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ORIGINAL PAPER

NMDA receptor-mediated Ca^{2+} influx triggers nucleocytoplasmic translocation of diacylglycerol kinase ζ under oxygen–glucose deprivation conditions, an in vitro model of ischemia, in rat hippocampal slices

Yusuke Suzuki · Yoshihiko Yamazaki · Yasukazu Hozumi · Masashi Okada · Toshiaki Tanaka · Ken Iseki · Nobuo Ohta · Masaru Aoyagi · Satoshi Fujii · Kaoru Goto

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Abstract Diacylglycerol kinase (DGK) plays a key role in pathophysiological cellular responses by regulating the levels of a lipid messenger diacylglycerol. Of DGK isozymes, DGKζ localizes to the nucleus in various cells such as neurons. We previously reported that DGKζ translocates from the nucleus to the cytoplasm in hippocampal CA1 pyramidal neurons after 20 min of transient forebrain ischemia. In this study, we examined the underlying mechanism of DGKζ translocation using hippocampal slices exposed to oxygen-glucose deprivation (OGD) to simulate an ischemic model of the brain. DGKζ-immunoreactivity gradually changed from the nucleus to the cytoplasm in CA1 pyramidal neurons after 20 min of OGD and was never detected in the nucleus after reoxygenation. Intriguingly, DGKζ was detected in the nucleus at 10 min OGD whereas the following 60 min reoxygenation induced complete cytoplasmic translocation of DGKζ. Morphometric analysis revealed that DGKζ cytoplasmic translocation correlated with nuclear shrinkage indicative of an early process of neuronal degeneration. The translocation under OGD conditions was blocked by NMDA receptor (NMDAR) inhibitor, and was induced by activation of NMDAR. Chelation of the extracellular Ca^{2+} blocked the translocation under OGD conditions. These results show that DGKζ cytoplasmic translocation is triggered by activation of NMDAR with subsequent extracellular Ca^{2+} influx. Furthermore, inhibition of PKC activity under OGD conditions led to nuclear retention of DGKζ in about one-third of the neurons, suggesting that PKC activity partially regulates DGKζ cytoplasmic translocation. These findings provide clues to guide further investigation of glutamate excitotoxicity mechanisms in hippocampal neurons.

Keywords Diacylglycerol kinase · Hippocampal slices · NMDA receptor · Ca^{2+} influx · OGD · PKC

Y. Suzuki · Y. Hozumi · M. Okada · T. Tanaka · K. Goto (✉)
Department of Anatomy and Cell Biology, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan
e-mail: kgoto@med.id.yamagata-u.ac.jp

Y. Suzuki · N. Ohta · M. Aoyagi
Department of Otolaryngology, Yamagata University School of Medicine, Yamagata 990-9585, Japan

Y. Yamazaki · S. Fujii
Department of Physiology, Yamagata University School of Medicine, Yamagata 990-9585, Japan

K. Iseki
Department of Emergency and Critical Care Medicine, Yamagata University School of Medicine, Yamagata 990-9585, Japan

Introduction

To maintain normal function and viability, the brain requires a continuous supply of oxygen and glucose. Cessation of blood supply attributable to arterial occlusion or cardiac arrest can engender neuronal death (Dirnagl et al. 1999). The molecular mechanisms responsible for neuronal damage are incompletely understood, although glutamate is widely accepted as playing a key role in the early phase of ischemic brain injury (Ikegaya et al. 2001; Lau and Tymianski 2010). Glutamate excitotoxicity causes a massive influx of Ca^{2+} that activates various catabolic processes, culminating in neuronal cell death (Choi 1985, 1995; Lau and Tymianski 2010). Furthermore, previous reports show that prominent changes in lipids are accompanied in the ischemic brain, in which a rapid decrease in phosphatidylinositol-4,5-bisphosphate (PIP2) levels together with a

parallel accumulation of diacylglycerol (DG) occurs (Kunievsky et al. 1992). These findings suggest that phosphoinositide (PI) pathway is involved in this process. Of the PI-related enzymes, DG kinase (DGK), an enzyme responsible for phosphorylation of second messenger DG to phosphatidic acid, plays a central role in controlling DG-dependent signaling pathways (Kanoh et al. 1990).

The DGK family constitutes of several isozymes, which show distinct properties including structural motif, enzymatic characteristics, subcellular localization, and binding partner (Goto et al. 2007; Merida et al. 2008; Sakane et al. 2007; Topham and Epand 2009). We have isolated several DGK isozymes from rats and examined their gene expression in the brain using in situ hybridization histochemistry (Goto et al. 1992, 1994, 2007; Goto and Kondo 1993, 1996, 1999; Ito et al. 2004). Intriguingly, the mRNA for each isozyme is expressed in a distinct pattern in the brain, suggesting that each isozyme plays a unique role in various signaling pathways under a distinct regulatory mechanism.

Of DGKs, DGKζ is characterized by the presence of a nuclear localization signal (NLS). It localizes primarily to the nucleus of neurons in various regions of the brain under normal conditions, including the olfactory bulb, cerebral and cerebellar cortices, and hippocampus (Goto and Kondo 1996; Hozumi et al. 2003). However, we previously reported that, in 20 min of transient forebrain ischemia, DGKζ translocates from the nucleus to the cytoplasm in pyramidal neurons of the hippocampal CA1 region and that it is never relocated to the nucleus during the time course of reperfusion (Ali et al. 2004). Delayed neuronal death in the hippocampal CA1 neurons is well known to occur after 48–72 h reperfusion in a transient ischemia model (Kirino 1982). Our data suggest that the translocation of DGKζ might be involved in the early process of selective vulnerability of hippocampal neurons in the post-ischemic brain. However, the detailed mechanism of DGKζ translocation and its functional significance remain to be elucidated.

In this study, we approached these issues using acute hippocampal slices, a common experimental system for a model of analyses such as histochemical, physiological, and pharmacological examinations. An acute slice system confers an advantage not only in maintaining neuronal connectivity and relation with glial cells but also in manipulating the environmental conditions (Gahwiler et al. 1997). We used hippocampal slices exposed to oxygen–glucose deprivation (OGD) to simulate an ischemic model of the brain, and evaluated the triggering cascade and detailed time course of DGKζ translocation by immunohistochemical analysis using pharmacological agents. Here we show that DGKζ cytoplasmic translocation is well recapitulated in hippocampal slices exposed to OGD, which reveals that the translocation is triggered by activation of *N*-methyl-D-aspartate (NMDA) receptor followed by extracellular Ca^{2+} influx. These results suggest that DGKζ cytoplasmic translocation is closely involved in glutamate excitotoxicity in hippocampal neurons.

Materials and methods

Acute hippocampal slices

All experiments were conducted in accordance with the Yamagata university guide for the care and use of laboratory animals and were approved by the Yamagata university institutional animal care and use committee. Male Sprague–Dawley rats (4–7 weeks old) were anesthetized with ether and decapitated. Immediately after decapitation, the brain was removed from the skull; within 2 min, the right hippocampus was placed in chilled (4°C) artificial cerebrospinal fluid (ACSF) equilibrated with 95% O_2 and 5% CO_2. The composition of the solution was (mM): NaCl 124, NaH_2PO_4 1.25, KCl 3.0, $MgSO_4$ 2.0, $NaHCO_3$ 22, $CaCl_2$ 2.5, and glucose 10. The hippocampus was sliced to thickness of 500 μm using a rotor slicer (DTY-7700; Dosaka EM Co. Ltd., Osaka), transferred to a regular chamber containing oxygenated ACSF, and incubated for 2 h at temperature of 30°C before use in experiments (Yamazaki et al. 2007). About ten slices were obtained from one hippocampus. At least eight slices were used for each experiment. Control experiments in each condition were performed on slices derived from the one and the same hippocampus. We used at least three slices in each group of the experiment. One slice provided 5–10 cryostat sections suitable for immunohistochemistry.

Oxygen–glucose deprivation

To simulate an ischemic model of the brain, the hippocampal slices were transferred to an oxygen–glucose deprivation (OGD) chamber containing glucose-deficient ACSF equilibrated with 95% N_2 and 5% CO_2 at 30°C. Subsequently, to simulate a reperfusion model of the brain, some hippocampal slices were returned to a regular chamber containing oxygenated (95% O_2 and 5% CO_2) ACSF with 10 mM glucose (designated simply as 'reoxygenation').

Glutamatergic receptor, VDCC, and enzyme inhibition

Hippocampal slices were exposed to 10 min OGD, with subsequent incubation in a regular chamber for 30 min, under which conditions DGKζ translocated to the cytoplasm. To investigate the relevance to glutamate signaling pathways, glutamate receptor antagonists, including D,L-2-amino-5-phosphonopentanoic acid (AP5, 50 μM) (Sigma, St. Louis, MO, USA), methyl-4-carboxyphenylglycine

(MCPG, 500 μM) (Tocris Bioscience, Bristol, UK), and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM) (Sigma, St. Louis, MO, USA) were added to the chamber (Yamazaki et al. 2005). $CdCl_2$ (200 μM) (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used to block voltage-dependent calcium channels (VDCC). The hippocampal slices were incubated with agents 10 min before the experiment was started. Ca^{2+} free conditions were established by superfusing the slices with ACSF in which $CaCl_2$ was replaced by $MgCl_2$. In some experiments, enzyme inhibitors such as 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62, 10 μM) (Biomol International, Plymouth Meeting, PA, USA), Tacrolimus (FK506, 10 μM) (Sigma, St. Louis, MO, USA) (Yamazaki et al. 2011), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7, 100 μM) (Calbiochem, San Diego, CA), and bisindolylmaleimide I (GF 109203X, 1 μM) (Calbiochem, San Diego, CA) were used.

Glutamatergic receptor stimulation and VDCC activation

Hippocampal slices were incubated in a regular chamber for 30 min, under which conditions DGKζ remained in the nucleus. To stimulate VDCC by depolarization, the concentration of potassium in ACSF was changed from 3.0 to 52 mM together with AP5 (50 μM) and DNQX (20 μM) to inhibit other receptors. To stimulate α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (AMPAR), AMPA (100 μM) was added together with $CdCl_2$ (200 μM), AP5 (50 μM) and tetrodotoxin (TTX, 0.5 μM) (Sigma, St. Louis, MO, USA) to inhibit other receptors. To stimulate NMDAR, NMDA (1 mM) was added together with $CdCl_2$ (200 μM), DNQX (20 μM), and TTX (0.5 μM) to inhibit other receptors. In addition, the concentration of $MgSO_4$ in ACSF was changed from 2.0 to 0.1 mM to relieve Mg^{2+} block of NMDAR (Yamazaki et al. 2006). Moreover, KCl was changed from 3.0 to 5.0 mM to maintain equilibrium. In some experiments, calcium ionophore (A23187, 1 μM) (Sigma, St. Louis, MO, USA) was added to raise the intracellular calcium.

Immunohistochemistry

Hippocampal slices were fixed by immersion for 2 h in 4% phosphate-buffered (pH 7.4) paraformaldehyde at 4°C immediately after the end of the experiment. They were kept in 30% sucrose in 0.1 M phosphate buffer until use. The slices were cut into sagittal sections (30 μm) on a cryostat (Leica CM1900; Leica Microsystems). Free-floating sections were soaked with 0.5% Triton-X in phosphate-buffered saline (PBS) for 15 min at room temperature (RT) to facilitate antibody penetration. Non-specific binding sites were blocked with 10% normal horse serum in PBS for 30 min at RT. The primary antibodies used were rabbit anti-DGKζ (1.0 μg/ml) (Hozumi et al. 2003) and goat anti-PKCγ (1.0 μg/ml) (Hozumi et al. 2010; Yoshida et al. 2006) antibodies in PBS containing 0.1% Tween-20 (PBS-T). Incubation was performed overnight at RT in a moist chamber. After washing in PBS-T several times, slices were incubated with anti-rabbit IgG-Alexa 546 (red) (Molecular Probes Inc., Eugene, OR, USA) for anti-DGKζ antibody, or anti-goat IgG-Alexa 488 (green) (Molecular Probes Inc.) for anti-PKCγ antibody in PBS-T for 2 h at RT. To stain nuclei, some slices were also incubated with TO-PRO-3 (2.5 μM) in the same solution for 2 h at RT in the dark. The images were taken under a confocal laser-scanning microscope (LSM 510META; Carl Zeiss Inc.) and processed using Adobe Photoshop (Adobe Systems Inc.). For morphometric analysis of nuclear shrinkage, a fluorescence photomicrograph was taken at high magnification within the CA1 pyramidal cell layer. The well-focused cell nuclei areas ($n > 100$) were delineated and measured using Image J software. Student's *t* test was used to compare the degrees of shrinkage.

Results

We previously reported that in a transient cerebral ischemia model of rats DGKζ translocates from the nucleus to the cytoplasm and is never relocated to the nucleus after reperfusion (Ali et al. 2004). To simulate an ischemic model of the brain, we used acute hippocampal slices exposed to oxygen–glucose deprivation (OGD) and evaluated the triggering cascade and detailed time course of DGKζ translocation using immunohistochemical analysis with pharmacological agents.

Cytoplasmic translocation of DGKζ induced by OGD

Hippocampal slices were incubated for 2 h in a chamber containing oxygenated ACSF with glucose at 30°C before the experiments. Under these conditions (control), DGKζ immunoreactivity was detected predominantly in the nucleus of hippocampal CA1 neurons in slice (Fig. 1a). This pattern of DGKζ subcellular localization is principally similar to that in the brain (Hozumi et al. 2003), but the immunoreactivity in hippocampal slices under normal conditions was also observed to some degree in the cytoplasm. A slight difference of subcellular localization pattern might simply reflect different conditions between in vivo and in slices.

Under continuous OGD conditions, DGKζ immunoreactivity remained nuclear at 10 min OGD (Fig. 1b). However, after 20 min OGD, the immunoreactivity changed gradually from the nucleus to the cytoplasm (Fig. 1c) and was

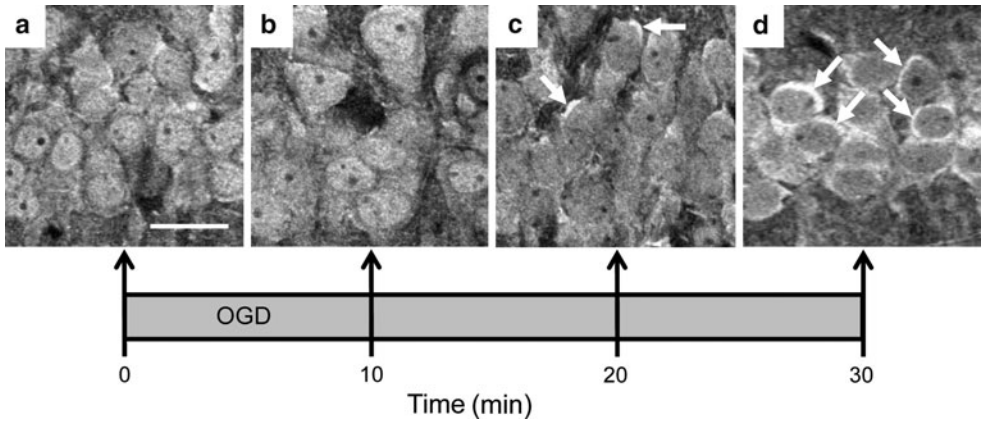

Fig. 1 Subcellular localization of DGKζ in hippocampal slices exposed to continuous oxygen-glucose deprivation (OGD). Immunofluorescent photomicrographs of DGKζ in hippocampal CA1 neurons exposed to OGD for 0 (**a**), 10 (**b**), 20 (**c**), and 30 (**d**) min. Under control conditions (**a**), DGKζ is detected predominantly in the nucleus in hippocampal CA1 neurons. After 20 min OGD DGKζ gradually translocates to the cytoplasm (**c**, *arrows*) and is predominantly detected in the cytoplasm at 30 min OGD (**d**, *arrows*). *Scale bars* 20 μm

detected predominantly in the cytoplasm at 30 min OGD (Fig. 1d). This result shows that nucleocytoplasmic translocation of DGKζ was induced after 20 min of continuous OGD in hippocampal CA1 neurons. It should be noted that DGKζ cytoplasmic translocation was not observed in CA2, CA3, and dentate gyrus neurons at least under 30 min OGD conditions (data not shown).

DGKζ cytoplasmic translocation induced by transient OGD followed by reoxygenation

Delayed neuronal cell death is well known to occur in CA1 neurons after 48–72 h reperfusion in a transient ischemia model (Kirino 1982). Therefore, we investigated whether DGKζ cytoplasmic translocation would be induced by transient OGD followed by reoxygenation (Fig. 2). Hippocampal slices were subjected to 10 min OGD, which was insufficient to induce translocation. They were subsequently returned to a regular chamber containing oxygenated ACSF with glucose (simply 'reoxygenation' hereinafter). As presented in Fig. 1b, DGKζ remained in the nucleus at 10 min OGD, although it gradually changed from the nucleus to the cytoplasm after 10 min reoxygenation (Fig. 2c, arrowheads). After 20 min reoxygenation, DGKζ was detected almost entirely in the cytoplasm (Fig. 2d, e, arrows). DGKζ cytoplasmic translocation under conditions of transient OGD followed by reoxygenation appeared more extensive than that under continuous OGD (Fig. 1c, d). Furthermore, it is noteworthy that DGKζ was never detected in the nucleus during the course of reoxygenation at least until 60 min (Fig. 2f), a phenomenon similar to that observed in our previous study of a transient ischemic brain (Ali et al. 2004).

Next, to investigate the minimum duration of OGD sufficient to induce DGKζ cytoplasmic translocation, hippocampal slices were subjected to varying times of OGD followed by 60 min reoxygenation. 6 min of OGD/reoxygenation did not induce translocation (Fig. 3b), although 8 min OGD induced the translocation in about half of the CA1 neurons after 60 min reoxygenation (Fig. 3c), and 10 min OGD was sufficient to induce the translocation in almost all the neurons (Fig. 3d). Taken together, these results can be summarized as follows: (1) 8–10 min OGD is sufficient to trigger the signaling cascade to induce DGKζ cytoplasmic translocation in CA1 neurons after 60 min reoxygenation in slices; (2) DGKζ is never relocated to the nucleus during the course of reoxygenation; and (3) its cytoplasmic translocation under conditions of transient OGD followed by reoxygenation appears more extensive than that under continuous OGD, suggesting that the translocation should be an energy-requiring process.

OGD-induced DGKζ cytoplasmic translocation correlates with nuclear shrinkage

OGD induces morphological and biochemical changes in hippocampal slices, which engender neuronal cell death (Bonde et al. 2005; Frantseva et al. 1999; Kass and Lipton 1982). Nuclear shrinkage is an early and easily quantifiable indicator of neuronal degeneration in hippocampal slices (Bonde et al. 2002). We therefore examined whether DGKζ translocation induced by OGD would correlate with neuronal degeneration. Hippocampal slices subjected to continuous or transient OGD were stained with nuclear marker TO-PRO-3, and cross-sectional areas of all well-focused neuronal nuclei in CA1 pyramidal layer were delineated and measured using morphometric analysis. Continuous

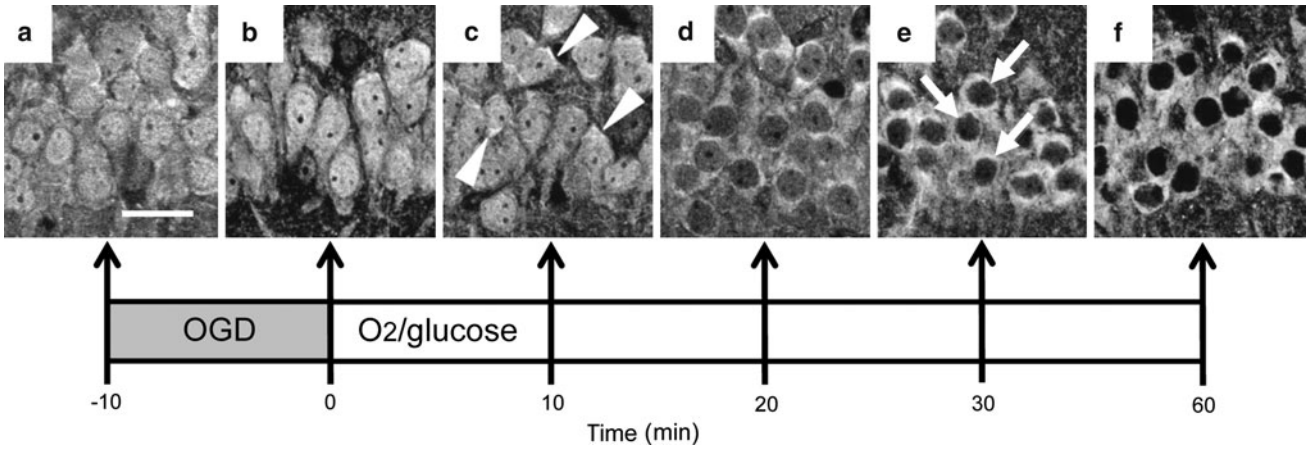

Fig. 2 Subcellular localization of DGKζ in hippocampal slices exposed to 10 min OGD followed by varying times of reoxygenation. Immunofluorescent photomicrographs of DGKζ in hippocampal CA1 neurons exposed to 10 min OGD followed by reoxygenation for 0 (**b**), 10 (**c**), 20 (**d**), 30 (**e**), and 60 (**f**) min. Under control conditions (**a**), DGKζ is detected predominantly in the nucleus in hippocampal CA1 neurons. After 10 min reoxygenation DGKζ gradually translocates to the cytoplasm (**c**, *arrowheads*) and is entirely detected in the cytoplasm at 30 min OGD (**e**, *arrows*). DGKζ is never relocated to the nucleus during the course of reoxygenation until 60 min (**f**). *Scale bars* 20 μm

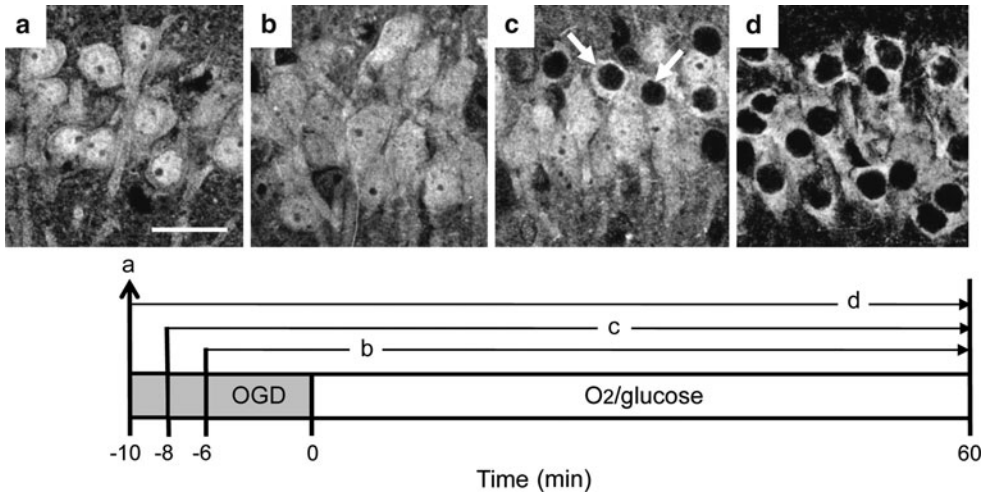

Fig. 3 Subcellular localization of DGKζ in hippocampal slices exposed to varying times of OGD followed by 60 min reoxygenation. Immunofluorescent photomicrographs of DGKζ in hippocampal CA1 neurons exposed to 6 (**b**), 8 (**c**), and 10 (**d**) min OGD followed by 60 min reoxygenation. Under control conditions (**a**), DGKζ is detected predominantly in the nucleus in hippocampal CA1 neurons. 6 min OGD/reoxygenation does not induce DGKζ cytoplasmic translocation (**b**), whereas 8 min OGD/reoxygenation induces the translocation in about half of the CA1 neurons (**c**, *arrows*). 10 min OGD/reoxygenation completely induces the translocation in almost all of the CA1 neurons. *Scale bars* 20 μm

OGD gradually induced nuclear shrinkage of CA1 neurons. At 15 min OGD, when DGKζ remained in the nucleus, nuclear shrinkage was not significant, although it became statistically evident at 30 and 45 min OGD when DGKζ was observed predominantly in cytoplasm (Fig. 4a, b; $p < 0.005$). In an experiment of transient OGD followed by 60 min reoxygenation, 5 min OGD/reoxygenation induced no changes in DGKζ localization and the cross-sectional area of CA1 pyramidal cell nuclei whereas 10 min OGD/reoxygenation clearly induced its cytoplasmic translocation and nuclear shrinkage of CA1 neurons (Fig. 4c, d; $p < 0.005$). These results demonstrate a correlation between DGKζ cytoplasmic translocation and nuclear shrinkage, suggesting a functional link between translocation and neuronal degeneration induced by OGD.

DGKζ cytoplasmic translocation is triggered by NMDA receptor activation followed by extracellular Ca^{2+} influx

Glutamate excitotoxicity is widely acknowledged to be linked inextricably to neuronal dysfunction and degeneration induced by ischemic brain injury and experimental OGD stress (Choi 1995; Lau and Tymianski 2010). We first

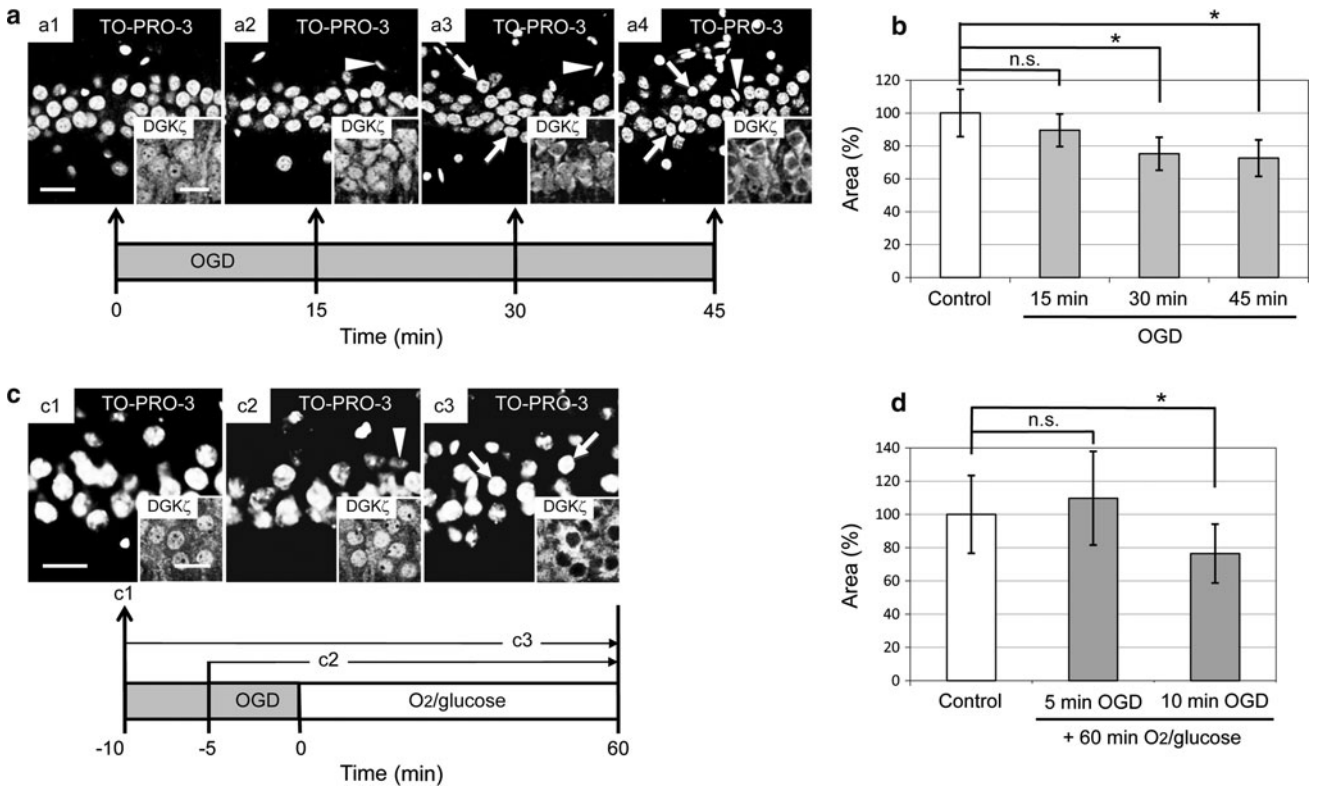

Fig. 4 Nuclear shrinkage correlates with DGKζ cytoplasmic translocation under OGD conditions. Nuclear staining (TO-PRO-3) of hippocampal CA1 neurons exposed to continuous OGD for 0 (*a1*), 15 (*a2*), 30 (*a3*), and 45 (*a4*) min or to 5 (*c2*) and 10 (*c3*) min OGD followed by 60 min reoxygenation. Cross-sectional areas of all well-focused neuronal nuclei ($n > 100$) in CA1 pyramidal layer were delineated and measured by morphometric analysis (**b** continuous OGD, **d** OGD/reoxygenation). Representative shrunk nuclei were indicated by *arrows*, and peculiar or elongated nuclei (*arrowheads*) were removed from the analysis. Inset shows DGKζ immunofluorescence in each condition. The control value was arbitrarily set 100%. The presented data are mean ± SD of at least three separate experiments. *Asterisks* indicate significance, $*p < 0.005$ (Student's *t* test); *n.s.* not significant. *Scale bars* 20 μm

examined which glutamate receptor is involved in DGKζ cytoplasmic translocation under OGD conditions using glutamate receptor antagonists. For this purpose, the experiment was performed under conditions of 10 min OGD followed by 30 min reoxygenation, which are confirmed to induce DGKζ cytoplasmic translocation (Fig. 2e). An application of metabotropic glutamate receptor (mGluR) antagonist MCPG failed to block the translocation (Fig. 5a), although AMPAR antagonist DNQX and NMDAR antagonist AP5 inhibited the translocation under OGD/reoxygenation conditions (Fig. 5b, c).

We next performed the reverse experiment and stimulated glutamate receptors using their agonists. For this purpose, hippocampal slices were incubated under normal conditions including oxygen and glucose. An application of NMDA clearly induced DGKζ cytoplasmic translocation (Fig. 6a), although AMPA failed to induce the translocation (Fig. 6b). These data suggest that NMDAR triggers activation DGKζ cytoplasmic translocation.

Once activated by excessive levels of glutamate, the NMDAR allows excessive Ca^{2+} influx (Lau and Tymianski 2010). Elevated intracellular Ca^{2+} levels engender overactivation of several deleterious enzymes and signaling processes that impair neuronal function, culminating in cell death (Choi 1985). Major routes of Ca^{2+} entry into neurons after ischemia are via NMDAR and voltage-dependent Ca^{2+} channel (VDCC) (Bertolino and Llinas 1992; Miller 1987). We next investigated whether DGKζ cytoplasmic translocation would also be induced by Ca^{2+} influx through the other routes using $CdCl_2$ (VDCC blocker) and A23187 (Ca^{2+} ionophore) or under conditions of high K^+ concentration ACSF sufficient to open VDCC. Results show that an incubation in Ca^{2+} eliminated ACSF inhibited translocation under OGD conditions (Fig. 7a, arrowheads), suggesting that the translocation process requires extracellular Ca^{2+} influx. An application of $CdCl_2$ failed to inhibit the translocation under OGD/reoxygenation conditions (Fig. 7b), and a high concentration of K^+ did not induce translocation under normal conditions (Fig. 7c), showing that the translocation process is not triggered by Ca^{2+} influx through VDCC. Similarly, an application of Ca^{2+} ionophore A23187 did not induce translocation under normal conditions (Fig. 7d). Collectively, these results suggest that DGKζ cytoplasmic translocation is triggered by extracellular Ca^{2+} influx almost entirely via NMDAR activation.

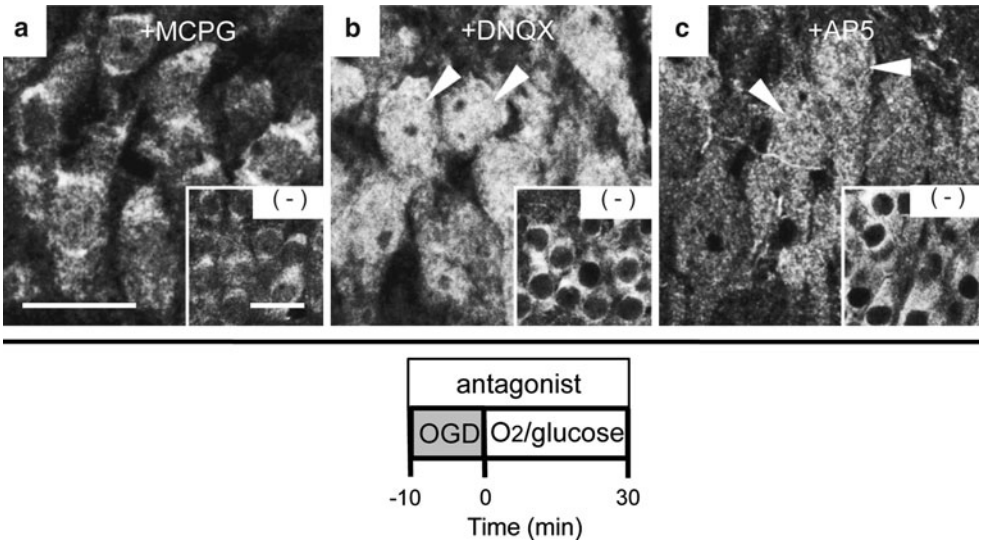

Fig. 5 Subcellular localization of DGKζ in hippocampal slices exposed to OGD/reoxygenation in the presence of glutamate receptor antagonists. Immunofluorescent photomicrographs of DGKζ in hippocampal CA1 neurons exposed to 10 min OGD followed by 30 min reoxygenation in the presence of MCPG (**a** mGluR antagonist), DNQX (**b** AMPAR antagonist), or AP5 (**c** MNDAR antagonist). Inset shows DGKζ immunofluorescence in each condition without antagonist (vehicle only). An application of MCPG (**a**) fails to block DGKζ cytoplasmic translocation, whereas DNQX (**b**) and AP5 (**c**) inhibit the translocation under OGD/reoxygenation conditions (*arrows*). *Scale bars* 20 μm

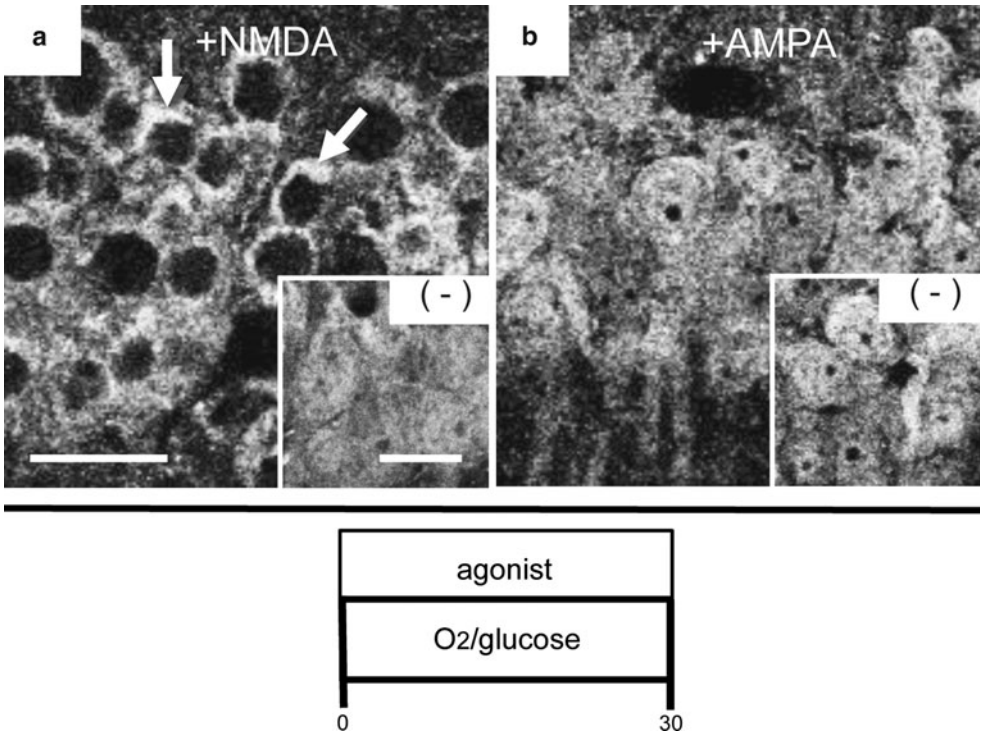

Fig. 6 Subcellular localization of DGKζ in hippocampal slices under normal conditions in the presence of glutamate receptor agonists. Immunofluorescent photomicrographs of DGKζ in hippocampal CA1 neurons under normal conditions for 30 min in the presence of NMDA (**a** NMDAR agonist) and AMPA (**b** AMPAR agonist). Inset shows DGKζ immunofluorescence in each condition without agonist (vehicle only). An application of NMDA clearly induces DGKζ cytoplasmic translocation (**a** *arrows*) whereas AMPA fails to induce the translocation (**b**). *Scale bars* 20 μm

DGKζ cytoplasmic translocation is partially regulated by PKC

Extracellular Ca^{2+} influx in neurons is well known to activate various molecules such as calmodulin (CaM), calcium–calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and calcineurin (CaN) (Orrenius et al. 2003; Szydlowska and Tymianski 2010). Therefore we examined the mechanisms of Ca^{2+} signal transduction pathway to induce DGKζ translocation using inhibitors. For this purpose, hippocampal slices were subjected to 10 min OGD followed by 30 min reoxygenation to induce DGKζ

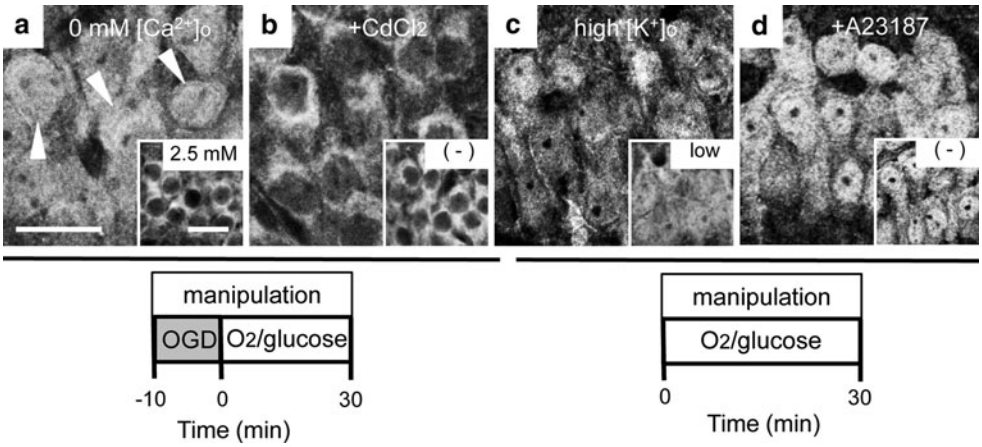

Fig. 7 Effect of Ca^{2+} on subcellular localization of DGKζ. **a** Immunofluorescent photomicrograph of DGKζ in hippocampal CA1 neurons exposed to 10 min OGD/30 min reoxygenation in Ca^{2+}-free medium. Inset shows DGKζ immunofluorescence under the same conditions in 2.5 mM Ca^{2+}-containing medium. **b** Immunofluorescent photomicrograph of DGKζ in hippocampal CA1 neurons exposed to 10 min OGD/30 min reoxygenation in the presence of $CdCl_2$ (VDCC inhibitor). Inset shows DGKζ immunofluorescence under the same conditions without inhibitor (vehicle only). **c** Immunofluorescent photomicrograph of DGKζ in hippocampal CA1 neurons under normal conditions in high (52 mM) K^+-containing medium. Inset shows DGKζ immunofluorescence under the same conditions in low (3.0 mM) K^+-containing medium. **d** Immunofluorescent photomicrograph of DGKζ in hippocampal CA1 neurons under normal conditions in the presence of A23187 (Ca^{2+} ionophore). Inset shows DGKζ immunofluorescence under the same conditions without A23187 (vehicle only). Note that chelation of extracellular Ca^{2+} inhibits DGKζ cytoplasmic translocation (**a** *arrowheads*). *Scale bars* 20 μm

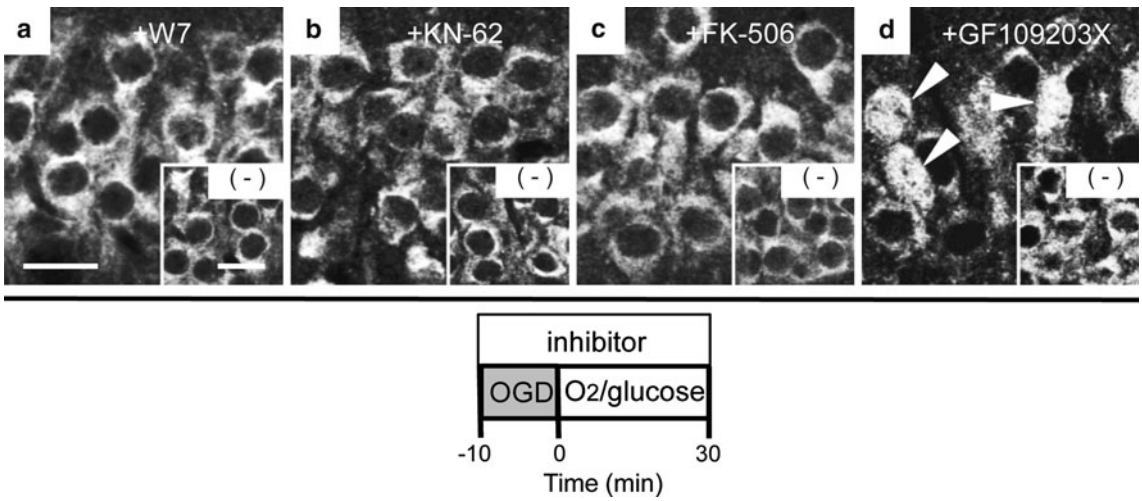

Fig. 8 Effect of intracellular Ca^{2+} signaling on subcellular localization of DGKζ. Immunofluorescent photomicrograph of DGKζ in hippocampal CA1 neurons exposed to 10 min OGD/30 min reoxygenation in the presence of W7 (**a** CaM inhibitor), KN-62 (**b** CaMKII inhibitor), FK-506 (**c** CaN inhibitor), and GF 109203X (**d** PKC inhibitor). Inset shows DGKζ immunofluorescence under the same conditions without inhibitor (vehicle only). Note that an application of W7 (**a**), KN-62 (**b**) or FK-506 (**c**) fails to inhibit DGKζ cytoplasmic translocation under OGD/reoxygenation conditions whereas GF109203X suppresses the translocation in about half of the CA1 neurons (**d**, *arrowheads*). *Scale bars* 20 μm

cytoplasmic translocation. As presented in Fig. 8, an application of CaM inhibitor W7 (a), CaMKII inhibitor KN-62 (b) or CaN inhibitor FK-506 (c) failed to inhibit the translocation under OGD/reoxygenation conditions. However, GF109203X, selective PKC inhibitor, suppressed the translocation in 34.1% of the CA1 neurons (Fig. 8d; $n > 200$), suggesting that DGKζ cytoplasmic translocation is partially, if not entirely, regulated by PKC activity under OGD conditions.

PKCγ disappears before DGKζ cytoplasmic translocation

Ca^{2+}-dependent PKCs, which are designated as classical PKCs, include PKCα, PKCβI, PKCβII, and PKCγ. They are fully activated in the presence of DG (Newton 1995; Nishizuka 1995). Because DGK is an enzyme responsible for phosphorylation of DG and because it plays a central role in controlling PKC activity (Sakane and Kanoh 1997),

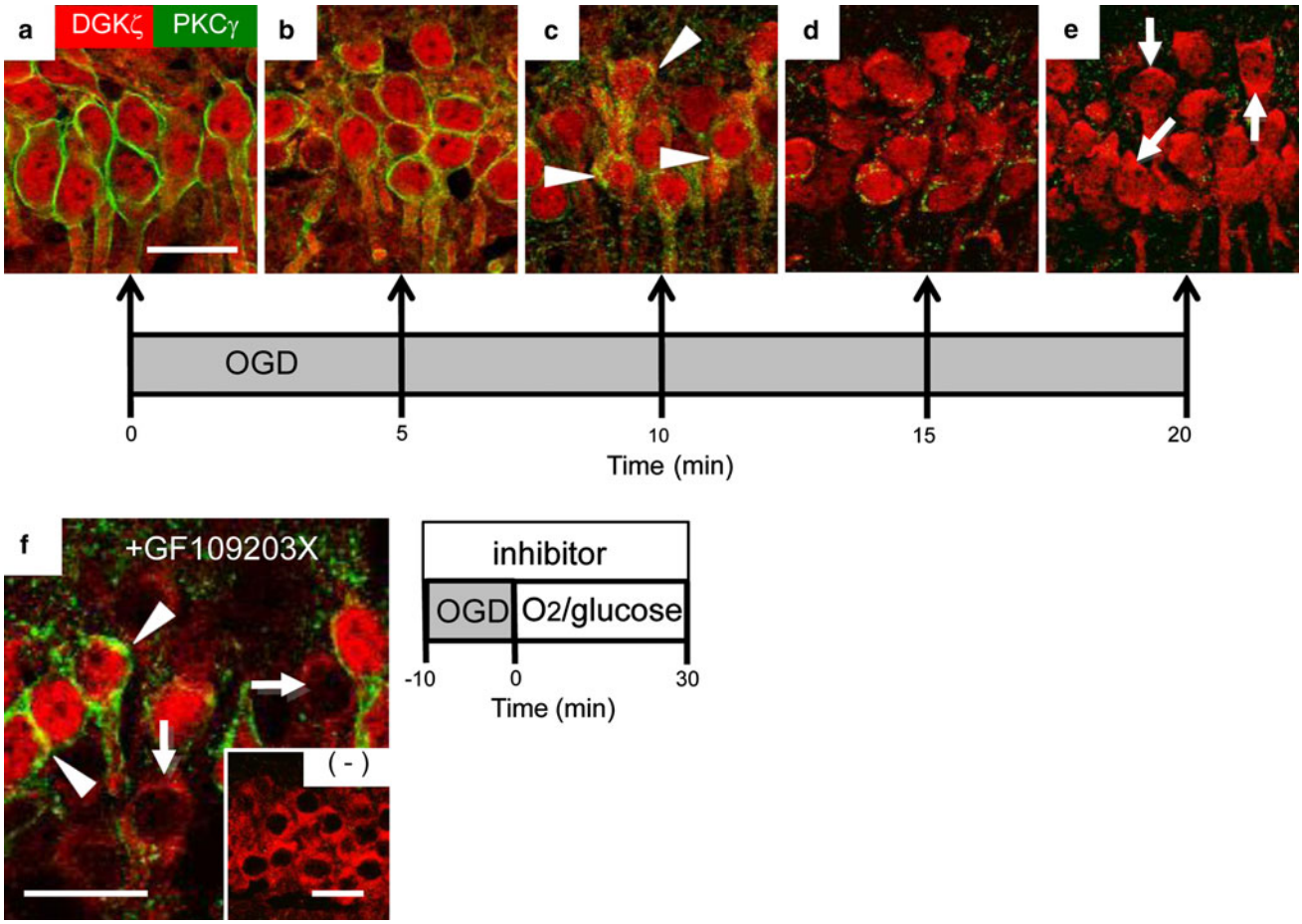

Fig. 9 Subcellular localization of DGKζ and PKCγ in hippocampal slices exposed to OGD. Double immunofluorescent photomicrographs of DGKζ (*red*) and PKCγ (*green*) in hippocampal CA1 neurons exposed to OGD for 0 (**a**), 5 (**b**), 10 (**c**), 15 (**d**), and 20 (**e**) min. Under control conditions (**a**), DGKζ is detected predominantly in the nucleus and PKCγ localizes to the plasma membrane in hippocampal CA1 neurons. PKCγ becomes gradually fragmented at 10 min OGD (**c** *arrowheads*) and almost disappears at 20 min OGD, where DGKζ is detected predominantly in the cytoplasm (**e** *arrows*). **f** Double immunofluorescent photomicrographs of DGKζ and PKCγ in hippocampal CA1 neurons exposed to 10 min OGD/30 min reoxygenation in the presence of GF 109203X (PKC inhibitor). Inset shows the immunofluorescence under the same conditions without inhibitor (vehicle only). Note that in the presence of PKC inhibitor PKCγ remains at the plasma membrane in about half of the CA1 neurons, in which DGKζ also remains in the nucleus (**f** *arrowheads*). In contrast, CA1 neurons, in which PKCγ disappears, exhibit cytoplasmic DGKζ (**f** *arrows*). *Scale bars* 20 μm

we examined a functional relation between the PKC pathway and DGKζ cytoplasmic translocation. Especially, PKCγ is expressed solely in neurons in the central nervous system (Saito and Shirai 2002) and is shown to be involved in ischemic brain injury (Aronowski et al. 2000; Aronowski and Labiche 2003; Wieloch et al. 1991). Therefore, we first investigated the time course of the immunoreactivities of DGKζ and PKCγ under continuous OGD conditions using immunohistochemical double staining (Fig. 9). Under control conditions, PKCγ was detected mainly at the plasma membrane of CA1 neurons and appeared to draw the outline of neuronal somata; DGKζ was detected predominantly in the nucleus (Fig. 9a). An initial change was observed in PKCγ-immunoreactivity, which became fragmented at 10 min OGD (Fig. 9c, arrowheads) and was greatly reduced at 15 min OGD, whereas DGKζ remained in the nucleus (Fig. 9d). At 20 min OGD, PKCγ-immunoreactivity almost disappeared and DGKζ was detected predominantly in the cytoplasm (Fig. 9e, arrows).

In an experiment related to OGD/reoxygenation, under conditions of 10 min OGD followed by 30 min reoxygenation, DGKζ translocated entirely to the cytoplasm, whereas PKCγ-immunoreactivity completely disappeared (Fig. 9f, inset). However, under identical conditions but in the presence of selective PKC inhibitor (GF109203X), PKCγ-immunoreactivity remained at the plasma membrane in about one-third of the CA1 neurons, in which DGKζ also remained in the nucleus (arrowheads). In contrast, CA1 neurons showing no PKCγ-immunoreactivity exhibited cytoplasmic DGKζ (arrows). These findings showed that PKCγ disappears prior to DGKζ cytoplasmic translocation under OGD conditions.

Discussion

Cessation of blood supply to the brain during ischemia results in oxygen and glucose deprivation and engenders depletion of cellular energy (Dirnagl et al. 1999). Neurons become unable to maintain the ion gradients for normal function and viability. Glutamate excitotoxicity is widely accepted as induced by massive release and reduced reuptake after ischemia/reperfusion, culminating in neuronal degeneration (Choi 1985, 1995; Lau and Tymianski 2010). We previously reported that DGKζ translocates from the nucleus to the cytoplasm in hippocampal CA1 neurons in 20 min of transient forebrain ischemia. It is never relocated to the nucleus during the course of reperfusion (Ali et al. 2004), suggesting that DGKζ is involved in processes of ischemic injury. However, the detailed mechanism of DGKζ translocation and its functional significance remained to be elucidated. In this study, we addressed these issues using hippocampal slices exposed to OGD to simulate an ischemic model of the brain.

Our immunohistochemical study reveals that DGKζ cytoplasmic translocation is well recapitulated in hippocampal slices exposed to transient OGD. Detailed time course analysis shows that a minimum of 8–10 min OGD is sufficient to induce the translocation in CA1 neurons after following reoxygenation. In addition, cytoplasmic DGKζ is never relocated to the nucleus after reoxygenation. These findings confirm that acute hippocampal slice system we employed here is suitable to simulate DGKζ cytoplasmic translocation observed in an animal model of transient ischemia (Ali et al. 2004). However, it should be noted that the cytoplasmic localization pattern of DGKζ is somewhat different in acute hippocampal slice model of OGD compared with animal model of ischemia. It is presumed to depend on the duration and severity of stress and energy deprivation.

How is DGKζ translocation implicated in neuronal cell death? A well-known histological marker for neuronal degeneration and cell death in hippocampal slices is cellular uptake of the fluorescent dye propidium iodide (PI), which enters cell nucleus after loss of cell membrane integrity (Macklis and Madison 1990). Other markers are staining of degenerating neurons with Fluoro-Jade B (Schmued and Hopkins 2000), release of cytosolic lactate dehydrogenase (LDH) (Noraberg et al. 1999; Ogura et al. 1994), and dendritic loss of immunostaining for microtubule-associated protein 2 (MAP2) (Kwei et al. 1993; Noraberg et al. 1999). However, significant loss of MAP2 staining is detected 6 h after OGD, and other markers can detect neuronal death well after 24 h after exposure to various toxic insults. They are inappropriate in this study to evaluate the correlation between neuronal degeneration and DGKζ translocation that occurs within an hour after OGD. In this regard, nuclear shrinkage is shown to be an early indicator of neuronal injury in hippocampal slices (Bonde et al. 2002). Our morphometric analysis reveals that DGKζ cytoplasmic translocation correlates with nuclear shrinkage, suggesting that DGKζ translocation is presumably an indicator of an early process of neuronal degeneration.

Glutamate excitotoxicity is well known to mediate neuronal cell death after ischemic injury, as explained above. In ischemic brain, overactivated NMDAR allows massive influx of Ca^{2+}, which results in uncontrolled, exaggerated activation of intracellular pathways culminating in neuronal cell death (Choi 1985, 1995; Lau and Tymianski 2010). The present pharmacological approach reveals that DGKζ cytoplasmic translocation is triggered by extracellular Ca^{2+} influx via NMDAR according to the findings that (1) NMDAR antagonist inhibits the translocation under OGD conditions; (2) NMDAR agonist induces the translocation under normal conditions; and (3) elimination of extracellular Ca^{2+} blocks translocation under OGD conditions. The reason that AMPAR antagonist DNQX alone inhibits the translocation under OGD conditions (Fig. 5b) might be that AMPAR mediates the fast excitatory component of glutamate. It is thought to relieve the Mg^{2+} block of NMDAR upon glutamate challenge (Burnashev et al. 1995; Hollmann et al. 1991). In this respect, it should be described that Ca^{2+} influx via other routes such as VDCC and Ca^{2+} ionophore fails to induce the translocation under normal conditions. These observations are compatible with the concept known as the 'source-specificity' hypothesis that Ca^{2+} toxicity is linked to the route of Ca^{2+} entry and the distinct second messenger pathways activated by Ca^{2+} entry (Arundine and Tymianski 2004; Tymianski et al. 1993). Some routes of Ca^{2+} entry, such as VDCC, did not elicit cell death, whereas others, such as NMDAR, were associated with significant Ca^{2+} dependent toxicity (Sattler et al. 1998).

How extracellular Ca^{2+} influx engenders DGKζ cytoplasmic translocation remains unclear. Ca^{2+} cytotoxicity is likely to be mediated through various pathways (Manev et al. 1989; Marcoux et al. 1990; Sattler et al. 1998; Tymianski et al. 1993). Major components activated by Ca^{2+} in neurons include CaMKII, CaN, and PKC. In this regard, we show that selective PKC inhibitor suppresses DGKζ cytoplasmic translocation in about one-third of the CA1 neurons under OGD conditions, suggesting that DGKζ cytoplasmic translocation is partially, if not all, regulated by PKC activity. One mechanism is suggested by a previous study: in cultured cells, phosphorylation of the myristoylated alanine-rich C-kinase substrates (MARCKS) domain that overlaps an NLS of DGKζ (Bunting et al. 1996; Goto and Kondo 1996) can affect its subcellular localization (Topham et al. 1998).

Another finding remains unexplained: disappearance of PKCγ-immunoreactivity from the plasma membrane in the course of OGD before DGKζ cytoplasmic translocation (Fig. 9). Considering that PKCγ is particularly susceptible to proteolytic degradation such as through massive activation of calpains by Ca^{2+}-overload after ischemia (Szydlowska and Tymianski 2010) and that irreversible inactivation of PKC always precedes neuronal damage (Aronowski et al. 2000; Wieloch et al. 1991), overactivation of PKCγ followed by its degradation might induce DGKζ cytoplasmic translocation through unknown mechanisms under OGD conditions.

The functional implication of DGKζ cytoplasmic translocation under ischemia/OGD conditions is expected to be closely related to perturbation of the homeostatic balance of DG metabolism in both the nucleus and the cytoplasm. As described, prominent changes in lipids are reportedly accompanied in the ischemic brain, where a rapid decrease in phosphatidylinositol-4,5-bisphosphate (PIP_2) levels together with a parallel accumulation of diacylglycerol (DG) is observed (Kunievsky et al. 1992). In addition, levels of PIP, PIP_2, and DG approach plateau values after 10 min of ischemia (Ikeda et al. 1986), a 30 min recirculation period after 15 min of ischemia engenders increases in PIP and PIP_2, whereas levels of DG decrease promptly toward control values (Moto et al. 1991; Yoshida et al. 1986). Detailed analysis of lipid changes in distinct subcellular compartments should help to determine how DGKζ cytoplasmic translocation is implicated in DG metabolism, although it might be difficult because of the rapid changes.

The pathophysiological role of DGKζ cytoplasmic translocation remains unclear. Does the translocation exert a protective or destructive effect under stressed conditions? In this regard, our present data on PKCγ might provide a clue to address the question. As described, PKCγ is particularly susceptible to proteolytic degradation after ischemia, and irreversible inactivation of PKC always precedes neuronal damage (Aronowski et al. 2000; Wieloch et al. 1991). Although a positive or negative role of PKCγ in neuroprotection after ischemic insults remains controversial at different stages of injury, PKCγ knockout mice demonstrate worsened injury after a transient ischemia, suggesting that PKCγ might mediate beneficial signaling processes during reperfusion injury (Bright and Mochly-Rosen 2005). Given that DGKζ cytoplasmic translocation correlates with nuclear shrinkage and that it is blocked by PKC inhibitor, presumably DGKζ translocation can be a protective stress response. In this respect, studies of transgenic mice show that cardiac-specific overexpression of DGKζ prevents pathological changes of cardiac muscle induced by Gq protein-coupled receptor agonist (Arimoto et al. 2006) and attenuates left ventricular remodeling after myocardial infarction (Niizeki et al. 2007) and pressure overload-induced cardiac hypertrophy (Harada et al. 2007) whereas these DGKζ-TG mice are indistinguishable from wild type mice in terms of appearance, physiological and histological analyses, and cardiac function under normal conditions.

In addition, pathophysiological implication of DGKζ disappearance from the nucleus should be discussed. Previous study shows that nuclear DGKζ is a negative regulator of cell cycle progression in C2C12 mouse myoblasts (Evangelisti et al. 2007). Furthermore, it is also demonstrated that nuclear DGKζ downregulates the expression of cyclin D through upregulation of BTG, a transcriptional regulator of cyclin D1 with a strong anti-proliferative function (Evangelisti et al. 2009). These studies suggest that downregulation of DGKζ would lead to cell cycle progression. In this regard, neurons are highly differentiated cells that normally never enter a cell cycle, and a number of studies suggests that cell cycle reentry of neurons usually causes cell death (Yang et al. 2003; Herrup and Yang 2007). Therefore it is plausible that DGKζ disappearance from the nucleus would promote cell cycle reentry in postmitotic neurons. Collectively, these findings suggest that DGKζ serves as a sentinel and exerts a beneficial effect on cellular pathophysiology by strictly controlling levels of DG produced in various stresses. Further studies are necessary to address the issues raised above.

In summary, we showed that DGKζ cytoplasmic translocation is well recapitulated in hippocampal slices exposed to OGD and revealed that the mechanism inducing DGKζ cytoplasmic translocation under OGD conditions is clearly compatible with that occurring in ischemic brain injury, i.e., NMDA-mediated Ca^{2+} influx, suggesting that DGKζ translocation is closely involved in glutamate excitotoxicity. The findings reported herein provide clues that will spur further examination and insight into the mechanism of glutamate excitotoxicity in hippocampal neurons.

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