquely positioned to contribute to short- and long-term synaptic plasticity.

Besides discovering a signaling pathway that is important in neurobiology, I believe our work toward understanding how PKD1 is regulated in neurons will have a broader impact on our understanding of kinase biology. Biochemically speaking, PKD1 appears to be an unusual kinase. Different kinase-inactive mutants of PKD1 have distinct phenotypes, depending on whether the inactivating mutations are in the activation loop (PKD1 SSAA) or the ATP binding domain (PKD1 K618N). Neurons seem to exploit at least one inactive form of PKD1, sending a non-phosphorylated form of PKD1 to AMPAR-containing endosomes. As this non-phosporylated PKD1 presumably has low kinase activity, its function at endosomes is unclear. Perhaps it has a function independent of phosphoryl transfer activity at endosomes. Alternatively, PKD1's role in AMPAR trafficking could require concomitant signals from mGluRs to activate it and send it to endosomes, respectively. Future work addressing both hypotheses will not only

aid in our understanding of PKD1's function in plasticity, but will help us understand the mechanisms by which PKD1 and related kinases behave on a biochemical level.

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