Protein Kinase D Controls the Integrity of Golgi Apparatus and the Maintenance of Dendritic Arborization in Hippocampal Neurons

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Protein kinase D (PKD) is known to participate in various cellular functions, including secretory vesicle fission from the Golgi and plasma membrane-directed transport. Here, we report on expression and function of PKD in hippocampal neurons. Expression of an enhanced green fluorescent protein (EGFP)-tagged PKD activity reporter in mouse embryonal hippocampal neurons revealed high endogenous PKD activity at the Golgi complex and in the dendrites, whereas PKD activity was excluded from the axon in parallel with axonal maturation. Expression of fluorescently tagged wild-type PKD1 and constitutively active PKD1S738/742E (caPKD1) in neurons revealed that both proteins were slightly enriched at the trans-Golgi network (TGN) and did not interfere with its thread-like morphology. By contrast, expression of dominant-negative kinase inactive PKD1K612W (kdPKD1) led to the disruption of the neuronal Golgi complex, with kdPKD1 strongly localized to the TGN fragments. Similar findings were obtained from transgenic mice with inducible, neuron-specific expression of kdPKD1-EGFP. As a prominent consequence of kdPKD1 expression, the dendritic tree of transfected neurons was reduced, whereas caPKD1 increased dendritic arborization. Our results thus provide direct evidence that PKD activity is selectively involved in the maintenance of dendritic arborization and Golgi structure of hippocampal neurons.

INTRODUCTION

Neurons are nondividing and extremely polarized cells, with enormous membrane surface compared with the size of the soma. These features assume a highly specialized secretory machinery, which resembles in certain parts the directed transport mechanisms described in polarized non-neuronal cells (Winckler and Mellman, 1999; Horton and Ehlers, 2004). The vast neuronal membrane surface is functionally and structurally divided into axonal and somatodendritic compartments, possessing specific lipid and protein components required for the spatially different functions, e.g., for pre- or postsynaptic activity (Dresbach et al., 2001; Sheng, 2001; Bresler et al., 2004). The constitutive and precisely directed supply of surface membrane components is indispensable for the function of neurons, especially when considering that postmitotic neurons normally serve throughout the life span of the organism. Up to now, we are far from understanding how the extreme polarization has been evolved and is maintained in neurons.

Despite a likely central role in neuronal morphogenesis and membrane trafficking, little is known about the special structure and transport features of the neuronal Golgi apparatus. A thread-like and reticular structure of the Golgi apparatus has been already described in many neuronal types (Takamine et al., 2000; Fujita and Okamoto, 2005; Horton et al., 2005). Central neuronal Golgi complex is localized in the soma and often extends into the principal dendrites, but Golgi elements were also found as discontinuous structures in the distal dendrites, often near synaptic contacts or in dendritic spines (Gardiol et al., 1999; Pierce et al., 2001; Sytnyk et al., 2002; Horton and Ehlers, 2003). The dispersed localization of the Golgi apparatus indicates unique spatial regulation of the neuronal secretory pathway compared with other mammalian cells. Recent investigations on hippocampal pyramidal cells indicated that Golgi outposts located in the dendritic shafts (Sytnyk et al., 2002; Horton and Ehlers, 2003) and the perinuclear Golgi apparatus oriented toward the longest dendrite (Horton et al., 2005) can provide the necessary membrane supply for intensive dendritic growth. In axonal outgrowth and extension, an increasing amount of data suggests that membrane supply and targeting of proteins are regulated in a different way compared with dendritic elongation (Silverman et al., 2001;
Animals

CD1, C57BL/6J rTA2 (Michalun et al., 2008), or kdpPKD1-enhanced green fluorescent protein (EGFP) transgenic mice were housed in the Animal Facility of the Biological Institute, Eötvös Loránd University, or of the Institute of Cell Biology and Immunology, University of Stuttgart, at 22 ± 1°C, with 12-h light/dark cycles and with ad libitum access to food and water. All procedures were performed under the supervision of Local Animal Care Committee, in agreement with the European Union and Hungarian legislation (Budapest) or approved by the Regierungsspräsidium Stuttgart. All experiments were conducted with local guidelines and regulations for the use of experimental animals (35-9185.81/2009, 33-9185.81/2007 and 878/003/2004 for the experiments carried out in Stuttgart or in Budapest, respectively).

Generation of kdpPKD1-EGFP Transgenic Mouse Line

An EGFP-tagged version of human kinase-dead (kd) PKD1 (K612W) cDNA was inserted into the multiple cloning site of pBLC (Baron et al., 1995) next to the tet-operon and human cytomegalovirus (hCMV) promoter sequences. The construct contained a downstream rabbit β-globin intron/polyadenylation signal. Vector sequences were excised by digestion with NotI and BsrBI. Pronuclear microinjection was performed by standard procedures (Nagy, 2003). Tail DNA from founder mice was digested with HindIII and analyzed by Southern blotting with a 577-bp EGFP probe. The 4.7-kb fragment indicated the integration of the transgene as head-to-tail array of multiple copies into the genome, whereas the ~5.8-kb fragment denoted adjacent integration into the original genome. Polymerase chain reaction (PCR) genotyping was performed with PKD1-specific primers (forward, 5'-GGT GGT GTC AGA AGA GGC ATT C' GC; reverse, 5'-CAC CAA GGC AGT TGG CTA CTT T-3'), giving an amplification product of 246 base pairs for transgenic kdpPKD1 and a fragment of 399 bp for endogenous PKD1. Frequency of transgenic littermates occurred in Mendelian manner.

To create a neuron-specific transgene expressing pattern, kdpPKD1-EGFP mice were bred with mice carrying rTA2 under the control of CamKIIα promoter (Michalun et al., 2005). Genotyping for rTA2 was carried out as described previously (Michalun et al., 2007).

MATERIALS AND METHODS

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Expression Constructs

Fluorescently tagged human wild-type PKD1-EGFP, kdpPKD1-EGFP (PKCa[1425S]EGFP), kdp1AP1-EGFP, or kdpPKD1-EGFP (PKD1T735W/742F) plasmids have been described previously (Hauser et al., 1999, 2002, 2005). pEGFP-C1, pEGFP-N1, and pmCherry-N1 vectors were from Clontech Europe (Saint-Germain-en-Laye, France) and A. Jerome (Institut Pasteur, Paris, France), respectively. The PKD activity reporter (PKDep) was generated by annealing oligonucleotides containing the cDNA sequence for the linker and phosphor- ylation substrate region of phosphatidylinositol 4-kinase III (PhKIIIp) (forward, 5'-ctg tta ggt gtt tct ggt ggt agc cag ctc ctg cca cag cgc gac gac cat aaag ggt gag agg ggt gtt act gct gg-3` and reverse, 5'-cgact acc acc gaa ctt aac ctc cct gta gtt act ggt gtt ggct ccc 3'). HindIII and SalI sites were incorporated in the multicloning site of the pEGFP-C1 vector (Clontech Europe) by using the HindIII and SalI restriction sites. Generation and characterization of PKD activity reporter constructs are described in detail elsewhere (Fuchs et al., 2009).

Cell Cultures

Primary cultures of embryonic hippocampal cells were prepared from CD1 mice on embryonic day 18. After aspiratively removing hippocampus from the skull, tissue was freed from meninges and incubated in 0.3% trypsin (EDTA) (Invitrogen, Cersy, Hungary) solution with 0.05% Dnasel in phosphate-buffered saline (PBS) for 15 min at 37°C. After a brief centrifugation, cells were triturated in Neurobasal (NB) medium supplemented with B27 (Invitrogen) containing 5% fetal calf serum (FCS; Sigma, Budapest, Hungary), 0.5 mM Glutamax (Invitrogen), 40 μg/ml gentamycin (Hunagorapharma, Budapest, Hungary), and 2.5 μg/ml amphotericin B and filtered through a sterile polyester mesh with 42-μm pore size (EmTek, Budapest, Hungary). Cell number was determined by trypan blue exclusion, and cells were seeded in NB culture medium onto poly-l-lysine (Sigma)-1 μg/cm² laminin (Sigma)-coated glass coverslips (13 mm in diameter) in 24-well plates at 6 × 10⁵ cells/cm² cell density for microscopic observations. For Western blot analyses, cells were seeded into 12-well plates at 4 × 10⁶ cells/well cell density; for live cell imaging experiments, 4 × 10⁵ cells were plated onto poly-l-lysine-laminin coated glass-bottomed 35-mm Petri dishes (Greiner, Frickenhausen, Germany). Cytosin-arabinofurosoramide (CAR, 10 μM; Sigma) was added to the cultures 6 h after plating, and then one third of the culture medium was changed to Neurobasal medium supplemented with B27 without FCS on the fourth day of cultivation. One third of the medium was replaced every 3–4 d thereafter. Cells were cultured for 10 d at 37°C in 5% CO₂, 95% air atmosphere. Immunocytochemical stainings revealed relatively low amount (~<5%) of interneurons containing GABA and a high level of vesicular glutamate transporter-1 (VGluT1) in our cultures (data not shown).

Nine days after plating, transfaction of hippocampal cells was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In 24-well plates, 0.3–0.5 μg of plasmid DNA was mixed with Lipofectamine in a 1 μg:2 μl ratio. Medium was changed 5–8 h after the transfection to the original cultivation medium, and cells were analyzed at the time indicated points after transfection. Phorbol ester treatment of cultures was carried out either by phorbol 12,13-dibutyrate (PDBu; 1 μM; 2 mM stock solution dissolved in dimethyl sulfoxide (DMSO); Sigma Aldrich Chemie Gmbh, Munich, Germany) or by the cell-permeable DAG analogue sn-1,2-dioctanoylglycerol (diC₈; 25 μM; 2 mM stock solution dissolved in DMSO; Sigma Aldrich Chemie Gmbh) treatment in the culture medium.

Human embryonic kidney (HEK) 293T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (all from Invitrogen, Karlsruhe, Germany). For transient transfections of the integrated plasmids, HEK293T cells were transfected with TransIT203 (Mirus Bio, Madison, WI) according to the manufacturers instructions.

Immunostaining

Hippocampal cultures were fixed on the 10th day after plating (days in vitro [DIV]) with 4% paraformaldehyde for 20 min with 0.5% Triton X-100 in PBS. After rinsing, cells were permeabilized with 0.1% Triton X-100 for 5 min. Nonspecific antibody binding was blocked by incubation with 2% bovine serum albumin (BSA), 0.1% Na-azide in PBS (PBS-BSA) for 1 h at room temperature. Primary antibodies such as anti-GM130 (mouse immunoglobul- lin G1G2G1, 1:250; BD Biosciences Transduction Laboratories, Lexington, KY), anti-vesicle-associated membrane protein (VAMP) 4 (rabbit, 1:300; Sigma), anti-pSer294 (rabbit, 1:700; Hauser et al., 2005), anti-FLAG (mouse, 1:500; Sigma),...
or anti-PKD1 (C20 rabbit, 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-pS910 (rabbit, 1:2000; Hauser et al., 2005; anti-IIlubulin (mouse, 1:5000; Exbio, Prague, Czech Republic), and anti-pT738/pS742 (rabbit, 1:100; Hauser et al., 2002). Proteins were visualized with horseradish peroxidase-coupled secondary antibodies (Dianova, Hamburg, Germany) by using the enhanced chemiluminescence system, (Roche Molecular Biochemical, Rockford, IL). Stripping of membranes was performed in 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 55°C. Membranes were then reprobed with the indicated antibodies.

RESULTS

PKD Is Localized at the Trans-Golgi Network in Neurons

We have visualized neuronal Golgi apparatus in cultured mouse hippocampal neurons using GM130 and VAMP4 immunostaining (Figure 1). GM130 has been widely used both in nonneuronal and neuronal cells to label cis-Golgi (Nakamura et al., 1995; Horton et al., 2005), whereas VAMP4, a member of the soluble N-ethyl maleimide-sensitive factor adaptor receptor complex, is known to be highly enriched at the TGN and to regulate the traffic and sorting of recycling endosomes (Steegmaier et al., 1999; Tran et al., 2007). Both GM130 and VAMP4 highlighted filamentous, perinuclear staining in cultivated neurons. The cis- and trans-Golgi compartments were located side by side, forming a reticular structure in the soma. Filamentous Golgi structures often entered one or two dendrites as well (see Supplemental Figure 1 and Supplemental Movie 1 for three-dimensional [3D] reconstruction).

In epithelial cells, PKD regulates transport processes and vesicle fission via binding to TGN membranes (Maeda et al., 2001; Diaz Anel and Malhotra, 2005; Ghanekar and Lowe, 2005). The intracellular localization of PKD was followed by introducing EGFP-tagged human wild-type PKD1 (wtPKD1-EGFP) into hippocampal neurons already possessing elaborate axonal arborization and well developed dendrites (9 d after plating; DIV9). Cultures were fixed 22–24 h after transfection and were processed for GM130 and VAMP4 immunochemistry (Figure 1A). Besides a rather homogeneous cytoplasmic distribution, wtPKD1-EGFP was significantly enriched at the Golgi apparatus (Table 1). On closer examination, wtPKD1-EGFP was localized side by side with GM130 but showed partially overlapping localization with VAMP4-stained structures (Figure 1A, enlargements).

Phorbol ester treatment is known to cause plasma membrane translocation and activation of PKD (Zugaza et al., 1996; Matthews et al., 1999; Rey et al., 2004). In live cell imaging of hippocampal neurons, 1 μM PDBu treatment led to a rapid recruitment of wtPKD1-EGFP to the plasma membrane, followed by the intracellular accumulation of the fluorescently tagged protein (Figure 1B). Immunocytochemical staining with VAMP4 confirmed that 15 min of PDBu treatment resulted in a decrease of Golgi marker GM130 and concomitant increase of VAMP4 and PKD localization, indicating activation of PKD (Figure 1B).
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Endogenous PKD Is Active at the Neuronal Golgi Complex

The consensus PKD target site has been mapped (Nishikawa et al., 1997), and antibodies raised against the phosphorylated serine of the consensus PKD target site have been successfully used to demonstrate PKD-mediated phosphorylation of various substrate molecules (Doppler et al., 2005; Hausser et al., 2005). PI4KIIIβ is a known target of PKD and gets selectively phosphorylated by PKD on Ser294 (Hausser et al., 2005). To visualize endogenous PKD activity at the Golgi complex, a Golgi-targeted EGFP-tagged PKD activity reporter construct (G-PKDrep) was created using the PKD-specific substrate sequence of PI4KIIIβ fused to the GRIP domain of p230, a known trans-Golgi localized protein (Fuchs et al., 2009). PKD-mediated phosphorylation of the transfected construct can be detected by an antibody specific for phosphorylated Ser294 (anti-pS294; Hausser et al., 2005). Specificity of the immunostaining was proven by transfecting a construct containing a serine to alanine mutation inside the PKD target sequence (G-PKDrep S/A).

The GRIP domain of p230 led to a selective enrichment of both EGFP-tagged constructs at the Golgi complex, the latter visualized by GM130 staining (Figure 2). As expected, pS294 antibody staining was detected only in those cells that were transfected with the PKD reporter containing the original serine site. GM130 immunostaining revealed a side-by-side localization with the pS294 signal, in accordance with the TGN-directed localization of the PKD activity reporter. Importantly, Golgi-targeted reporter was highly phosphorylated at the TGN in transfected neurons, even without phorbol ester treatment.

Nontargeted PKD Activity Reporter Is a Suitable Tool to Track Endogenous PKD Activity

The nontargeted versions of PKD activity reporter containing either serine or alanine within the PKD target site (PKDrep or PKDrep S/A, respectively) were also created (Figure 3A). Similarly to the Golgi-targeted PKD activity reporter, described in detail by Fuchs et al. (2009), PKDrep was shown to be a sensitive and specific tool to follow dynamics of PKD activation. On the cotransfection of HEK293T cells with the reporter and the indicated PKD1 constructs, mutant PKD1 lacking the inhibitory PH domain led to increased phosphorylation of the PKD target site compared with wtPKD1, whereas alanine mutation of PKDrep abolished pS294 reactivity (Figure 3B). Rapid and reversible phosphorylation of PKDrep was shown after FCS treatment of serum-starved HEK293T cells (Figure 3C). Application of diC8 (25 μM), a cell-permeable DAG analogue, induced rapid changes in the localization and intensity of pS294 signal in hippocampal neurons as well (Figure 3, D–F). Thus, alike a natural substrate, phosphorylation of the reporter allows the dynamic monitoring of endogenous PKD activity.

PKD Is Highly Active in Nonpolarized Neurons and in the Somatodendritic Compartments of Mature Neurons

Endogenous activity of mouse PKD1 was detected by an antibody recognizing autophosphorylated human PKD1 (pS910; Haworth and Avkiran, 2001; Hausser et al., 2002). This result was confirmed by the increased signal intensity upon phorbol ester stimulus as well as by reprobing with a PKD1-specific antibody (Figure 4, A and C). The relative amount of PKD1 remained similar during the first 4 d of neuronal development in the hippocampal cultures, but it decreased slightly after the second week of plating. In contrast, the relative amount of activated PKD1 decreased rapidly in our mouse hippocampal cultures, to a much higher extent than the decline observed in total PKD1 levels (Figure 4B). These results confirm that endogenous PKD1 is present in cultured hippocampal neurons and is highly active during initial neurite elongation.

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Developmental changes in the intracellular activity of endogenous PKD were also investigated using the nontargeted version of PKD activity reporters (PKDrep or PKDrep S/A; Figure 4, D–H). Both the control, alanine containing construct (PKDrep S/A; data not shown) and PKDrep were evenly distributed in the cytoplasm of the transfected neurons. In nonpolarized stage 2 neurons, PKDrep phosphorylation was evident in all neurites and in the soma (Figure 4D). In contrast, phosphorylation of PKDrep was gradually diminished in parallel with axonal maturation: stage 3 neurons with already elongated axons possessed endogenous PKD activity only in the proximal axon (Figure 4E, asterisk). In mature neurons, pS294 signal was excluded from the axons, both from the axonal initial segment (Figure 4F) or from the thinner axon collaterals (Figure 4G, arrowheads). When neurons were cotransfected with PKDrep and the constitutive active mutant PKD1 (caPKD1-mCherry), phosphorylation of PKDrep was detected in axons coexpressing caPKD1 (Figure 4H, arrowheads). Additionally, axonal pS294 signal was not increased even after 15 min of diC8 treatment (Figure 3F).

Using a monoclonal PKD1 antibody for immunostaining, PKD1 signal was clearly detected in the axons (Supplemental Figure 2). Specificity of the immunostaining was strengthened by increased signal intensity upon overexpressing wtPKD1-mCherry in the axon (Supplemental Figure 2C). Therefore, the lack of pS294 staining in the axons is not due to lack of endogenous PKD protein but rather to the selective lack of endogenous PKD activity in mature axons. This indicates that PKD has a polarized activity in the somatodendritic compartment of cultured hippocampal neurons.

We also tested the consequences of overexpressing wtPKD2-mCherry or wtPKD3-FLAG on axonal PKDrep phosphorylation (Supplemental Figure 3, A and B, respectively). In both cases, pS294 signal was clearly detected in the axons of the cotransfected neurons. Additionally, interaction between the different isoforms was proved by showing that kinase inactive PKD1K612W acts in a dominant-negative way of the cotransfected neurons. Additionally, interaction between average Golgi complex or cytoplasmic intensity values reflects relative enrichment at the Golgi apparatus. In comparison with values obtained from EGFP-transfected neurons, wtPKD1-EGFP and caPKD1-EGFP were significantly enriched at the TGN, whereas the kdPKD1-EGFP signal was reduced in the cytoplasm but highly trapped at the TGN. Data are given as mean ± SEM. Asterisks indicate significant changes compared with the corresponding EGFP values (*p < 0.05; **p < 0.001).

**Table 1. PKD1 constructs are enriched at the TGN of the transfected neurons**

<table>
<thead>
<tr>
<th>Transfection</th>
<th>No. of analyzed cells</th>
<th>Golgi apparatus enrichment index (GI)</th>
<th>Average fluorescence intensity in the cytoplasm</th>
<th>Average fluorescence intensity at the TGN</th>
<th>Average background fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP</td>
<td>13</td>
<td>1.27 ± 0.05</td>
<td>213.58 ± 18.19</td>
<td>267.65 ± 19.57</td>
<td>2.83 ± 0.50</td>
</tr>
<tr>
<td>wtPKD1-EGFP</td>
<td>13</td>
<td>1.91 ± 0.10*</td>
<td>253.05 ± 28.49</td>
<td>463.01 ± 44.30*</td>
<td>2.12 ± 0.26</td>
</tr>
<tr>
<td>caPKD1-EGFP</td>
<td>20</td>
<td>1.89 ± 0.08*</td>
<td>246.36 ± 19.38</td>
<td>445.63 ± 36.47*</td>
<td>3.29 ± 0.41</td>
</tr>
<tr>
<td>kdPKD1-EGFP</td>
<td>16</td>
<td>8.24 ± 0.83**</td>
<td>120.39 ± 17.36**</td>
<td>692.81 ± 41.63**</td>
<td>4.12 ± 0.42</td>
</tr>
</tbody>
</table>

Background intensity values, average fluorescence intensity over the cytoplasmic region or over the VAMP4-positive TGN were calculated as described in Materials and Methods, at 16 h after transfection, by using single z-stacks from 12-bit Apotome images. Exposure settings for the individual cells were made to avoid saturation of the pixels. Because GI was calculated for each cell individually, relative increase between average Golgi complex or cytoplasmic intensity values reflects relative enrichment at the Golgi apparatus. In comparison with values obtained from EGFP-transfected neurons, wtPKD1-EGFP and caPKD1-EGFP were significantly enriched at the TGN, whereas the kdPKD1-EGFP signal was reduced in the cytoplasm but highly trapped at the TGN. Data are given as mean ± SEM. Asterisks indicate significant changes compared with the corresponding EGFP values (*p < 0.05; **p < 0.001).
tion of the TGN (Liljedahl et al., 2001). To investigate the effects of different PKD mutations on neuronal Golgi complex morphology, DIV9 hippocampal neurons were transfected with PKDrep and the S/A variant, respectively, along with an empty vector or the indicated PKD1 expression plasmids. Cells were lysed, and reporter phosphorylation was assessed by immunoblotting with the pS294-specific antibody. Expression of the PKDrep and PKD1-GFP fusion proteins was verified with a GFP-specific antibody. (C) HEK293T cells transiently expressing PKDrep were serum starved overnight followed by stimulation with 10% serum for the indicated time. Phosphorylation and expression of the PKD reporter and phosphorylation of PKD on S910 were analyzed as described in B. (D–F) diC8 (25 μM) treatment rapidly induced PKDrep phosphorylation in DIV10 hippocampal neurons. Strong, plasma membrane localized pS294 staining was detected already upon 2 min of diC8 treatment (E). By 15 min of diC8 treatment, elevated pS294 signal was in intracellular compartments as well (F). Images are single z-stacks recorded by the Apotome system under identical illumination and exposure settings and with similar processing. Axons are indicated by arrowheads on E and F (on D, the axon is out of focus). Note that axonal pS294 signal is hardly detectable even after 15 min of diC8 treatment (F). Bars, 10 μm.

Figure 3. PKDrep is a sensitive, specific, and reversible tool to measure endogenous PKD activity. (A) Outline of the nontargeted PKDrep construct. (B) HEK293T cells were transfected with PKDrep and the S/A variant, respectively, along with an empty vector or the indicated PKD1 expression plasmids. Cells were lysed, and reporter phosphorylation was assessed by immunoblotting with the pS294-specific antibody. Expression of the PKDrep and PKD1-GFP fusion proteins was verified with a GFP-specific antibody. (C) HEK293T cells transiently expressing PKDrep were serum starved overnight followed by stimulation with 10% serum for the indicated time. Phosphorylation and expression of the PKD reporter and phosphorylation of PKD on S910 were analyzed as described in B. (D–F) diC8 (25 μM) treatment rapidly induced PKDrep phosphorylation in DIV10 hippocampal neurons. Strong, plasma membrane localized pS294 staining was detected already upon 2 min of diC8 treatment (E). By 15 min of diC8 treatment, elevated pS294 signal was in intracellular compartments as well (F). Images are single z-stacks recorded by the Apotome system under identical illumination and exposure settings and with similar processing. Axons are indicated by arrowheads on E and F (on D, the axon is out of focus). Note that axonal pS294 signal is hardly detectable even after 15 min of diC8 treatment (F). Bars, 10 μm.

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differ between the vector transfected neurons and wtPKD1-EGFP or caPKD1-EGFP expressing neurons (Figure 5D).

The extent of Golgi complex dispersal (data not shown) and the ratio of affected neurons were in good correlation with the expression level of kdPKD1-EGFP or with the post-transfection time (Supplemental Table 1). These data indicate that dominant-negative effects of kdPKD1-EGFP develop gradually and emphasize the importance of PKD activity in the maintenance of the normal Golgi apparatus structure in neurons.

Doxycycline induced kdPKD1-EGFP Expression in Hippocampal Neurons Leads to Disruption of Golgi Morphology In Vivo

To confirm our in vitro results, we generated mice expressing human kdPKD1-EGFP under the control of the tetracycline-responsive (TetO) hCMV promoter by standard transgenic techniques and pronucleus injection (kdPKD1-EGFP mice; Figure 6). Several transgenic lines were established from founder animals. The presence of the transgene in the founder mice as well as in the offsprings was verified both by Southern analysis and with genomic PCR (Figure 6B; data not shown). kdPKD1-EGFP mice were crossed to mice carrying rtTA2 under the control of CaMKII/H9251 promoter, known to provide a forebrain and hippocampus-specific expression pattern of the transactivator (Michalon et al., 2005). Neither single transgenic, nor double transgenic mice showed any signs of abnormal development or behavioral effects (data not shown).

Expression of kdPKD1-EGFP was induced in adult CaMKII\alpha rtTA2 × kdPKD1-EGFP mice only upon administration of Dox (Figure 6, C and D). The appearance of kdPKD1-EGFP signal was already visible upon 5 d of Dox treatment (data not shown). By 4 wk of feeding the animals with Dox, kdPKD1-EGFP expression exceeded the level of endogenous PKD expression in the hippocampus (Figure
On the second week of Dox treatment, transgene expression was evident in the perinuclear region and in the proximal dendrites of CA3 hippocampal neurons (Figure 6E). As expected, no EGFP-expressing cells were found in wild-type animals (Figure 6F). Continuous Dox treatment for 4 wk led to high level of transgene expression in the dentate gyrus (DG) and CA3, with somewhat lower signal in the CA1 region of double transgenic hippocampi (Figure 6G). Because of the partial activation of rtTA2 in the CaMKIIα/rtTA2 mice (Michalon et al., 2005), our observations revealed nonhomogenous expression patterns. However, most of the Dox-treated double transgenic hippocampal neurons were found to be kdPKD1-EGFP positive.

To visualize neuronal Golgi structure in vivo, VAMP4 immunostaining was used. Besides weakly labeling small, dot-like structures in the dendrites, resembling most likely recycling endosomes, VAMP4 staining was highly enriched at the neuronal Golgi complex (Figure 6H). In non-transgenic hippocampal sections, regardless of the length of Dox treatment, VAMP4 was localized to thread-like, perinuclear structures often extending into the apical dendrites (Figure 6H). These results show that even long-term Dox treatment did not disturb normal, thread-like TGN morphology in wild-type neurons. In contrast, in CaMKIIα rtTA2 × kdPKD1-EGFP double transgenic mice treated with Dox for 4 wk, kdPKD1-EGFP was enriched at VAMP4-positive TGN structures, which were strongly dispersed (Figure 6I). Thus, in vivo observations are in close agreement with the findings obtained from transfected hippocampal neuronal cultures.

PKD Controls Dendritic Arborization

Our experiments have already shown that besides being active at the Golgi complex, endogenous PKD was selectively active in the dendrites (Figure 4). To investigate the effect of PKD1 mutants on dendritic arrangement, morphology of EGFP, wtPKD1-GFP, caPKD-GFP, or kdPKD1-GFP-transfected neurons was quantitatively analyzed in DIV10 hippocampal neurons, 22–24 h after transfection. Modified Sholl analysis was carried out to determine the extent of dendritic branching by calculating the intersection numbers at concentric circles located over the soma of the transfected neurons with a 20-μm stepwise increase in radius (Figure 7A shows the outline and dendritic intersections of representative neurons). The average number of intersections as a function of distance from the soma characterizes well the dendritic arborization of the transfected neurons (Figure 7, B and C).
Control, vector-only–transfected neurons had the highest number of intersections in the close (within 40 μm) vicinity of the soma, with dendrites reaching up to a 300-μm distance from the soma. The extent of arborization gradually decreased in parallel with the increasing distance. The presence of kdPKD1-EGFP led to a significant decrease in the intersection numbers at almost all levels (Figure 7B, asterisks), and dendrites did not extend further than a 200-μm distance. Introducing caPKD1-EGFP into transfected neurons, in contrast, led to a significant increase in dendritic arborization. Not only did the distribution of the highest intersection numbers shift toward outer regions around the soma (60–80 μm) but also the average number of cross-points was also significantly elevated (Figure 7B, + signs). Expression of wtPKD1-EGFP led to similar changes, but to a lesser extent.

To reveal potential correlation between dendritic arborization and Golgi complex morphology, high-magnification pictures were recorded from the soma region of EGFP and kdPKD1-EGFP–transfected neurons, followed by evaluating dendritic arborization of the same cells. In neurons transfected with EGFP, no correlation was found between the extent of dendritic arborization and Golgi complex morphology (average number of dendritic intersections...
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DISCUSSION

Studies in polarized epithelial cells revealed that PKD is selectively involved in the basolateral membrane protein transport (Yeaman et al., 2004). In contrast, PKD-mediated effects and transport processes in neurons are still to be understood. In line with recent publications (Horton et al., 2005; Bisbal et al., 2008), our work provides additional evidence on the polarized, somatodendritic activity as well as on Golgi-localized action of PKD in neurons.

Our data clearly show that endogenous PKD activity is gradually restricted to the somatodendritic compartment of developing hippocampal neurons, leading to a highly polarized activity in mature, DIV10 cells. In neurons activated by diC8, a cell-permeable DAG analogue, or upon expression of caPKD1, pS294 immunostaining was highly increased at the plasma membrane in the soma and in the dendrites, reflecting local recruitment of activated PKD. Despite the presence of endogenous PKD1 in the axons (Supplemental Figure 2; Bisbal et al., 2008; Yin et al., 2008), treatment with diC8 did not increase axonal pS294 signal. In contrast, axonal PKD activity became detectable upon overexpression of exogenous PKDs, with caPKD1, wtPKD2, or wtPKD3 constructs all being evenly distributed throughout the neurons.

Initially, increased PKDrep phosphorylation upon wtPKD3 overexpression seems to be surprising because PI4KIIIβ is known to be selectively phosphorylated on S294 by both PKD1 and PKD2 but not by PKD3 (Haussier et al., 2005). However, PKD isoforms have been shown to form dimeric complexes (Bossard et al., 2007), suggesting that PKD3 action on PKDrep phosphorylation was probably mediated via interaction with PKD1 and/or PKD2. Our PKD activity reporter constructs cannot be used to directly differentiate between PKD1- or PKD2-mediated processes. Nevertheless, based on PKD1 being expressed to a much higher extent in mature hippocampal neuronal cultures than PKD2 (data not shown; Bisbal et al., 2008), both Golgi-targeted and nontargeted PKD activity reporters indicated mostly the activation of endogenous PKD1. It is important to note that mutation in the ATP binding domain of PKD1 (K612W) was shown to exert a dominant-negative effect over cotransfected wtPKD1 and wtPKD2 as well. Therefore, kdPKD1 expression in neurons can inhibit endogenous PKD activity (regardless to the PKD isoforms) in a dominant manner.

As a feasible explanation to the lack of endogenous PKD activity in the axon, we can assume that inactivation of PKD, e.g., via sequestration by 14-3-3 adapter proteins (Hausser et al., 1999; Taya et al., 2007) is selectively increased in the axons. At the same time, selective dephosphorylation of PKD, the reporter, or both (Uetani et al., 2006) has to be also taken into consideration. Irrespective of the underlying mechanisms, our findings are in accordance with recent data showing that kdPKD1 expression had no axonal effects in DIV5 hippocampal neurons (Horton et al., 2005) or on the localization of axonal membrane proteins (Bisbal et al., 2008).

In young, nonpolarized neuronal cultures, even intracellular phosphorylation of PKDrep and high level of pS910 autophosphorylation was observed. The restriction of PKD activity to the somatodendritic compartments corresponded to the relative decrease in pS910 levels. In accordance with

and SEM values were 76.45 ± 6.92 and 85.63 ± 7.11 for neurons having normal, thread-like or dispersed Golgi complex, respectively; 29 cells investigated). In kdPKD1-EGFP–expressing neurons, in contrast, strong correlation was found (Figure 7C; 37 cells investigated). Those neurons that possessed highly dispersed Golgi apparatus had an even more severely reduced dendritic arborization compared with those cells, where Golgi complex was at least partially preserved in a filamentous, thread-like state. Similar findings were observed already during shorter (12 and 18h) posttransfection times, when kdPKD1-EGFP–transfected neurons possessing dispersed Golgi apparatus had severely reduced dendritic tree, as well (data not shown). Importantly, the ratio of these cells increased with time (Supplemental Table 1).
recent publications (Watkins et al., 2008; Yin et al., 2008), these results indicate that PKD is involved in early neuronal polarization processes. The multiple axon formation upon decreased PKD activity described by Yin et al. (2008), however, is in contrast to published data (Horton et al., 2005; Watkins et al., 2008) and also to our observations (Czöndör and Schlett, unpublished data). Thus, further work is needed to clarify these apparent inconsistencies.

Recently, impaired PKD activity in mature neurons was shown to induce increased endocytosis of dendritic membrane proteins (Bisbal et al., 2008), indicating that PKD activity is required for the selective maintenance of the dendritic membrane surface. In accordance with these findings, our results showed dramatic shrinkage of the dendritic tree upon kdPKD1 expression within 24 h. In contrast, caPKD1 led to enhanced arborization in transfected neurons that can be a consequence of increased membrane surface due to inhibited dendritic endocytosis.

Besides endocytosis as a potential explanation for the observed changes in dendritic arborization, direct PKD action on Golgi-mediated secretory processes also has to be considered. Golgi-mediated secretory processes are known to play highly important role in the supply of cargo necessary for the maintenance of the dendritic surface machinery (Ye et al., 2007; Hanus and Ehlers, 2008; Tang, 2008). Therefore, impaired dendrite-directed secretory transport can also result in the shrinkage of the dendritic tree in kdPKD1-transfected neurons.

The importance of the Golgi structure necessary for the high turnover of dendritic membrane surface is reflected by the presence of so-called dendritic outposts in the main dendrites (Syntyk et al., 2002; Horton and Ehlers, 2003). In our hippocampal cultures, Golgi-targeted PKD reporter revealed high endogenous PKD activity at the neuronal Golgi complex. Moreover, PKD1 constructs showed enrichment at the TGN, especially in kdPKD1 expression. It is known that the K612W mutation in the kinase domain of PKD1 does neither interfere with substrate binding nor with DAG-dependent membrane recruitment of the kinase (Johannes et al., 1998; Haussser et al., 2002). However, detection from membranes seems impaired, resulting in an accumulation at the TGN (Maeda et al., 2001; Bard and Malhotra, 2006). Accordingly, accumulation of kdPKD1-GFP at the VAMP4-positive TGN structures was observed both in vitro, in transfected neurons, and in vivo, upon Dox-induced PKD mutant expression in CaMKIIrtTA2 × kdPKD1-EGFP double transgenic mice.

Importantly, kdPKD1 accumulation led to the disruption of the neuronal Golgi apparatus via the formation of smaller fragments, still possessing cis- and trans-Golgi elements. Prolonged or elevated kdPKD1 expression level led to increased ratio of cells with impaired Golgi complex morphology in the investigated cultures as well as in vivo. These data indicate that a certain level of kdPKD1 expression is needed to affect Golgi morphology, likely because of the development of the dominant-negative effect over endogenous PKD isoforms. Differences in the expression level of kdPKD1 and the time point of analyses can also explain the discrepancy between our results and that of Bisbal et al. (2008), who did not report morphological changes in the Golgi apparatus upon interference with neuronal PKD activity.

Fragmentation of the Golgi apparatus is at variance with the well-described tubulation of the TGN in kdPKD-expressing nonneuronal cells, which is widely accepted as a consequence of impaired vesicle fission (Bard and Malhotra, 2006; De Matteis and Luini, 2008). However, morphology of the Golgi complex in neuronal cells differs largely from those of the typical transformed cell lines of epithelial origin. Therefore, despite potential differences in phenotypical changes, our results presented here and in earlier studies carried out in nonneuronal cells share the findings that 1) PKD activity is required for the maintenance of the cell type-specific Golgi complex architecture and that 2) interfering with PKD activity rapidly changes Golgi complex integrity. Because the extent of Golgi apparatus disruption in kdPKD1-transfected neurons correlated well with the extent of impaired dendritic arborization, we assume that PKD activity regulates the maintenance of dendritic tree both by acting at the Golgi apparatus as well as by influencing local endocytotic processes in the dendrites.

In addition, PKD activity is involved in the regulation of cytoskeletal organization affecting cell migration and invasion in nonneuronal cells (Van Lint et al., 2002; Qiang et al., 2004). PKD was also reported to interact with F-actin and cortactin (Eiseler et al., 2007) and to participate in nocodazole-mediated disruption of the Golgi complex (Fuchs et al., 2009). As the morphology of the Golgi complex depends to a large extent on the integrity of the surrounding cytoskeletal elements (Bard and Malhotra, 2006; Egea et al., 2006; De Matteis and Luini, 2008), the observed disruption of the Golgi complex could be also a consequence of altered cytoskeletal organization upon kdPKD1 expression. Additionally, cytoskeletal rearrangements fundamentally influence the formation and maintenance of dendritic structures, including elongation and branching of dendrites or spine formation (Gauthier-Campbell et al., 2004; Kim et al., 2006; Hayashi et al., 2007; Tada et al., 2007; Zhang and Macara, 2008). Accordingly, direct or indirect cytoskeletal effects of PKD can also participate in the observed dendritic rearrangements in transfected neurons, especially when taking into account that high level of PKD activity was observed not only around the neuronal Golgi but also in the cytoplasm of dendrites and even in the dendritic spines.

Together, our investigations have provided evidence that PKD acts selectively in the somatodendritic compartment of polarized neurons and is needed for the integrity of Golgi apparatus and for the maintenance of dendritic arborization.

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