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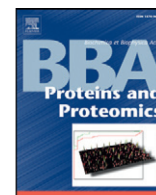

Distinct 1-monoacylglycerol and 2-monoacylglycerol kinase activities of diacylglycerol kinase isozymes

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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 <3.0%. Both the 1-MGK and 2-MGK activities of the type IV DGKs (ζ and ι) were <1% relative to their DGK activities. Intriguingly, type V DGKθ has approximately 6% 1-MGK activity and <2% 2-MGK activity compared to its DGK activity. Purified DGKθ 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 DGKθ and the 2-MGK activity of DGKα 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.

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1. Introduction

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) in biomembranes to produce phosphatidic acid (PA) [1–6]. To date, ten mammalian DGK isozymes (α, β, γ, δ, ε, ζ, η, θ, ι 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, DGKα (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]. DGKβ (type I) is an important regulator in neurite spine formation [13]. DGKγ (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], DGKδ (type II) is an important factor in hyperglycemia-induced peripheral insulin resistance, thereby exacerbating the severity of type-2 diabetes [18,19]. DGKη (type II) enhances C-Raf activity and B-Raf/C-Raf heterodimerization in cancer cells [20]. It was also reported that the gene encoding DGKη is implicated in the etiology of bipolar disorder [21]. DGKκ (type II) is associated with risk of hypospadias [22]. DGKε (type III) controls seizure susceptibility and long-term potentiation through modulating arachidonoyl-inositol lipid signaling [23]. DGKζ (type IV) is an important regulator of dendritic spine maintenance [24]. This isozyme is also known to regulate endothelin-1-induced cardiomyocyte hypertrophy [25]. DGKι (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]. DGKθ (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 DGKα 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 <4% relative to its DGK activity. Gantayet et al. reported that DGKε 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.

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

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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 <3%. Both the 2-MGK and 1-MGK activities of the type IV DGKs (ζ and ι) were <1% relative to their DGK activity. Interestingly, the type V DGKθ has approximately 6% 1-MGK activity and <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, p3×FLAG-CMV-pig DGKα [14], -rat DGKβ [14], -human DGKγ [14], -human DGKδ2 [32,33], -human DGKη1 [34], -human DGKκ [35], -human DGKε [36], -human DGKζ1 [36], -human DGKι1 [36], and -human DGKθ [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: CCGTCGAC TCACTGGGATGTGCTC. To express 6×His-tagged protein, human DGKθ 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% CO_2. The cells were transfected with p3×FLAG-CMV-DGK isozymes 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. After transfection for 48 h, the harvested cells were suspended in 300 μl ice-cold lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM 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-DGKθ 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 μl) contained 50 mM MOPS (pH 7.2), 50 mM *n*-octyl-β-D-glucoside, 1 mM dithiothreitol, 20 mM NaF, 10 mM $MgCl_2$, 27 mol% PS, 5.4 mol% 1.2-dioleoyl-*sn*-glycerol or 1-oleoyl-*rac*-glycerol or 2-oleoylglycerol and 1 mM [γ-^{32}P]ATP (approximately 100,000 cpm/nmol). When the activities of the Ca^{2+}-dependent isozymes, DGKα, β and γ, were measured, 1 μM $CaCl_2$ was added. When the activity of the arachidonoyl DG-selective isozyme, DGKε, 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 μg 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 N_2 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-^{32}P]ATP (100,000 cpm/nmol, 1 mM) containing $MgCl_2$ (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 6×histidine-tagged DGKθ

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 3×FLAG-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, DGKα, expressed in COS-7 cells. We confirmed that the DGK activity of DGKα in the COS-7 cell lysates increased lineally in a time-dependent manner over 30 min and with the increasing protein concentration (0–5 μg/50 μl of reaction mixture) [36]. As shown in Fig. 1A and Table 1, DGKα exhibited 12.0 ± 0.5% ($n = 3$) 2-MGK activity compared to its DGK activity, whereas its 1-MGK activity was 0.6 ± 0.0%. These results are essentially the same as those obtained with purified DGKα 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 DGKα is intrinsic, we expressed 6×His-tagged DGKα in COS-7 cells and purified it using Ni-affinity chromatography (Suppl. Fig. 3A). The purified DGKα also

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