Distinct 1-monoacylglycerol and 2-monoacylglycerol kinase activities of diacylglycerol kinase isozymes

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ABSTRACT

Diacylglycerol kinase (DGK) consists of ten isozymes and is involved in a wide variety of patho-physiological events. However, the enzymological properties of DGKs have not been fully understood. In this study, we performed a comprehensive analysis on the 1-monoacylglycerol kinase (MGK) and 2-MGK activities of ten DGK isozymes. We revealed that type I (α , β and γ), type II (δ , η and κ) and type III (ϵ) DGKs have 7.9–19.2% 2-MGK activity compared to their DGK activities, whereas their 1-MGK activities were less than 3.0%. Both the 1-MGK and 2-MGK activities of the type IV DGKs (ζ and ι) were less than 1% relative to their DGK activities. Intriguingly, type V DGK θ has approximately 6% 1-MGK activity and less than 2% 2-MGK activity compared to its DGK activity. Purified DGK θ exhibited the same results, indicating that its 1-MGK activity is intrinsic. Therefore, DGK isozymes are categorized into three types with respect to their 1-MGK and 2-MGK activities: those having (1) 2-MGK activity relatively stronger than their 1-MGK activity (types I–III), (2) only negligible 1-MGK and 2-MGK activities (type IV), and (3) 1-MGK activity stronger than its 2-MGK activity (type V). The 1-MGK activity of DGK θ and the 2-MGK activity of DGK α were stronger than those of the acylglycerol kinase reported as 1-MGK and 2-MGK to date. The presence or absence of 1-MGK and 2-MGK activities may be essential to the patho-physiological functions of each DGK isozyme.

Keywords

Diacylglycerol kinase; Monoacylglycerol kinase; Lysophosphatidic acid; Phosphatidic acid

Footnote**Abbreviations**

AGK, acylglycerol kinase; DG, diacylglycerol; DGK, diacylglycerol kinase; LPA, lysophosphatidic acid; MG, monoacylglycerol; MGK, monoacylglycerol kinase; PA, phosphatidic acid; PS, phosphatidylserine

1. Introduction

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) in biomembranes to produce phosphatidic acid (PA) [1-6]. To date, ten mammalian DGK isozymes ($\alpha, \beta, \gamma, \delta, \epsilon, \zeta, \eta, \theta, \iota$ and κ) have been identified. These DGK isozymes are divided into five groups (type I: α, β and γ ; type II: δ, η and κ ; type III: ϵ ; type IV: ζ and ι ; type V: θ) according to their structural features [1-6]. DGK isozymes are known to be involved in a wide variety of patho-physiological functions. For example, DGK α (type I) induces clonal anergy [7, 8]. On the other hand, this isozyme also prevents melanoma apoptosis [9] and promotes hepatocellular carcinoma proliferation [10], 3D cancer cell growth [11] and angiogenesis [12]. DGK β (type I) is an important regulator in neurite spine formation [13]. DGK γ (type I) serves as an upstream suppressor of Rac1 and lamellipodium formation [14]. This isozyme regulates allergic reactions [15] and insulin secretion [16]. In addition to epidermal growth factor signaling [17], DGK δ (type II) is an important factor in hyperglycemia-induced peripheral insulin resistance, thereby exacerbating the severity of type-2 diabetes [18, 19]. DGK η (type II) enhances C-Raf activity and B-Raf/C-Raf heterodimerization in cancer cells [20]. It was also reported that the gene encoding DGK η is implicated in the etiology of bipolar disorder [21]. DGK κ (type II) is associated with risk of hypospadias [22]. DGK ϵ (type III) controls seizure susceptibility and long-term potentiation through modulating arachidonoyl-inositol lipid signaling [23]. DGK ζ (type IV) is an important regulator of dendritic spine maintenance [24]. This isozyme is also known to regulate endothelin-1-induced cardiomyocyte hypertrophy [25]. DGK ι (type IV) regulates Ras guanyl-releasing protein 3 and inhibits Rap1 signaling [26] and presynaptic release during metabotropic glutamate receptor-dependent long-term depression [27]. DGK θ (type V) has been implicated in familial Parkinson disease [28] and bile acid signaling [29]. Although DGKs have been established as important biomembrane-related modulators as described above, their enzymological properties have not been fully

elucidated.

30 years ago, Kanoh *et al.* purified DGK α from porcine liver and demonstrated that the enzyme had 10–20% 2-monoacylglycerol (MG) kinase (MGK) activity compared with its DGK activity [30]. However, its 1-MGK activity was less than 4% relative to its DGK activity. Gantayat *et al.* reported that DGK ϵ also exhibited 6.4% 2-MGK activity compared with its DGK activity [31]. However, because a comprehensive analysis has not been done, the MGK activities of the other isozymes are presently unknown.

In this study, we measured the 1-MGK and 2-MGK activities of all ten DGK isozymes. We revealed that the type I DGKs (α , β and γ), type II DGKs (δ , η and κ) and type III DGK (ϵ) have 8–19% 2-MGK activity compared to their DGK activities, whereas their 1-MGK activities were less than 3%. Both the 2-MGK and 1-MGK activities of the type IV DGKs (ζ and ι) were less than 1% relative to their DGK activity. Interestingly, the type V DGK θ has approximately 6% 1-MGK activity and less than 2% 2-MGK activity compared to its DGK activity. These results indicate that the ten DGK isozymes have enzymological diversity beyond our expectation.

2. Materials and Methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycerol, 2-arachidonoyl glycerol, 1,2-dioleoyl-*sn*-glycero-3-phosphate and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Stearoyl-2-arachidonoyl-*sn*-glycerol, 1-oleoyl-*rac*-glycerol and 2-oleoylglycerol were obtained from Sigma-Aldrich (Tokyo, Japan). 1-Oleoyl lysophosphatidic acid was obtained from Cayman Chemical (Ann Arbor, MI).

2.2. cDNA constructs

The expression plasmids, p3xFLAG-CMV-pig DGK α [14], -rat DGK β [14], -human DGK γ [14], -human DGK δ [32, 33], -human DGK η [34], -human DGK κ [35], -human DGK ϵ [36], -human DGK ζ [36], -human DGK ι [36], and -human DGK θ [36] were generated as described previously. Acylglycerol kinase (AGK) cDNA was amplified by PCR from mouse muscle cDNA using the AGK forward primer: GGGAATTCGATGACTGCATTCTTTA, and the reverse primer: CCGTCGACTCACTGGGATGTGCTC. To express 6xHis-tagged protein, human DGK θ and mouse AGK cDNAs were cloned into the pSF-CMV-NH2-His-EKT3 vector (Oxford Genetics, Begbroke, UK). The amplified cDNAs were verified by sequencing carried out by Eurofins Genomics (Tokyo, Japan).

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 (Corning, Corning, NY) at 37 °C in an atmosphere containing 5% CO₂. The cells were transfected with p3xFLAG-CMV-DGK isozymes by electroporation (1 x10⁶ 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. After transfection for 48 h, the harvested cells were suspended in 300 μ l ice-cold lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM MgCl₂, 1mM dithiothreitol, cOmplete™ EDTA-free protease inhibitor (Roche Diagnostics, Tokyo, Japan)) and then sonicated. The mixtures were centrifuged at 1000 g for 10 min at 4 °C to yield the cell lysates. The expression of the DGK isozymes and AGK was confirmed by Western blotting using anti-FLAG monoclonal antibody (Sigma-Aldrich, Tokyo, Japan) and anti-DGK θ monoclonal antibody (BD biosciences, San Jose, CA).

2.4. DGK, 1-MGK and 2-MGK activity assays

The octylglucoside mixed micellar assay of kinase activity was performed as described previously [37]. In brief, the assay mixture (50 μ l) contained 50 mM MOPS (pH 7.2), 50 mM *n*-octyl- β -D-glucoside, 1 mM dithiothreitol, 20 mM NaF, 10 mM MgCl₂, 27 mol% PS, 5.4 mol% 1,2-dioleoyl-*sn*-glycerol or 1-oleoyl-*rac*-glycerol or 2-oleoylglycerol and 1 mM [γ -³²P]ATP (approximately 100,000 cpm/nmol). When the activities of the Ca²⁺-dependent isozymes, DGK α , β and γ , were measured, 1 μ M CaCl₂ was added. When the activity of the arachidonoyl DG-selective isozyme, DGK ϵ , was assessed, 1-stearoyl-2-arachidonoyl-*sn*-glycerol and 2-arachidonoyl glycerol were used as the substrate in place of 1,2-dioleoyl-*sn*-glycerol and 2-oleoylglycerol. The reaction was initiated by adding the cell lysates (5 μ g protein), and continued for 30 min at 30 °C. Lipids were extracted from the mixture, and PA and lysoPA (LPA) separated by thin layer chromatography were scraped and counted by a liquid scintillation spectrophotometer.

Suppl. Fig. 1 shows that the expression levels of the DGK isozymes were comparable to each other. Suppl. Fig. 2 exhibits the DGK, 1-MGK and 2-MGK activities of the DGK isoforms that were able to be measured above the vector control, indicating

that they could be quantitatively analyzed. We confirmed that the assays were linear with respect to protein concentration (0 – 5 µg/50 µl) and time (0 – 30 min).

2.5. MGK activity assay in the presence of deoxycholate

The kinase activity assay without octylglucoside was performed as described previously [38]. Lipids (50 nmol) were dried under N₂ and resuspended in 30 µl of buffer containing 100 mM MOPS, pH 7.2, 2 mM EGTA, 15 mM NaF, 2 mM orthovanadate, 50 mM NaCl, 250 mM sucrose, 0.03% deoxycholate, and 1:5 diluted cOmplete™ EDTA-free protease inhibitor. After a brief sonication, 10 µl lysates (5 µg) and 10 µl [γ -³²P]ATP (100,000 cpm/nmol, 1 mM) containing MgCl₂ (10 mM) were added, and the reactions were performed for 30 min at 30 °C. Lipids were extracted from the mixture, and the LPA separated by thin layer chromatography was scraped and counted by a liquid scintillation spectrophotometer.

2.6. Purification of 6xhistidine-tagged DGK θ

200 µl Ni Sepharose 6 Fast Flow (GE Healthcare, Pittsburgh, PA) was equilibrated with 10 ml binding buffer containing 5 mM imidazole. The cell lysate (2 mg protein) was loaded onto the column, and the column was washed with 10 ml wash buffer containing 10 mM imidazole to remove unbound proteins. Finally, the binding protein was eluted with 800 µl of elution buffer containing 50 mM imidazole.

2.7. Western blot analysis

COS-7 cell lysates expressing the 3xFLAG-tagged proteins were separated using SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Tokyo, Japan) and blocked with Block Ace (Dainippon Pharmaceutical, Tokyo, Japan). The membrane was incubated with an

anti-FLAG antibody (Sigma-Aldrich) in 10% Block Ace for 1 h. The immunoreactive bands were then visualized using a peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) and the Enhanced Chemiluminescence Western Blotting Detection System (GE Healthcare).

3. Results

3.1. 1-MGK and 2-MGK activities of type I DGKs (α , β and γ)

We first examined the 1-MGK and 2-MGK activities of a type I isozyme, DGK α , expressed in COS-7 cells. We confirmed that the DGK activity of DGK α in the COS-7 cell lysates increased linearly in a time-dependent manner over 30 min and with the increasing protein concentration (0–5 μ g/50 μ l of reaction mixture) [36]. As shown in Fig. 1A and Table 1, DGK α exhibited $12.0 \pm 0.5\%$ ($n = 3$) 2-MGK activity compared to its DGK activity, whereas its 1-MGK activity was $0.6 \pm 0.0\%$. These results are essentially the same as those obtained with purified DGK α in a previous report (10–20% of 2-MGK and 1–4% of 1-MGK activities compared to DGK activity) [30]. Therefore, it is suggested that COS-7 cell lysates do not contain substantial DGK/1-MGK/2-MGK inhibitors or enhancers.

To confirm whether the 2-MGK activity of DGK α is intrinsic, we expressed 6xHis-tagged DGK α in COS-7 cells and purified it using Ni-affinity chromatography (Suppl. Fig. 3A). The purified DGK α also showed 12.0% 2-MGK activity compared to its DGK activity, and its relative 1-MGK activity was 0.4% (Suppl. Fig. 3B). These results indicate that DGK α has intrinsic 2-MGK activity in addition to DGK activity.

We next compared the affinities of DGK α for DG and 2-MG. The activity of DGK α was increased in a DG or 2-MG dose-dependent manner (Suppl. Fig. 4A). A double reciprocal plot gave apparent K_m values for DG and 2-MG of 1.4 and 11.0 mol%, respectively (Suppl. Fig. 4B). The apparent K_m value for 2-MG was 7.7-fold higher than that of DG. The K_m value for DG (1.4 mol%) is almost the same as that (2.1 mol%) of purified DGK α [39], further suggesting that the COS-7 lysates do not significantly disturb the DGK activity.

The second type I isozyme, DGK β , showed $16.8 \pm 0.3\%$ and $1.4 \pm 0.3\%$ ($n = 3$) 2-MGK and 1-MGK activities, respectively, compared to its DGK activity (Fig. 1B and Table 1). Likewise, the third type I isozyme, DGK γ , exhibited $19.2 \pm 6.3\%$ and $0.7 \pm$

1.2% 2-MGK and 1-MGK activities, respectively, compared to its DGK activity (Fig. 1C and Table 1). These results indicate that type I DGK isozymes have 12 – 19% 2-MGK activity relative to their DGK activities, while their 1-MGK activities are markedly lower than their 2-MGK activities (0.6 – 1.4%). Of the three type I isozymes, DGK γ has the highest relative 2-MGK activity (approximately 20%).

3.2. 1-MGK and 2-MGK activities of type II DGKs (δ , η and κ)

The first type II isozyme, DGK δ 2, showed $13.3 \pm 0.3\%$ 2-MGK activity compared to its DGK activity, whereas its 1-MGK activity was $1.0 \pm 0.2\%$ (Fig. 2A and Table 1). The same results were obtained with an alternative splicing product, DGK δ 1 (data not shown). The second type II isozyme, DGK η 1, exhibited $12.6 \pm 0.3\%$ and $3.0 \pm 0.2\%$ 2-MGK and 1-MGK activities compared to its DGK activity (Fig. 2B and Table 1), respectively. The same results were obtained with an alternative splicing product, DGK η 2 (data not shown). Similarly, the third type II isozyme, DGK κ , had $12.4 \pm 2.4\%$ and $2.0 \pm 0.9\%$ 2-MGK and 1-MGK activities relative to its DGK activity (Fig. 2C and Table 1). These results indicate that type II DGK isozymes have approximately 13% 2-MGK activity relative to their DGK activities, whereas their 1-MGK activities are markedly lower (less than 3.0%). Therefore, the 2-MGK and 1-MGK activities of type II DGK isozymes, relative to their DGK activities are similar to those of the type I isozymes.

3.3. 1-MGK and 2-MGK activities of type III DGK (ϵ)

The type III isozyme, DGK ϵ , had $7.9 \pm 2.3\%$ 2-MGK activity compared to its DGK activity (Fig. 3 and Table 1). This value is almost the same as that of a previous report by Gantayet *et al.* (6.4% relative to DGK activity)[31]. On the other hand, its 1-MGK activity was negligible ($0.0 \pm 0.0\%$ DGK activity) (Fig. 3 and Table 1). We also examined DGK ϵ activity in the absence of PS and obtained essentially the same results

(data not shown). Therefore, similar to the type I and II isozymes, DGK ϵ is a 2-MGK-positive and 1-MGK-negative enzyme. However, its relative 2-MGK activity was moderately lower than those of the type I and II isozymes.

3.4. 1-MGK and 2-MGK activities of type IV DGKs (ζ and ι)

As shown in Fig. 4A and Table 1, the 1-MGK and 2-MGK activities of the first type IV isozyme DGK ζ 1 were very weak ($0.3 \pm 0.0\%$ and $0.7 \pm 0.1\%$ relative to DGK activity, respectively). The activities of the second type IV isozyme DGK ι 1 were also negligible ($0.0 \pm 0.0\%$ and $0.0 \pm 0.0\%$ relative to DGK activity, respectively) (Fig. 54B and Table 1). Therefore, in contrast to the type I, II and III DGK isozymes, type IV isozymes are 1-MGK- and 2-MGK-negative enzymes.

3.5. 1-MGK and 2-MGK activities of type V DGK (θ)

In contrast to the type I–IV DGK isozymes, DGK θ (type V) showed $6.0 \pm 0.2\%$ 1-MGK activity compared to its DGK activity (Fig. 5 and Table 1). However, its 2-MGK activity was $1.9 \pm 0.3\%$ relative to its DGK activity. These results indicate that, unlike the other DGK isozymes, DGK θ is a 1-MGK-positive and 2-MGK-negative enzyme.

Compared with the ratios (12.0–19.2%) of relative 2-MGK activities of type I and II DGK isozymes, that (6.0%) of the 1-MGK activity of DGK θ is low. However, the comparison of the 1-MGK activity of DGK θ with the 2-MGK activity of DGK η indicates that these specific activities were comparable each other (Suppl. Fig. 5).

We cannot deny the possibility that the overexpression of DGK θ might induce the expression of enzyme(s) having 1-MGK activity. To confirm whether the 1-MGK activity of DGK θ is intrinsic, we expressed 6xHis-tagged DGK θ in COS-7 cells and purified it using Ni-affinity chromatography (Fig. 6A). The purified DGK θ also showed 4.9% 1-MGK activity compared to its DGK activity, and its relative 2-MGK activity was

0.5% (Fig. 6B). These results indicate that DGK θ possesses intrinsic 1-MGK activity in addition to DGK activity.

We next compared the affinities of DGK θ for DG and 1-MG. The activity of DGK θ was increased in a DG or 1-MG dose-dependent manner (Suppl. Fig. 6A). A double reciprocal plot provided the apparent K_m values for the DG and 1-MG of DGK θ of 1.2 and 19.6 mol%, respectively (Suppl. Fig. 6B). The apparent K_m value for 1-MG was 16.3-fold higher than that of DG. The apparent K_m values of DGK θ for DG (1.2 mol%) and 1-MG (19.6 mol%) were comparable to those of DGK α for DG and 2-MG (1.4 and 11.0 mol%), respectively (Suppl. Fig. 4).

3.6. Comparison of 2-MGK and 1-MGK activities of DGK α and DGK θ with those of AGK

AGK was also reported to have 2-MGK and 1-MGK activities in addition to DGK activity [38]. Thus, we compared these activities of AGK with the 2-MGK activity of DGK α and the 1-MGK activity of DGK θ using an octylglucoside-mixed micellar assay. We confirmed that the expression levels of AGK and DGK α were comparable to each other (Fig. 7A). As shown in Fig. 7B, DGK α exhibited a markedly higher 2-MGK activity (approximately 5-fold) than AGK. As shown in Fig. 8A, the expression levels of AGK and DGK θ were also comparable to each other. Moreover, DGK θ exhibited a significantly higher 1-MGK activity (approximately 11-fold) than AGK (Fig. 8B). Although the deoxycholate assay was reported to be more suitable for the measurement of the 1-MGK and 2-MGK activities of AGK than the octylglucoside-mixed micellar assay [38], we did not detect a marked enhancement of these activities of AGK, DGK α and DGK θ using the deoxycholate assay (data not shown).

4. Discussion

30 years ago, Kanoh *et al.* demonstrated that purified DGK α had high 2-MGK activity (10–20% compared to DGK activity), whereas its 1-MGK activity was less than 4% [30]. DGK ϵ was also reported to have 2-MGK activity (6.4% of DGK activity) by Gantayat *et al.* [31]. In the present study, DGK α and ϵ exhibited approximately 12 and 8% 2-MGK activities compared to their DGK activities (Figs. 1 and 3 and Table 1). Therefore, we reproduced their results in this study. Moreover, we newly revealed that DGK β , γ , δ , η and κ are also 2-MGK-positive and 1-MGK-negative enzymes (Figs. 1 and 2 and Table 1) like DGK α and ϵ . Among them, DGK γ has the highest 2-MGK activity (19.2%) relative to its DGK activity (Fig. 1). Intriguingly, we demonstrated for the first time that DGK θ has a high 1-MGK activity (approximately 5 – 6% of DGK activity), but not 2-MGK activity (Figs. 5 and 6 and Table 1). Notably, DGK ζ and ι are both 1-MGK- and 2-MGK-negative (Fig. 4 and Table 1). The existence of 1-MGK- and 2-MGK-negative isozymes emphasizes the importance of 1-MGK and 2-MGK-positive isozymes. These results indicate that the ten DGK isozymes have great enzymological diversity beyond our expectation.

We elucidated that the ten DGK isozymes have different relative 1-MGK and 2-MGK activities vs DGK activity under the same conditions. However, because the octylglucoside-mixed micellar assay was developed for DGK, it is not known whether the DGKs exhibit maximum 1-MGK and 2-MGK activities in this assay. Although the deoxycholate assay was reported to be more suitable for the measurement of the 1-MGK and 2-MGK activities of AGK than the octylglucoside-mixed micellar assay [38], we did not detect a marked enhancement of these activities of AGK and DGK using the deoxycholate assay (data not shown). If an improved MGK assay method is developed, higher intrinsic 1-MGK and 2-MGK activities of DGK isozymes may be found.

The 1-MGK activity of DGK θ and the 2-MGK activity of DGK α were stronger than those of AGK (Figs. 7 and 8), which has DGK, 1-MGK and 2-MGK activities[38], under several assay conditions. Therefore, it is possible that the type I–III DGKs and type V DGK act as primary 2-MGK and 1-MGK in cells, respectively. AGK lacks a corresponding region homologous to the C-terminal half of the catalytic domain of DGK[38]. Rittiner *et al.* [40] and we (Murakami, E. and Sakane, F. unpublished work) revealed that DGK η lacking the C-terminal half of the catalytic domain did not show DGK activity. Therefore, this deletion may cause the low DGK, 1-MGK and 2-MGK activities of AGK.

DG content represented 0.7 – 4 mol% of total lipids in isolated cell membranes [41]. The concentrations of MG are 2–10-fold higher than those of DG in macrophages[42] and 3T3-L1 adipocytes[43]. Therefore, the contribution of the 1-MGK and 2-MGK activities of DGK may be considerably great in these MG-rich cells, macrophages and adipocytes. Moreover, it is possible that the concentrations of 1-MG and 2-MG are high in a focal manner. In the 1-MG- and 2-MG-rich regions, DGK isozymes (type I–III and V) would generate substantial amounts of 1-LPA and 2-LPA.

2-Arachidonoyl-glycerol acts as endogenous cannabinoid[44, 45], so its metabolism must be strictly regulated. Because DGK ϵ prefers DG species containing arachidonic acid at the *sn*-2 position[46, 47], this isozyme may play an important role in 2-arachidonoyl-glycerol kinase. In addition to DGK ϵ [23], DGK α [48, 49], β [50], γ [51, 52], δ [53] and η [34, 53, 54] are also highly expressed in the brain. Moreover, DGK α , β , γ , δ and η have broad selectivities to fatty acids at the *sn*-2 position of DG[3]. For example, DGK γ indeed showed comparable phosphorylation activities against 2-oleoyl(18:1)-glycerol and 2-arachidonoyl-glycerol (Suppl. Fig. 7). Therefore, these type I and II isozymes may also act as 2-arachidonoyl-glycerol kinase in the brain.

Our preliminary experiments showed that DGK δ -specific siRNA attenuated a high glucose-dependent increase of LPA (Sakai, H. and Sakane, F., unpublished work).

However, a phospholipase A inhibitor (methyl arachidonylfluorophosphonate) [55, 56] did not inhibit the high glucose-dependent increase of LPA (Sato, Y., Mizuno, S. and Sakane, F. unpublished work). These results suggest that DGK δ produces LPA through the direct phosphorylation of MG, but not the PA hydrolysis by phospholipase A. Thus, although further works are needed to confirm, it is possible that DGK δ serves as MGK in cells.

The ten DGK isozymes are divided into five groups (type I: α , β and γ ; type II: δ , η and κ ; type III: ϵ ; type IV: ζ and ι ; type V: θ) according to their structural features [1-6]. Arachidonic acid-containing DG-selectivity can also be used to categorize the isozymes into two groups: an arachidonoyl DG-selective isozyme (ϵ) versus non-selective isozymes (α , β , γ , δ , η , κ , ζ , ι and θ) [1-6]. DGK isozymes can also be grouped into high DG affinity type (η) versus low DG affinity type (α , ϵ and ζ) (the affinities of the other isozymes for DG are presently unknown) [57]. In addition to these categorizations, the results of this study provide a new classification based on the different activities for 1-MG and 2-MG: DGK isozyme(s) having (1) 2-MGK activity relatively stronger than their 1-MGK activity (type I–III DGKs), (2) only negligible 1-MGK and 2-MGK activities (type IV DGK), and (3) 1-MGK activity stronger than its 2-MGK (type V DGK). These differences in regard to the relative 1-MGK and 2-MGK activities are probably essential for the DGK isozymes in serving their individual functions.

LPA is known to regulate the peroxisome proliferator-activated receptor γ and the reorganization of actin cytoskeleton [58]. However, the physiological functions of LPA in cells are still poorly understood. The simultaneous production of PA with/without 1-LPA or 2-LPA may be important to maximize a variety of physiological functions of DGKs [1-6]. This study provides a new aspect of DGK and will enhance the elucidation of the physiological functions of LPA in cells.

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Author contributions

FS conceived and coordinated the study and wrote the paper. YS, CM, AY, SM and HS designed, performed and analyzed the experiments.

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Figure Legends

Fig. 1. MGK activities of type I DGK isozymes. 1,000xg supernatants (5 µg) of extracts from COS-7 cells expressing each DGK isozyme (α (A), β (B) and γ (C)) were incubated with DG, 1-MG and 2-MG for 30 min in the presence of 1 µM CaCl_2 . The results are presented as percentages of the value of the control (DGK activity) and represent the means $\pm$ SDs of the values obtained in three separate experiments. The statistical significance was determined using Student's t test (***) $p < 0.005$.

Fig. 2. MGK activities of type II DGK isozymes. 1,000xg supernatants (5 µg) of extracts from COS-7 cells expressing each DGK isozyme ($\delta 2$ (A), $\eta 1$ (B) and κ (C)) were incubated with DG, 1-MG and 2-MG for 30 min. The results are presented as percentages of the value of the control (DGK activity) and represent the means $\pm$ SDs of the values obtained in three separate experiments. The statistical significance was determined using Student's t test (***) $p < 0.005$.

Fig. 3. MGK activity of type III DGK isozyme. 1,000xg supernatants (5 µg) of extracts from COS-7 cells expressing type III DGK isozyme (ϵ) were incubated with DG, 1-MG and 2-MG for 30 min. Because $\text{DGK}\epsilon$ is the 2-arachidonoyl DG-selective isozyme, 1-stearoyl-2-arachidonoyl-*sn*-glycerol and 2-arachidonoyl glycerol were used as substrates in place of 1,2-dioleoyl-*sn*-glycerol and 2-oleoylglycerol. The results are presented as percentages of the value of the control (DGK activity) and represent the means $\pm$ SDs of the values obtained in three separate experiments. The statistical significance was determined using Student's t test (***) $p < 0.005$.

Fig. 4. MGK activities of type IV DGK isozymes. (A, B) 1,000xg supernatants (5 µg) of extracts from COS-7 cells expressing type IV DGK isozymes ($\zeta 1$ (A) and $\iota 1$ (B))

were incubated with DG, 1-MG and 2-MG for 30 min. The results are presented as percentages of the value of the control (DGK activity) and represent the means $\pm$ SDs of the values obtained in three separate experiments. The statistical significance was determined using Student's t test (** $p < 0.005$).

Fig. 5. MGK activity of type V DGK isozyme. 1,000xg supernatants (5 μ g) of extracts from COS-7 cells expressing type V DGK isozyme (θ) was incubated with DG, 1-MG and 2-MG for 30 min. The results are presented as percentages of the value of the control (DGK activity), and represent the means $\pm$ SDs of the values obtained in three separate experiments. The statistical significance was determined using Student's t test (** $p < 0.005$).

Fig. 6. 1-MGK activity of purified DGK θ . (A) 6xHis-tagged DGK θ was purified by Ni Sepharose affinity chromatography. The 6xHis-tagged DGK θ in each fraction was detected by Western blotting using an anti-DGK θ monoclonal antibody. (B) Kinase activities of purified DGK θ for DG, 1-MG and 2-MG were examined. The results are presented as percentages of the value of the control (DGK activity) and represent the means $\pm$ SDs of the values obtained in three separate experiments. The statistical significance was determined using Student's t test (** $p < 0.005$).

Fig. 7. 2-MGK activities of AGK and DGK α . (A) The expression of the 3xFLAG-tagged AGK and DGK α . The 3xFLAG-tagged AGK and DGK α expressed in COS-7 cells were detected by Western blotting using an anti-FLAG monoclonal antibody. (B) 1,000xg supernatants (5 μ g) of extracts from COS-7 cells expressing 3xFLAG-AGK and 3xFLAG-DGK α were incubated with 2-MG for 30 min. The results are presented as relative to the control (2-MG kinase activity of 3xFLAG-AGK). The 2-MGK activity of 3xFLAG-DGK α was normalized for the expression levels of

3xFLAG-AGK. Representative results of three independent experiments are shown.

Fig. 8. 1-MGK activities of AGK and DGK θ . (A) The expression of the 3xFLAG-tagged AGK and DGK θ . The 3xFLAG-tagged AGK and DGK θ expressed in COS-7 cells were detected by Western blotting using an anti-FLAG monoclonal antibody. (B) 1,000 $\times$ g supernatants (5 μ g) of extracts from COS-7 cells expressing 3xFLAG-AGK and 3xFLAG-DGK θ were incubated with 1-MG for 30 min. The results are presented as relative to the control (1-MGK activity of 3xFLAG-AGK). The 1-MGK activity of 3xFLAG-DGK θ was normalized for the expression levels of 3xFLAG-AGK. Representative results of three independent experiments are shown.

Table 1

1-MGK and 2-MGK activities of ten DGK isozymes

Type	Isozyme	Relative activity (%)		
		DG	1-MG	2-MG
I	α	100	0.6 $\pm$ 0.0	12.0 $\pm$ 0.5
	β	100	1.4 $\pm$ 0.3	16.8 $\pm$ 0.3
	γ	100	0.7 $\pm$ 1.2	19.2 $\pm$ 6.3
II	$\delta 2$	100	1.0 $\pm$ 0.2	13.3 $\pm$ 0.3
	$\eta 1$	100	3.0 $\pm$ 0.2	12.6 $\pm$ 0.3
	κ	100	2.0 $\pm$ 0.9	12.4 $\pm$ 2.4
III	ϵ	100	0.0 $\pm$ 0.0	7.9 $\pm$ 2.3
IV	$\zeta 1$	100	0.3 $\pm$ 0.0	0.7 $\pm$ 0.1
	$\iota 1$	100	0.0 $\pm$ 0.0	0.0 $\pm$ 0.1
V	θ	100	6.0 $\pm$ 0.2	1.9 $\pm$ 0.3

1,000xg supernatants (5 μ g) of extracts from COS-7 cells expressing each DGK isozyme (α – θ) were incubated with DG, 1-MG and 2-MG for 30 min, and the kinase activities of the DGK isozyme for DG, 1-MG and 2-MG were examined (Figs. 1–5). The data are shown as the percentages of the value of the control (DGK activity), and the means $\pm$ SDs of the values obtained in three separate experiments.

graphical abstract

Fig. 1

A

B

C

Fig. 2

A

B

C

Fig. 3

Fig. 4

Fig. 5

Fig 6

A

B

Fig 7

A

B

Fig 8

A

B