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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

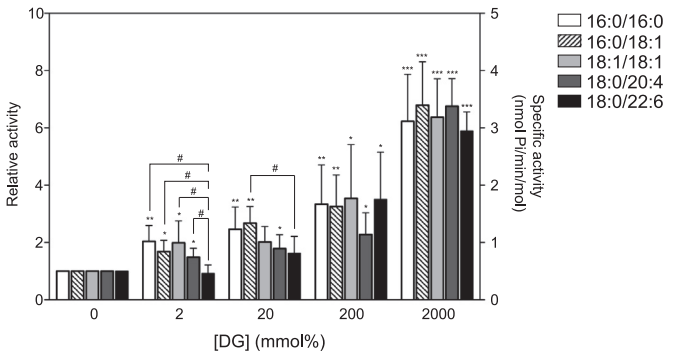

Fig. 1. Effects of different DG molecular species on PKCα activation. Lipid vesicles were prepared with different DG molecular species (16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/20:4- and 18:0/22:6-DG) and PKC activity toward MBP was determined in vesicles as a function of increasing concentrations of 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 ($^{\#}P < 0.05$).