Cellular expression and subcellular localization of diacylglycerol kinase γ in rat brain

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ABSTRACT
Gq protein-coupled receptors lead to activation of phospholipase C, which triggers phosphoinositide signaling. Diacylglycerol (DG) is one of the phosphoinositide metabolites and serves as a second messenger. Diacylglycerol kinase (DGK) phosphorylates DG to produce another second messenger phosphatidic acid. Of the DGK family, DGKγ is predominantly expressed in the brain at the mRNA level. Recent studies have shown the expression of DGKγ in vascular endothelial cells and adrenal medullary cells at the protein level, although its detailed cellular expression pattern and subcellular localization in the brain remain to be determined. In the present study, we addressed this point using specific DGKγ antibody. DGKγ was expressed in both projection neurons and interneurons in the cerebral cortex, hippocampal formation, and cerebellum. In cerebellar Purkinje cells, DGKγ was distributed to the soma and dendrites. Fractionation study revealed that DGKγ was enriched in the internal membranes containing the endoplasmic reticulum and Golgi complex. In immunoelectron microscopy, DGKγ was localized throughout the smooth endoplasmic reticulum system. These findings suggest that DGKγ shows unique cellular expression pattern in the brain and distinct subcellular localization different from other DGK isozymes.

INTRODUCTION
External stimuli trigger activation of phospholipase C (PLC) through Gq protein-coupled receptors, which yields two messengers, namely diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP3), in phosphoinositide signaling. In this system, diacylglycerol kinase (DGK) phosphorylates DG to produce phosphatidic acid. DG regulates a number of proteins containing DG-binding C1 domain, for which DG acts as an allosteric activator. Those proteins include protein kinase C (PKC) (Nishizuka 1992; Ron and Kazanietz 1999; Martelli et al. 2004), protein kinase D (PKD) (Baron and Malhotra 2002), chimaerin (Caloca et al. 1999), and RasGRP (Ebinu et al. 1998). Moreover, phosphatidic acid, the product of DGK, is also shown to act as a messenger to regulate other signaling molecules (Merida et al. 2008; Zhang and Du 2009). Therefore, DGK is thought to regulate the two lipid messenger pathways by modulating the attenuation of DG and the production of phosphatidic acid signals.

So far 10 DGK isozymes have been identified in mammals (Goto et al. 2007). Accumulating evidence has shown characteristic patterns of expression and localization of DGKs in various organs, including the central and peripheral nervous systems, heart, vascular smooth muscle and endothelial cells, lung, liver, female reproductive organs, pituitary gland, pancreas, adrenal gland, and retina under pathophysiological conditions (Goto et al. 2006, 2007, 2014; Nakano et al. 2009, 2012; Hozumi et al. 2010, 2013, 2015, 2016). These observations
suggest that the DGK family is involved in widely diverse functions of tissues and cells.

Intriguingly, most of the DGK isozymes are expressed abundantly in the brain, suggesting the physiological importance of this enzyme family for proper brain function (Goto and Kondo 2004; Goto et al. 2006). Of the isozymes, DGKγ is characterized by predominant expression in cerebellar Purkinje cells (Goto et al. 1994). Recent studies show that DGKγ is compartmentalized to the Golgi complex in cDNA-transfected cells, rat aortic endothelial cells, adrenal zona glomerulosa cells, and adrenomedullary cells (Kobayashi et al. 2007; Nakano et al. 2012; Hozumi et al. 2015). Despite the potential importance of DGKγ in neurons, fundamental issues of its cellular expression and subcellular localization in the brain remain undetermined.

In this study, we performed light and electron microscopic immunohistochemistry to investigate the expression and localization of DGKγ in rat brain. We found that DGKγ is expressed in both projection neurons and interneurons in various brain regions. At the electron microscopic level, DGKγ was localized widely to the smooth endoplasmic reticulum (sER) in dendrites of Purkinje cells.

MATERIALS AND METHODS

Tissue and section preparation. All experiments were performed according to the guidelines laid down by the animal welfare committee of Yamagata University and under institutional approval. Adult Wistar rats at 9 weeks of age (Japan SLC Inc., Hamamatsu, Japan) were used in the present study. For immunohistochemistry, rats were anesthetized by intraperitoneal injection of medetomidine hydrochloride (0.375 mg/kg), midazolam (2 mg/kg), and butorphanol (2.5 mg/kg). They were fixed transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min at 4°C to remove debris. The whole brains of 9-week-old rats (Japan SLC Inc., Hamamatsu, Japan) were used in the present study. For immunohistochemistry, rats were anesthetized by intraperitoneal injection of medetomidine hydrochloride (0.375 mg/kg), midazolam (2 mg/kg), and butorphanol (2.5 mg/kg). They were fixed transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for light and immunofluorescence microscopies or 4% paraformaldehyde/0.1% glutaraldehyde in 1 M sodium phosphate buffer (pH 7.2) for immunoelectron microscopy. Microslicer sections (50 μm; VT1200S; Leica, Nussloch, Germany) were prepared for immunoperoxidase, immunofluorescence, and pre-embedding immunogold electron microscopy.

Immunoblotting. The whole brains of 9-week-old rats were homogenized with 4 volumes of a buffer containing 10 mM Tris-HCl (pH 7.4), 20 mM KCl, 0.1 mM EDTA and 0.25 M sucrose, and centrifuged at 1000 ×g for 10 min at 4°C to remove debris. Protein concentration was determined using BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL). Values were the means of triplicate determinations. The resulting supernatant (10 μg) was boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer (New England Biolabs, Inc., Ipswich, MA) and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were then electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA). After blocking the non-specific binding sites with 4% non-fat dry milk (w/v) in phosphate-buffered saline (PBS) containing 0.02% sodium azide and 0.2% Tween 20, the membrane was incubated for 1 h at room temperature with rabbit anti-DGKγ antibody (0.5 μg/mL) (Nakano et al. 2012; Hozumi et al. 2015) in PBS containing 0.1% Tween 20 and 2% non-fat dry milk (w/v) and then with peroxidase-linked secondary antibody (1:5000; GE Healthcare UK Ltd, Buckinghamshire, England) for 30 min. Sites of antigen-antibody reaction were visualized using the chemiluminescent Immobilon Western blotting detection system (Millipore).

Biochemical subcellular fractionation was performed as reported previously (Hozumi et al. 2017). The cerebella of adult Wistar rats were excised and homogenized using a Potter homogenizer with 15 strokes at 800 rpm in 10 volumes of ice-cold homogenize buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl (pH 7.0) and 0.4 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1000 ×g for 10 min to remove nuclei and large debris. The supernatant (S1, postnuclear fraction) was centrifuged at 10000 ×g for 20 min to obtain a crude synaptosomal fraction (P2), lysed hypo-osmotically and centrifuged at 25000 ×g for 30 min. The pellet (LP1, synaptosomal membranes) was suspended with 0.5% Triton X-100 in the homogenize buffer for 15 min and centrifuged at 111000 ×g for 1 h to separate a postsynaptic density (PSD) fraction (PSD, pellet) and a Triton-soluble synaptosomal membrane fraction (TSM, supernatant). The supernatant (S2) after centrifugation of S1 was further centrifuged at 165000 ×g for 1 h to obtain a cytosolic fraction (S3) and a light membrane/microsome-enriched fraction (P3). After SDS–PAGE and electroblotting of the S3, P3, TSM, and PSD fractions, membranes were incubated with 4% non-fat dry milk (w/v) in PBS containing 0.02% sodium azide and 0.2% Tween 20, followed by a 1-h incubation with the following primary antibodies; rabbit anti-DGKγ (0.5 μg/mL), rabbit anti-Gαq/11 (0.5 μg/mL; sc-392, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-postsynaptic density 95 kDa (PSD-
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95, 1:2000; MA1-045, Affinity BioReagents, Inc., Golden, CO), goat anti-calreticulin (1 μg/mL; sc-7431, Santa Cruz Biotechnology Inc.), or goat anti-calbindin (0.5 μg/mL) (Nakamura et al. 2004).

Immunohistochemistry. All immunohistochemical incubations were performed at room temperature (−18°C) by the floating method, using phosphate-buffered saline (PBS) containing 0.1% Triton X-100 as antibody diluent and washing buffer. Immunoperoxidase staining was performed by overnight incubation with rabbit anti-DGKγ antibody (0.5 μg/mL). Sections were further incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 min and avidin-biotin-peroxidase complex for 30 min using the avidin-biotine-peroxidase complex (ABC) system (Vector Laboratories). Immunoreaction was visualized with 3,3-diaminobenzidine (DAB) and photographs were taken by a light microscope (Leica).

For immunofluorescence, sections were incubated successively with 10% normal donkey serum for 20 min, primary antibodies overnight, and Alexa Fluor 488- or Alexa Fluor 594-linked species-specific secondary antibodies (1:200 for each; Molecular Probes, Eugene, OR) for 2 h. For multiple labelings, we used primary antibodies against the following molecules: goat anti-calbindin (1 μg/mL) (Miura et al. 2006), rabbit anti-DGKγ antibody (1 μg/mL) (Nakano et al. 2012; Hozumi et al. 2015), mouse anti-glutamate decarboxylase (GAD; 1 μg/mL; MAB5406, Millipore), goat anti-microtubule associated protein-2 (MAP2; 1 μg/mL) (Miura et al. 2006), and guinea pig anti-parvalbumin (1 μg/mL) (Nakamura et al. 2004). Sections were scanned using a confocal laser scanning microscope (LSM700; Carl Zeiss, Göttingen, Germany) in multitrack mode. In light and fluorescent microscopic examinations, we could not observe any animal-to-animal variability of DGKγ immunoreactivity.

For pre-embedding immunoelectron microscopy, microslicer sections were dipped in 5% bovine serum albumin (BSA)/0.02% saponin/Tris-buffered saline (TBS) for 30 min, and incubated overnight with DGKγ antibody diluted with 1.25% BSA/0.004% saponin/TBS and then with anti-rabbit IgG linked to 1.4 nm gold particles (Nanogold; Nanoprobes Inc., Stony Brook, NY) for 2 h. Immunogolds were intensified using the silver enhancement system (R-GENT SE-EM; Aurion, Wageningen, Netherlands). Sections labeled by silver-enhanced immunogold were treated with 2% osmium tetroxide for 30 min, stained in block with 2% uranyl acetate for 30 min, dehydrated, and embedded in Epon 812.

RESULTS

Expression and localization in the brain

We used polyclonal antibody that was raised in a rabbit against rat DGKγ (a.a. 75-108) as reported previously (Nakano et al. 2012; Hozumi et al. 2015). Immunoblot analysis showed that the antibody detects a single band at the predicted size (88 kDa) of DGKγ (Fig. 1A) (Goto et al. 1994).

First, biochemical fractionation was performed to examine the subcellular distribution of DGKγ using protein samples from the cerebellum (Fig. 1B). The fractionation quality was estimated using specific markers. These include cytosolic protein calbindin in the cytosolic fraction (S3), endoplasmic reticulum (ER)-resident protein calreticulin in the light membrane/microsome-enriched fraction (P3), plasma membrane-attached G protein Gαq/11 in the Triton-soluble synaptosomal membrane (TSM) fraction, and postsynaptic protein PSD-95 in the postsynaptic density fraction (PSD). After fractionation, DGKγ was highly enriched in the P3 fraction as well as in the S3 fraction at a much lower level. DGKγ was hardly recovered in the TSM and PSD fractions (Fig. 1B). The P3 fraction is shown to contain internal membranes such as the ER and Golgi complex together with fragmented plasma membrane (Nakamura et al. 2004). These results suggest that DGKγ is selectively targeted to membranous compartment. This feature closely resembled that of DGKε, which was analyzed by the same protocol (Hozumi et al. 2017).

To examine the distribution of DGKγ in rat brain, we performed immunoperoxidase histochemistry. We observed intense labeling for DGKγ in the olfactory bulb, hippocampus, and cerebellum, and weak labeling in most other gray matter regions (Fig. 1C). The overall distribution of DGKγ immunoreactivity (Fig. 1C) reproduced that of DGKε mRNA by in situ hybridization (Goto et al. 1994). This distribution pattern of DGKγ in the brain differed from that of DGKα, -β, -ζ, and -ε (Goto et al. 2007; Hozumi and Goto 2012; Hozumi et al. 2017), suggesting a unique functional assignment of this isozyme.

Neuronal expression in the cerebral cortex and hippocampus

We next examined cellular expression of DGKγ in highly immunoreactive brain regions. In the cerebral cortex, DGKγ expression levels were moderate in cortical layers II/III, and weak in cortical layers I,
IV, V and VI (Fig. 2A, 2B). DGKγ immunolabeling was detected as granular structures in somata of neurons in the cerebral cortex (Fig. 2C). In cortical layers II/III, the immunoreactivity was observed intensely in a MAP2-negative interneuron (asterisk in Fig. 2D) and moderately in MAP2-positive cortical projection neurons (daggers in Fig. 2D).

In the hippocampus, the CA1, CA2, CA3, CA4, and dentate gyrus were immunostained with DGKγ antibody (Fig. 2A). In the CA1, DGKγ immunoreactivity was observed strongly in the stratum oriens, pyramidal layer, and stratum radiatum (Fig. 3A, 3B). In the CA1 and CA3 subfields, the immunohistochemical signals were seen as dense tiny puncta occupying the neuropil (Fig. 3B, 3C). In the dentate gyrus, DGKγ was observed as granular structures in somata of granule cells (Fig. 3D). When compared by double immunofluorescence in the CA1 region, DGKγ was detected in both MAP2-positive pyramidal neurons (daggers in Fig. 4A) and a MAP2-negative interneuron (asterisk in Fig. 4A). By double immunostaining, DGKγ was detected in both MAP2-
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Neuronal expression in the cerebellum

In the cerebellum, DGKγ immunoreactivity was intensely observed in the molecular layer, Purkinje cell layer, and granular layer (Fig. 5A, 5B). At the cellular level, DGKγ was detected in Purkinje cells and granule cells (Fig. 5B). In Purkinje cells, DGKγ was observed as particulate or granular structures in somata or dendrites (Fig. 5C). More detailed distribution profile was obtained by using neurochemical markers (Fig. 5D–F). By double immunostaining with antibodies for calbindin (a marker for Purkinje cells in the cerebellum) or parvalbumin (a marker for Purkinje cells and molecular layer interneurons), DGKγ was observed in both calbindin-positive Purkinje cells (Fig. 5D) and parvalbumin-positive interneurons in the molecular layer (asterisks in Fig. 5E). In the granular layer and white matter, the immunoreactivity was moderately observed in somata of granule cells, but was absent in calbindin-positive Purkinje cell axons (Fig. 5F). This pattern differs from DGKε immunoreactivity, which is clearly detected in Purkinje cell axons but is absent in granule cells (Hozumi et al. 2017).

Fig. 2 Immunohistochemistry showing DGKγ distribution and DGKγ-expressing neurons in the cerebral cortex. A A low magnification image of DGKγ immunoperoxidase in coronal section of rat brain. Note intense immunolabeling for DGKγ in the hippocampal formation and moderate immunolabeling for DGKγ in the cortical surface. B, C Low and high magnification images of DGKγ immunoperoxidase in cortical layers II/III. D Immunoreactivity for DGKγ is observed in both MAP2-positive cortical projection neurons (†) and a MAP2-negative interneuron (*). Cx, cerebral cortex; Hi, hippocampus; St, striatum; Th thalamus. Scale bars: 1 mm (A); 20 μm (B, C, D).
As a next step, we performed immunoelectron microscopic analysis to examine subcellular localization of DGKγ. We employed electron microscopy with pre-embedding silver-enhanced immunogold labeling method. Immunogold particles were principally associated with the sER in proximal dendrites of Purkinje cells (Fig. 5G, 5H), although the particles were occasionally found in the cytosol (arrows in Fig. 5H). The immunoparticles were distributed throughout the sER as observed in the confocal image (Fig. 5E1). This immunolabeling pattern was abolished when using pre-immune immunoglobulin or pre-absorbed antibody instead of the primary antibody (data not shown). Therefore, the results indicate that DGKγ mainly localizes widely to the sER in Purkinje cell dendrites.

DISCUSSION

This study reveals detailed cellular expression and subcellular localization of DGKγ in the brain at the protein level using a specific antibody. We show clearly that DGKγ is expressed in both projection neurons and interneurons in the brain, including cerebral cortex, hippocampal formation, and cerebellum. DGKγ protein is enriched in the internal membranes by fractionation analysis and is localized widely throughout the sER system in dendrites of cerebellar Purkinje cells at the immunoelectron microscopy.

Recently, of phosphoinositide signaling molecules, Gq protein α subunits Gaq and Gα11, PLCβ3, and PLCβ4 localize to the sER in dendrites of Purkinje cells (Tanaka et al. 2000; Nakamura et al. 2004; Nomura et al. 2007). In Purkinje cells, PLCβ4 forms a coimmunoprecipitable complex with mGluR1α in the sER (Nakamura et al. 2004), suggesting the possibility that these phosphoinositide signaling molecules, including DGKγ, are localized to the sER as a component of a physically linked phosphoinositide signaling complex, mainly in the mGluR1α signaling pathway in Purkinje cells. In addition, DGKγ di-

![Figure 3](image-url)
Fig. 4 Immunofluorescence for characterization of DGKγ-expressing neurons in the hippocampal formation. A In the CA1 region, DGKγ is detected in both MAP2-positive projection neurons (†) and a MAP2-negative interneuron (*). B, C In the dentate gyrus, immunoreactivity for DGKγ is observed in both MAP2-positive dentate granule cells and a GAD-positive interneuron (*). DG, dentate gyrus; Or, stratum oriens; Py, pyramidal layer; Ra, stratum radiatum. Scale bars: 20 μm.

rectly interacts with PKCγ (Yamaguchi et al. 2006), which belongs to DG-dependent subtype of the PKC family and is abundantly expressed in Purkinje cells (Kose et al. 1988). These findings suggest a functional coupling among DG-generating PLCβ4, DG-dependent PKCγ, and DG-catalyzing DGKγ, which facilitates an efficient and regulated signal transduction in Purkinje cells. In fact, PKCγ phosphorylates DGKγ, thereby exerting a negative effect on this lipid kinase activity (Yamaguchi et al. 2006).

With respect to Purkinje cells, DGKε is also shown to reside to Purkinje cells with features similar, but not identical, to DGKγ (Hozumi et al. 2017). Our previous study shows that DGKε localizes selectively to the subsurface cisterns in Purkinje cell dendrites. On the other hand, DGKγ is distributed throughout the sER in Purkinje cell dendrites. Both of these DGKs are enriched in the light membrane/microsome-enriched (P3) fraction, although immunoelectron microscopic examination reveals characteristic differences in subcellular localization. In Purkinje cells, DGKε localizes selectively to the subsurface cisterns, and colocalizes with IP₃ receptor-1 (IP₃R1) in dendrites. In addition, DGKε is closely apposed to DG lipase α (DGLα) that preferentially accumulates on the plasma membrane of the spine neck in Purkinje cells (Yoshida et al. 2006). DGKε and DGLα share the same substrate, i.e. DG containing arachidonate at sn-2 position (arachidonoyl-DG), and generate arachidonoyl-phosphatidic acid and 2-arachidonoyl glycerol, respectively. Therefore, crosstalk of signaling pathways is supposed to occur at the sites of this close apposition of the ER with the cell membrane (Divet et al. 2005), which represent the subsurface cisterns in neurons (Henkart et al. 1976; Kaufmann et al. 2009). Since 2-arachidonoyl glycerol serves as an endocannabinoid, this crosstalk may contribute to the regulation of endocannabinoid pathway.

From a functional point of view at the animal level, both DGKε and DGKγ ablations are shown to influence behavioral activities: DGKε-KO mice ex-
Fig. 5  Expression and subcellular localization of DGKγ in the cerebellum. A A low magnification image of DGKγ immunoperoxidase in sagittal section of the cerebellum. B DGKγ immunoreactivity is detected in the molecular layer, Purkinje cell layer, and granular layer. C In Purkinje cells, particulate or granular labeling for DGKγ is present in both somata and dendrites. D DGKγ is detected in calbindin-positive Purkinje cells. E At high magnification, particulate or granular labeling for DGKγ is present in both Purkinje cells and parvalbumin-positive interneurons (*). F In the granular layer and white matter, immunoreactivity against DGKγ is present in granule cells, but not in calbindin-positive axons of Purkinje cells. G, H Pre-embedding immunoelectron microscopy for DGKγ in Purkinje cells. In proximal dendrites of Purkinje cells, metal particles are associated with the sER and sometimes found in the cytosol (arrows). Cb, cerebellum; CB, calbindin; CN cerebellar nuclei; Dn dendrite; GL, granular layer; ML, molecular layer; PC, Purkinje cell layer; PV, parvalbumin; WM, white matter. Scale bars: 1 mm (A); 100 μm (B); 20 μm (C, D, E, F); 500 nm (G, H).
hibit hyperlocomotive activities in the open field test and impaired motor coordination and learning in the accelerating rotarod test (Hozumi et al. 2017). Similarly, DGKγ-KO mice show motor coordination impairments as assessed by the rotarod, footprint and beam tests (Tsumagari et al. 2020a, 2020b). Although detailed comparison of behaviors of these KO mice awaits to be examined, it should be noted that DGKγ ablation leads to impaired long-term depression (LTD) in Purkinje cells, which is caused by upregulated PKCγ activity. In addition, DGKγ ablation induces abnormalities in the dendritic development of Purkinje cells, although climbing fiber (CF) innervation in mature Purkinje cells remains intact (Tsumagari et al. 2020a, 2020b). Proper CF innervation is shown to be regulated by mGluR1-PKCγ signaling (Kano et al. 1995, 1997, 1998; Offermanns et al. 1997). An unanswered question is why mGluR1-PKCγ-mediated CF innervation remains intact in Purkinje cells of DGKγ-deficient mice. Increased PKCγ activity due to DGKγ ablation may not exert an apparent effect on mGluR1-PKCγ pathway that innervates CF for unknown reasons. Or mGluR1-PKCγ-mediated CF innervation is modulated by some other mechanisms in DGKγ-null mice.

In this regard, DGKγ is widely distributed to sER system in dendrites and is not detected in axons. The ER generally represents a synthetic site of the major membrane bilayer-forming phospholipids, including phosphatidic acid, phosphoinositide, phosphatidylserine and phosphatidylcholine (Gaspar et al. 2007). Wide distribution throughout the sER may suggest that DGKγ is involved in the homeostasis of membrane phospholipids, which might result in disturbed regulation of LTD in Purkinje cells of DGKγ-deficient mice. Further study is warranted to elucidate the functional implications of DGKγ.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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