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Activation of conventional and novel protein kinase C isozymes by different diacylglycerol molecular species

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ABSTRACT

A variety of diacylglycerol (DG) molecular species are produced in stimulated cells. Conventional (α, βII and γ) and novel (δ, ε, η and θ) protein kinase C (PKC) isoforms are known to be activated by DG. However, a comprehensive analysis has not been performed. In this study, we analyzed activation of the PKC isozymes in the presence of 2–2000 mmol% 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- or 18:0/22:6-DG species. PKCα activity was strongly increased by DG and exhibited less of a preference for 18:0/22:6-DG at 2 mmol%. PKCβII activity was moderately increased by DG and did not have significant preference for DG species. PKCγ activity was moderately increased by DG and exhibited a moderate preference for 18:0/22:6-DG at 2 mmol%. PKCδ activity was moderately increased by DG and exhibited a preference for 18:0/22:6-DG at 20 and 200 mmol%. PKCε activity moderately increased by DG and showed a moderate preference for 18:0/22:6-DG at 2000 mmol%. PKCη was not markedly activated by DG. PKCθ activity was the most strongly increased by DG and exhibited a preference for 18:0/22:6-DG at 2 and 20 mmol% DG. These results indicate that conventional and novel PKCs have different sensitivities and dependences on DG and a distinct preference for shorter and saturated fatty acid-containing and longer and polyunsaturated fatty acid-containing DG species, respectively. This differential regulation would be important for their physiological functions.

1. Introduction

Diacylglycerol (DG) serves to activate a variety of signaling proteins, including protein kinase C (PKC) [1–5]. PKC is involved in receptor desensitization, in modulating membrane structure events, in regulating transcription, in mediating immune responses, in regulating cell growth, and in learning and memory among many other functions. PKC is a family of closely related serine/threonine kinases, and at least ten different isoforms have been discovered to date. The isoforms can be split into three families according to their requirement for different co-factors: the conventional or classical (c) PKCs: α, βI/βII and γ; novel (n) PKCs: δ, ε, η and θ; and the atypical (a) PKCs: ζ and λ. cPKCs can be activated by Ca^{2+} and by DG. nPKCs can also be activated by DG. However, aPKCs are unresponsive to Ca^{2+} or DG.

DG kinase (DGK) phosphorylates DG to produce phosphatidic acid [6–9]. To date, 10 mammalian DGK isozymes have been identified, and these isozymes are subdivided into five groups according to their structural features. DGK isozymes are also involved in regulating cell growth [10–12] and angiogenesis [13], mediating immune responses [14–16], exacerbating the severity of type-2 diabetes [17–19] and controlling neuronal network formation [20–23]. Because DGK consumes an activator of PKC, DG, DGK regulates (attenuates) cPKC and nPKC activities. Indeed, many functional linkages between PKC isozymes and DGK isozymes have been reported [6–9].

Mammalian cells contain at least 50 structurally distinct DG molecular species, which are supplied from a variety of lipid metabolic pathways, such as phosphatidylinositol turnover [24], phosphatidylcholine (PC) hydrolysis by PC-specific phospholipase C [25,26] and PC hydrolysis by phospholipase D followed by dephosphorylation by phosphatidic acid phosphatase [27] in a cell stimulation-dependent manner. The DG species generated through phosphatidylinositol turnover mainly consists of 18:0/20:4-DG. The DG species derived from PC predominantly contain saturated and monounsaturated fatty acids.

DGKε has preference for 18:0/20:4-DG *in vitro* [28,29]. Our recent studies reported that several isozymes of DGK selectively metabolize different DG species within the cell. For example, DGKα was suggested to phosphorylate 18:0/20:4-DG and 18:0/22:6-DG in T-lymphocytes and COS-7 cells [30,31]. DGKδ utilized PC-derived 14:0/16:0-, 14:0/16:1-, 16:0/16:0-, 16:0/16:1-, 16:0/

Abbreviations: cPKC, conventional protein kinase C; DG, diacylglycerol; DGK, diacylglycerol kinase; MBP, myelin basic protein fragment 4-14; nPKC, novel protein kinase C; PKC, protein kinase C; TPA, 12-O-Tetradecanoylphorbol-13-acetate

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18:0-, and 16:0/18:1-DG in C2C12 myoblasts [19]. Based on these results, the possibility that each DGK isozymes metabolizes limited DG species and regulates the activity of restricted PKC isozymes has been suggested. However, a comprehensive analysis of the DG dependency of all cPKC and nPKC isozymes has not been performed. It was reported that the *in vitro* activation of different PKC isozymes varies in response to different DG species [32]. However, in this report, only PKCα, βI, γ, δ and ε were analyzed, and only 8:0/8:0-, which is not a natural product, 18:0/20:4-, 18:0/20:5- and 18:0/22:6-DG (50–1000 mmol%) were used.

Therefore, in this study, we more comprehensively re-evaluated the effects of 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- or 18:0/22:6-DG species in concentrations ranging from 2 to 2000 mmol% on all cPKC and nPKC isozymes side by side under the same conditions and aimed to obtain fundamental knowledge to explore the relationship between PKC isozyme activation and DG-related enzymes, including DGK isozymes. The obtained results indicate that, beyond our expectations, the modes of activation of cPKC and nPKC isozymes by DG molecular species varied considerably.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-*sn*-glycerol (16:0/16:0-DG), 1-palmitoyl-2-oleoyl-*sn*-glycerol (16:0/18:1-DG), 1,2-dioleoyl-*sn*-glycerol (18:1/18:1-DG), 1-stearoyl-2-docosahexaenoyl-*sn*-glycerol (18:0/22:6-DG) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL).1-stearoyl-2-arachidonoyl-*sn*-glycerol (18:0/20:4-DG) and the PKC substrate, the [pGlu^4]-Myelin basic protein fragment 4-14 (MBP) was obtained from Sigma-Aldrich (St. Louis, MO). PKC isoforms (α (product number: 01-133), βII (01-165), γ (01-137), δ (01-135), ε (01-136), η (01-138) and θ (01-140)) were obtained from Carna Biosciences (Kobe, Japan). These PKC isoforms were expressed as N-terminal Glutathione S-transferase-fusion proteins using baculovirus expression system and were highly purified by using glutathione Sepharose chromatography. P81 phosphocellulose squares were obtained from Merck Millipore (Darmstadt, Germany).

2.2. PKC activity assay

PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P] ATP into [pGlu^4]-Myelin basic protein fragment 4-14 (pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu). The reaction mixture (12.5 μl) contained 24 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 0.2 mM $CaCl_2$ (for α, βII, and γ) or 0.5 mM EDTA (for δ, ε, η and θ), 56 mg/ml MBP, 0.05 μg of PKC, 2 mol% PS, and 2–2000 mmol% DG. PS and DG were first mixed in chloroform/methanol and then dried under nitrogen. The residue was then sonicated in a buffer solution containing 255 mM Triton X-100 and 10 mM Tris–HCl to prepare lipid vesicles. The reaction was started by the addition of 0.1 mM [γ-^{32}P] ATP (~300 cpm/pmol), continued for 10 min at 30 °C and stopped by spotting 10 μl of samples on 0.5 × 0.5 cm squares of Whatman P81 phosphocellulose paper. The papers were dried, washed four times for 15 min each time with 1% H_3PO_4, and transferred to a scintillation counter to determine the radioactivity of [^{32}P] MBP.

2.3. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. The cells were transfected with pEGFP-N3-PKCη by electroporation (1×10^6 cells/2 mm gap cuvette, 110 V, 20.0 ms pulse length, one pulse) with the Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Tokyo, Japan), according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were harvested and suspended in 500 μl of ice-cold lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and cOmplete™ EDTA-free protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan)) and then sonicated. The mixtures were centrifuged at 10,000 × *g* for 5 min at 4 °C to yield cell lysates. EGFP-PKCη expression was confirmed by Western blotting using an anti-GFP antibody (sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA).

2.4. Statistical analysis

Statistical comparisons were performed using one-way ANOVA followed by Tukey's *post hoc* test.

3. Results

3.1. Effects of different DG species on PKCα activation

We measured the activation of highly purified cPKC and nPKC isozymes in the presence of 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- or 18:0/22:6-DG species in concentrations ranging from 2 to 2000 mmol%. The reason in choosing the concentration range of DG in the study is that DG produced by cell stimulation is approximately 20 mmol% [33]. We analyzed (1) the sensitivity of cPKC and nPKC isozymes to DG (DG concentration for PKC activation), (2) their dependence on DG (fold increase of PKC activity by DG), and (3) preference for DG species.

We first examined effects of different DG species on the activity of a cPKC, PKCα. PKCα activity was increased approximately 2-fold in the presence of 2 mmol% 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4-DG (Fig. 1). However, 2 mmol% 18:0/22:6-DG did not significantly activate PKCα. PKCα activity was increased by DG in a dose dependent manner and was increased approximately 2.5-, 3.5- and 7-fold by 20, 200 and 2000 mmol% DG, respectively. However, PKCα did not exhibit preference for DG species at 20–2000 mmol%. Taken together, these results indicate that PKCα has

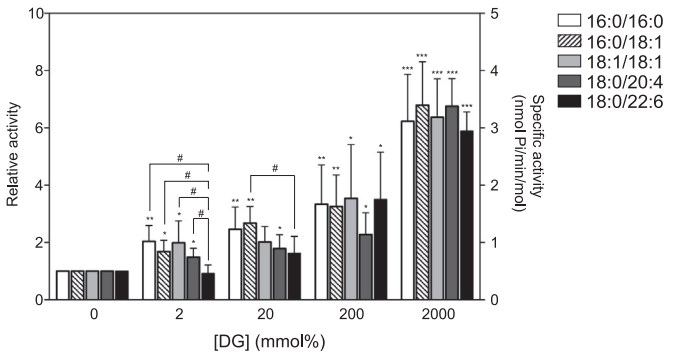

Fig. 1. Effects of different DG molecular species on PKCα activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs, as described in Section 2.The results are the means ± SD of four independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG) and the right axis shows the specific activity. The data are significantly different from the control, 0 mmol% DG ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$), and among the DG molecular species ($^{\#}P < 0.05$).

Table 1
Summary of the effects of different DG molecular species on the activity of cPKC and nPKC isozymes.

		[DG] (mmol%)			
		2	20	200	2000
PKCα	Fold increase	+	+	++	+++
	Preference	DP > SD	–	–	–
PKCβII	Fold increase	+	+	+	++
	Preference	–	–	–	–
PKCγ	Fold increase	+	+	+	++
	Preference	DP < SD	–	–	–
PKCδ	Fold increase	–	+	++	++
	Preference	–	DP < SD	DP < SD	–
PKCε	Fold increase	–	+	+	++
	Preference	–	–	–	DP < SD
PKCη	Fold increase	–	–	–	–
	Preference	–	–	–	–
PKCη expressed in COS-7 cells	Fold increase	–	+	+	+
	Preference	–	DP < SD	–	–
PKCθ	Fold increase	++	+++	++++	++++
	Preference	DP < SD	–	–	–

Fold increase: –, less than 1.5-fold; +, more than 1.5-fold; ++, more than 3-fold; +++, more than 6-fold; ++++, more than 12-fold.
Preference: different preference for 16:0/16:0-DG (1,2-dipalmitoyl-*sn*-glycerol (DP)) and 18:0/22:6-DG (1-stearyl-2-docosahexanoyl-*sn*-glycerol (SD)).

(1) a high sensitivity to DG, (2) a strong dependence on DG and (3) less of a preference for 18:0/22:6-DG at low DG concentrations (Table 1).

3.2. Effects of different DG species on PKCβII activation

Differential splicing gives rise to the two forms of PKCβ, βI and βII, which only differ in their extreme C-terminal ends [34]. We next tested the effects of different DG species on the activity of a cPKC, PKCβII. PKCβII activity was increased approximately 1.5-fold by 2 mmol% DG, and its activity was increased approximately 1.5-, 2- and 4-fold by 20, 200 and 2000 mmol% DG, respectively (Fig. 2). However, PKCβII did not show significant preference for either 18:0/22:6-DG or 16:0/16:0-DG at 2–2000 mmol%. Taken together, these results indicate that PKCβII has (1) a high sensitivity to DG, (2) a moderate dependence on DG and (3) no significant preference for DG molecular species (Table 1).

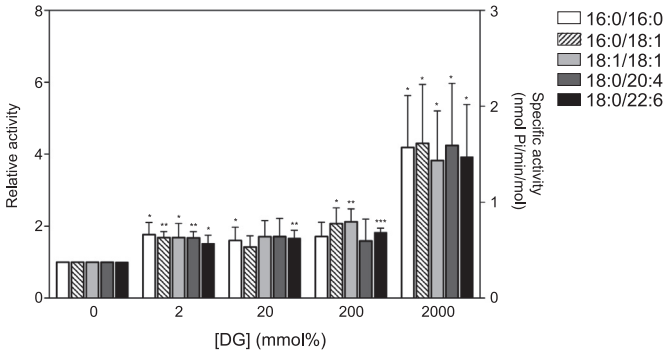

Fig. 2. Effects of different DG molecular species on PKCβII activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs, as described in Section 2. The results are the means ± SD of three independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG) and the right axis shows the specific activity. The data are significantly different from the control, 0 mmol% DG ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

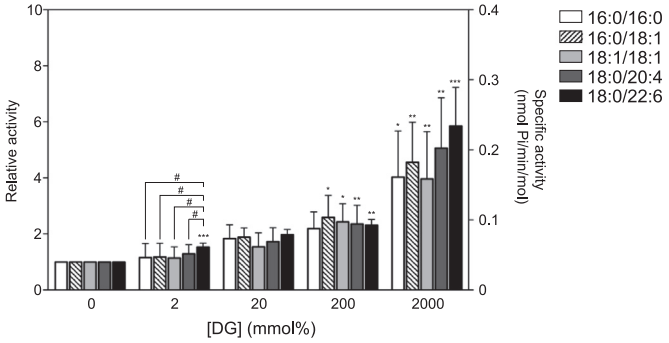

Fig. 3. Effects of different DG molecular species on PKCγ activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs, as described in Section 2. The results are the means ± SD of five independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG) and the right axis shows the specific activity. The data are significantly different from the control, 0 mmol% DG ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$), and among the DG molecular species ($^{\#}P < 0.05$).

3.3. Effects of different DG species on PKCγ activation

PKCγ (cPKC) activity was increased approximately 1.5-fold in the presence of 2 mmol% 18:0/22:6-DG (Fig. 3). However, 2 mmol% 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, and 18:0/20:4-DG failed to significantly activate PKCγ. PKCγ activity was increased approximately 2-, 2.5- and 6-fold by 20, 200 and 2000 mmol% DG, respectively. However, PKCγ did not exhibit a significant preference for DG species at 20–2000 mmol%. These results indicate that PKCγ has (1) a high sensitivity to DG, (2) a moderate dependence on DG and, (3) in contrast to PKCα, a moderate preference for 18:0/22:6-DG at low DG concentrations (Table 1).

3.4. Effects of different DG species on activation of PKCδ

We next tested the effects of different DG species on the activity of an nPKC, PKCδ. PKCδ was not significantly activated by 2 mmol% DG (Fig. 4). However, the activity of this isozyme was increased approximately 2.5-fold by 20 mmol% DG, and its activity was increased approximately 5- and 5.5-fold by 200 and 2000 mmol% DG, respectively. 16:0/18:1-, 18:0/20:4- and 18:0/22:6-DG strongly activated PKCδ compared to 16:0/16:0-DG at

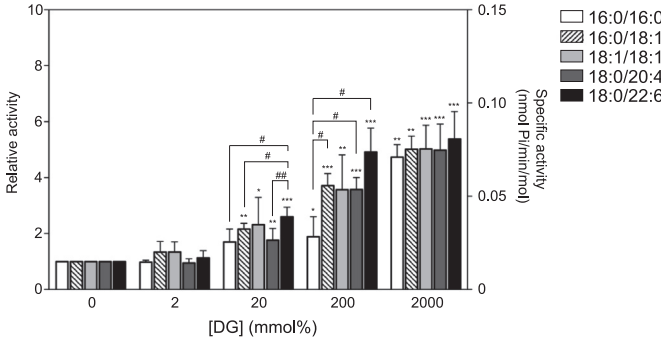

Fig. 4. Effects of different DG molecular species on PKCδ activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs, as described in Section 2. The results are the means ± SD of four independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG) and the right axis shows the specific activity. The data are significantly different from the control, 0 mmol% DG ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$), and among the DG molecular species ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

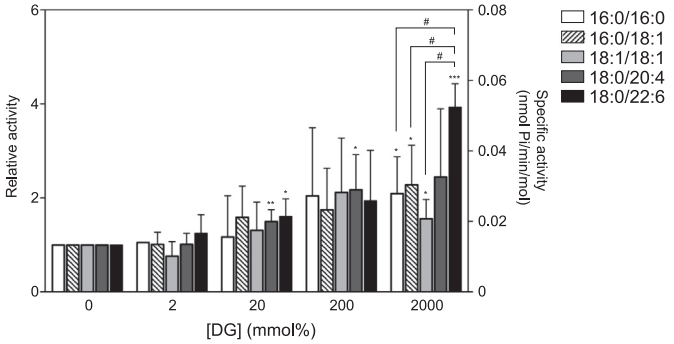

Fig. 5. Effects of different DG molecular species on PKCε activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs, as described in Section 2. The results are the means ± SD of four independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG) and the right axis shows the specific activity. The data are significantly different from the control, 0 mmol% DG ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$), and among the DG molecular species ($^{\#}P < 0.05$).

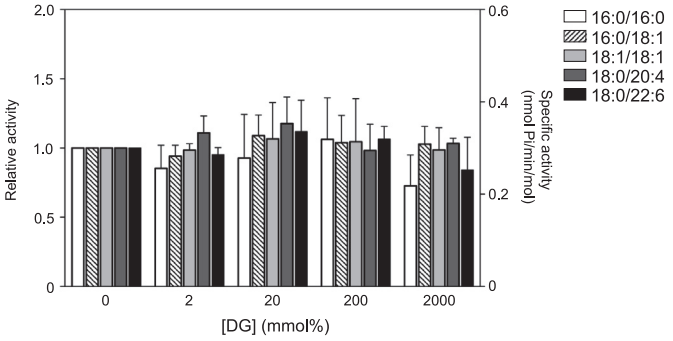

Fig. 6. Effects of different DG molecular species on PKCη activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs, as described in Section 2. The results are the means ± SD of four independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG) and the right axis shows the specific activity.

200 mmol%. At 20 mmol%, the activity of this isozyme was more strongly enhanced by 18:0/22:6-DG. However, PKCδ did not exhibit a significant difference in the degree of activation by DG species at 2000 mmol%. These results indicate that PKCδ has (1) a moderate sensitivity to DG, (2) a moderate dependence on DG, (3) a preference for 18:0/22:6-DG, and less of a preference for 16:0/16:0-DG at medium concentrations (Table 1).

3.5. Effects of different DG species on PKCε activation

PKCε (nPKC) was not significantly activated by 2 mmol% DG (Fig. 5). However, the activity of this isoform was increased approximately 1.5-fold by 20 mmol% DG and was increased approximately 2- and 4-fold by 200 and 2000 mmol% DG, respectively. Although PKCε did not exhibit a significant preference for DG species at 20 and 200 mmol%, this isozyme showed a preference for 18:0/22:6-DG at 2000 mmol%. These results indicate that PKCε has (1) a moderate sensitivity to DG, (2) a moderate dependence on DG and (3) a preference for 18:0/22:6-DG at high concentrations (Table 1).

3.6. Effects of different DG species on PKCη activation

PKCη (nPKC) was not markedly activated by any DG molecular species at any concentrations (Fig. 6). 12-O-Tetradecanoylphorbol-13-acetate (TPA), which mimics DG, is known to strongly activate cPKC and nPKC isozymes [35]. Although the effect of TPA on the activity of PKCη was examined, TPA also did not significantly activate this enzyme (Suppl. Fig. 1).

Given that PKCη expressed in insect cells is not responsive to DG, we next determined the effects of DG species on this isozyme expressed in mammalian cells (COS-7 cells) instead of insect cells. PKCη activation by DG species (approximately 1.5-fold increase) was detected at 20 mmol% (Fig. 7). This isozyme showed a weak preference for 18:0/22:6-DG at 20 mmol%. The activity of this isoform was increased approximately 1.5- and 2.5-fold by 200 and 2000 mmol% DG, respectively. However, PKCη did not show a significant preference for DG molecular species at 200 and 2000 mmol%.

These results indicate that PKCη expressed in insect cells has (1) no sensitivity to DG, (2) no dependence on DG and (3) no preference to DG species, even at high concentrations (Table 1). On the other hand, PKCη expressed in mammalian cells has (1) a moderate sensitivity to DG, (2) a weak dependence on DG and

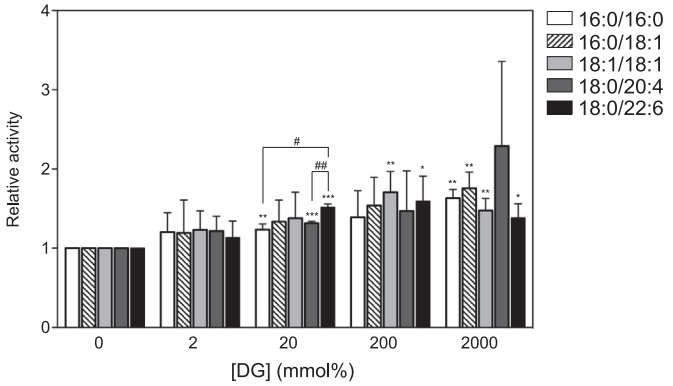

Fig. 7. Effects of different DG molecular species on the activation of PKCη expressed in COS-7 cells. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs, as described in Section 2. The activities of vector-transfected cells were subtracted. The results are the means ± SD of three independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG). The data are significantly different from the control, 0 mmol% DG ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$), and among the DG molecular species ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

(3) a weak preference for 18:0/22:6-DG at medium concentrations (Table 1).

3.7. Effects of different DG species on activation of PKCθ

Finally, we tested the effects of different DG species on the activity of an nPKC, PKCθ. PKCθ activity was increased approximately 4-fold by 18:0/22:6-DG at 2 mmol% DG, and its activity was increased approximately 8-, 21- and 25-fold by all DG species at 20, 200 and 2000 mmol% DG, respectively (Fig. 8). PKCθ was more strongly activated by 16:0/18:1-DG, 18:0/20:4- and 18:0/22:6-DG than by 16:0/16:0-DG and by 18:0/22:6-DG than by 16:0/18:1-, 18:1/18:1 and 18:0/20:4-DG at 2 and 20 mmol% DG, respectively. However, PKCθ did not exhibit a significant preference for DG species at 200 and 2000 mmol%. These results indicate that PKCθ has (1) a high sensitivity to DG, (2) a very strong dependence on DG and (3) a preference for 18:0/22:6-DG at relatively low DG concentrations (Table 1).

Overall, these results indicate that cPKC and nPKC isozymes have different dependencies/sensitivities to DG and distinct preferences for shorter and saturated fatty acid-containing DG species

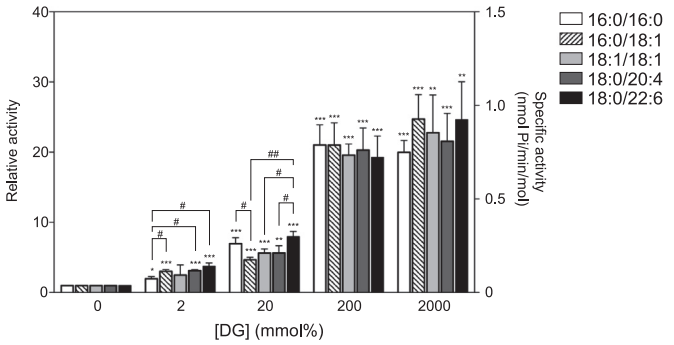

Fig. 8. Effects of different DG molecular species on PKCθ activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of DGs as described in Section 2. The results are the means ± SD of three independent experiments. The left axis shows the relative activity compared to the control (0 mmol% DG) and the right axis shows the specific activity. The data are significantly different from the control, 0 mmol% DG ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$), and among the DG molecular species ($^{\#}P<0.05$, $^{\#\#}P<0.01$).

(16:0/16:0-DG) and longer and polyunsaturated fatty acid-containing DG species (18:0/22:6-DG), respectively.

4. Discussion

A variety of DG molecular species are produced in stimulated cells. cPKC (α, βII and γ) and nPKC (δ, ε, η and θ) isozymes are known to be activated by DG [1–5]. However, a comprehensive analysis of the DG dependency of these PKCs has not been performed. Therefore, we performed comprehensive comparison of the effects of DG molecular species (2–2000 mmol% 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- or 18:0/22:6-DG species) on the activities of these PKCs side by side under the same conditions. We found that beyond our expectation, the modes of activation of cPKC and nPKC isozymes by DG molecular species varied considerably.

We analyzed (1) the sensitivity to DG (DG concentration for PKC activation), (2) dependence on DG (fold increase in PKC activity in the presence of DG), and (3) preference for DG molecular species of highly purified cPKC and nPKC isozymes expressed in the baculovirus-insect cell expression system. (1) Regarding sensitivity to DG, the highly sensitive isozymes are PKCα, PKCβII, PKCγ and PKCθ (Table 1). (2) PKCθ was the most strongly activated and PKCα was strongly activated (Table 1). The activities of cPKC and nPKC isozymes in the presence of DG species were comparable to those in the presence of TPA (Suppl. Fig. 1). Therefore, the activities at 2000 mmol% DG are roughly maximal values. (3) PKCα showed less preference to long/unsaturated fatty acid-containing DG species (18:0/22:6-DG) (Table 1). PKCγ, On the other hand, PKCδ, PKCε and PKCθ showed a preference for long/unsaturated fatty acid-containing DG species (18:0/22:6-DG) (Table 1). Moreover, these preferences appear at different DG concentrations. The activation capacity of saturated and polyunsaturated fatty acid-containing DG molecular species is suggested to rely on the fluidity state of the micelles and membranes [36]. Taken together, these results indicate that c/nPKCs have different sensitivities and dependences on DG and a distinct preference for shorter and saturated fatty acid-containing and longer and polyunsaturated fatty acid-containing DG species, respectively (Table 1).

PKCη was not activated by 2–2000 mmol% DG in our assay conditions (Fig. 6). In addition, this isozyme was not activated by TPA (data not shown). Because PKCη showed relatively high specific activity in the absence of DG compared with the other isozymes (Fig. 6), this isoform may be already activated in the absence of DG/TPA. Unlike the insect cell-expressed enzyme, the mammalian cell-expressed PKCη was activated by DG (Fig. 7). Essentially the same results (activated at 2 mmol% DG and approximately 6-fold activated at 2000 mmol% DG) were obtained using PKCα expressed in mammalian cells (data not shown) compared to the insect cell-expressed enzyme (Fig. 1). The results suggest that in the case of PKCη, some modification in mammalian COS-7 cells, but not in insect cells, is needed for the enzyme to exhibit DG-dependent activation.

It has been claimed that DGs with saturated chains were less effective on triggering PKC activation than those bearing polyunsaturated chains [36–41]. Moreover, Madani et al. [32] reported that the *in vitro* activation of different PKC isozymes varies in response to different DG species. However, in this report, only PKCα, βI, γ, δ and ε were analyzed. Moreover, only 8:0/8:0-, which is not a natural product, 18:0/20:4-, 18:0/20:5- and 18:0/22:6-DG were used. Furthermore, approximately 50–1000 mmol% of DG species were tested. In the present study, we more comprehensively analyzed the effects of a wide range of physiological DG molecular species and concentrations (2–2000 mmol% 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- or 18:0/22:6-DG species) on the activities of cPKC (α, βII and γ) and nPKC (δ, ε, η and θ) isozymes (Table 1) side by side under the same conditions, and identified several additional intriguing differences, as described above. For example, we showed, for the first time, that PKCα showed less of a preference for polyunsaturated fatty acid-containing DG species (18:0/22:6-DG) at 2 mmol% and that PKCθ has a higher sensitivity and stronger dependence on DG than the other isozymes.

The results obtained in the present study allow us to speculate a correlation between the PKC isozymes and DGK isozymes. DGKε has preference for polyunsaturated fatty acid-containing DG such as 18:0/20:4-DG [28,29], suggesting possible linkages with PKCγ, δ, ε and θ, which have preferences for polyunsaturated fatty acid-containing DG species. DGKα was shown to phosphorylate 18:0/20:4-DG and 18:0/22:6-DG in cells [30,31]. Therefore, DGKα may be associated with PKCγ, δ, ε and θ. DGKα is predominantly expressed in T-lymphocytes [42] and regulates T-cell activation [15,16]. Of PKCγ, δ, ε and θ, PKCθ is predominantly expressed in T-lymphocytes and is a key molecule in T-cell activation and differentiation [43], implying an intimate linkage between DGKα and PKCθ. However, further studies are required to definitely indicate the relation between DGKs and PKCs.

DGKδ contributes to hyperglycemia-induced peripheral insulin resistance [17] and regulates the activities of PKCα [18] and PKCδ [17] in myotubes. Palmitic acid (16:0)-containing DG species are known to accumulate in skeletal muscle of type 2 diabetes patients [44]. We showed that DGKδ indeed utilized PC-derived, palmitic acid-containing DG species (14:0/16:0-, 16:0/16:0-, 16:0/16:1-, 16:0/18:0-, and 16:0/18:1-DG) [19]. The results are not inconsistent with the DG species preferences of PKCα and PKCδ (Figs. 1 and 4 and Table 1).

In the present study, we comprehensively analyzed the effects of different DG species on cPKC and nPKC isozymes side by side under the same conditions and revealed that these PKCs have different dependencies/sensitivities to DG and distinct preferences for shorter and saturated fatty acid- and longer and polyunsaturated fatty acid-containing DG molecular species, respectively. This differential regulation would be important for their physiological functions. The comprehensive *in vitro* results obtained in the present study could be a useful guide for interpreting the activation and functional significance of cPKC and nPKC isozymes *in vivo*.

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Appendix A. Transparency document

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