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Endocannabinoids and diacylglycerol kinase activity

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ABSTRACT

Mammalian diacylglycerol kinases are a family of enzymes that catalyze the phosphorylation of diacylglycerol to produce phosphatidic acid. The extent of interaction of these enzymes with monoacylglycerols is the focus of the present study. Because of the structural relationship between mono- and diacylglycerols, one might expect the monoacylglycerols to be either substrates or inhibitors of diacylglycerol kinases. This would have some consequence to lipid metabolism. One of the lipid metabolites that would be affected is 2-arachidonoyl glycerol, which is an endogenous ligand for the CB1 cannabinoid receptor. We determined if the monoglycerides 2-arachidonoyl glycerol or 2-oleoyl glycerol affected diacylglycerol kinase activity. We found that 2-arachidonoyl glycerol is a very poor substrate for either the epsilon or the zeta isoforms of diacylglycerol kinases. Moreover, 2-arachidonoyl glycerol is an inhibitor for both of these diacylglycerol kinase isoforms. 2-oleoyl glycerol is also a poor substrate for these two isoforms of diacylglycerol kinases. As an inhibitor, 2-oleoyl glycerol inhibits diacylglycerol kinase ε less than does 2-arachidonoyl glycerol, while for diacylglycerol kinase ζ, these two monoglycerides have similar inhibitory potency. These results have implications for the known role of diacylglycerol kinase ε in neuronal function and in epilepsy since the action of this enzyme will remove 1-stearoyl-2-arachidonoylglycerol, the precursor of the endocannabinoid 2-arachidonoyl glycerol.

1. Introduction

Diacylglycerol kinase (DGK) from different species exhibits different specificities. Thus, bacterial forms of DGK can phosphorylate ceramide as well as diacylglycerol (DAG), while DGK from yeast utilizes CTP, rather than ATP, as the source of phosphate [1]. Mammalian DGKs are a family of enzymes comprised of at least 10 isoforms [2]. We undertook this study to evaluate the interactions of 2-acyl-glycerols with isoforms of DGK in order to assess the possible role of this enzyme family in affecting the concentration of these signaling lipids in cells as well as to further understand the nature of substrate and lipid interactions with binding sites on DGKs. Mammalian isoforms of DGK have only been shown to catalyze the phosphorylation of one class of lipid substrates, DAG, using only ATP as the source of phosphate. In the present study, we determined if structurally related monoacylglycerols are either substrates or inhibitors of mammalian DGKs.

A particularly important acyl chain for monoacyl- and diacylglycerols is arachidonic acid. DAG having arachidonic acid at the *sn*-2 position is an intermediate in phosphatidylinositol cycling. Arachidonoyl-DAG is preferentially phosphorylated by the isoform DGKε [2]. The monoglyceride with arachidonic acid at the *sn*-2 position is 2-arachidonoyl glycerol (2-AG). This monoglyceride is an important ligand for the CB1 cannabinoid receptor [3]. 2-AG is known to be generated in the brain by the enzyme diacylglycerol (DAG) lipase [4] and is one of the most abundant molecular species of monoacylglycerols in the brain [5]. The concentration of DAG in brain synaptosomes is at least an order of magnitude higher than that of 2-AG [6]. Interestingly, even in organisms lacking known cannabinoid receptors, such as nematodes, 2-AG has been identified [7]. This suggests that 2-AG, in addition to being a cannabinoid receptor ligand, is also an intermediate in lipid metabolism in organisms without developed endocannabinoid systems. The expression of DAG lipase, that converts DAG to a monoglyceride, is required for axonal growth during development and for retrograde synaptic signaling at mature synapses. Endocannabinoid signaling is a key regulator of synaptic communication throughout the central nervous system [8]. The lysolipid 2-Arachidonoyl-sn-glycero-3-phosphate, an arachidonic acid-containing lysophosphatidic acid is found in rat brain and can

Abbreviations: DGK, diacylglycerol kinase; 2-AG, 2-arachidonoyl glycerol; 2-OG, 2-oleoyl glycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; SAPA, 1-stearoyl-2-arachidonoyl phosphatidic acid; DOG, 1,2-dioleoylglycerol; SAG, 1-stearoyl-2-arachidonoylglycerol; DAG, diacylglycerol; DTT, dithiothreitol; BHT, butylated hydroxytoluene

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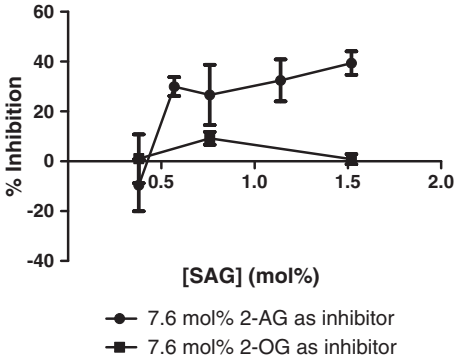

Fig. 1. Inhibition of DGKε by 2-AG and 2-OG. Lipid films were created with 0.37, 0.57, 0.76, 1.14 and 1.52 mol% SAG as substrate and 7.6 mol% 2-AG or 2-OG as inhibitor and used in a mixed micelle activity assay. The data are presented as percent inhibition calculated by taking the ratio of the difference in activity due to inhibition over the activity without the inhibitor. The data points are mean ± SEM, $n = 3$.

be rapidly converted to 2-AG. However, the metabolic fate of 2-AG is not known. A principle metabolic fate of this lipid is its hydrolysis by monoacylglycerol lipase. However, an additional possibility is that 2-AG is also a substrate for diacylglycerol kinase (DGK) to reform lysophosphatidic acid that is also a signaling lipid [9–12]. This possibility was tested in the present study.

2. Materials and methods

2.1. Materials

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and were dissolved in 2:1 $CHCl_3/CH_3OH$ or in pure $CHCl_3$. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was stored in 2:1 $CHCl_3/CH_3OH$ and 0.1% (wt./vol.) butylated hydroxytoluene (BHT). 2-AG and 2-OG were stored in C_2H_5OH. [γ-^{32}P]ATP (50 μCi/mL) was purchased from Perkin Elmer Life Sciences. All other chemicals and reagents were purchased from Sigma or BioShop Canada.

2.2. Enzyme preparation for DGK enzymatic activity assay

Human DGKε with a C-terminal hexahistidine tag fusion or DGKζ with a C-terminal FLAG epitope tag fusion were overexpressed in baculovirus-infected Sf21 cells. The cell pellets were resuspended in cold lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.4 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate and 1:1000 dilution of protease inhibitor (Sigma)) and detergent 1% (vol./vol.) Nonidet P-40 and kept on ice for 10 min to lyse. The lysates were centrifuged at 100,000*g* for 30 min at 4 °C to solubilize DGK and the supernatant was utilized in the mixed micelle activity assays.

2.3. Detergent-phospholipid-mixed micelle-based DGK enzymatic activity assay

Inhibition of DGKε and DGKζ by 2-AG or 2-OG was studied using enzymatic activity assays following a previously established protocol [13–15]. Lipid films were prepared by evaporation of organic solvent of lipid solutions of the substrate 1-stearoyl-2-arachidonoyl glycerol (SAG) for DGKε or 1,2-dioleoyl glycerol (DOG) for DGKζ. In addition to the substrate, the phospholipid DOPC was added for DGKε or 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) for DGKζ. The monoglycerides, 2-AG and 2-OG, were tested alone as substrates or tested as inhibitors with SAG or DOG as substrates. When 2-AG and 2-OG were tested as substrates, SAG and DOG were not included in the lipid films. DOPC or DOPS were added in addition to other lipids so as to maintain the total concentration of all lipids at 24.1 mol% (19 mM). The lipids were dried under stream of nitrogen gas to remove the organic solvent and then further dried in a vacuum dessicator for 2 h. All lipids and lipid films were covered with Argon gas to avoid oxidation by air. The films were hydrated with 50 μL of 4× assay buffer (200 mM Tris–HCl (pH 7.5), 400 mM NaCl, 20 mM $MgCl_2$, 4 mM EGTA, 30 mM Triton X-100 and 30 mM Triton X-114) and vortexed for 2 min, followed by addition of 105 μL ddH_2O, 20 μL of 10 mM DTT and 5 μL of Sf21 insect cell lysates expressing either DGKε or DGKζ or empty vector controls, to obtain a final volume of 180 μL. The reaction was initiated with 20 μL of 1 mM [γ-^{32}P]ATP (50 μCi/mL) and was stopped after 10 min at 25 °C with the addition of 2 mL of stop solution (1:1 $CHCl_3/CH_3OH$ and 0.25 mg/mL dihexadecyl phosphate). The organic layer was washed three times. To allow for maximal separation of the organic and aqueous phases, the mixture was allowed to stand for 2 h, 20 and 5 min, after the first, second and third wash, respectively, with 2 mL of wash solution (7:1 H_2O/CH_3OH, 1% $HClO_4$, 0.1% H_3PO_4) used for each wash. The aqueous layer was removed after each wash. Four hundred microliters of the organic layer was collected in a scintillation vial and incorporation of radioactive phosphate into the organic phase was measured by Cerenkov counting using a scintillation counter (Beckman Coulter). The counts were corrected for a blank reaction in which no enzyme was added. The activity is presented as % relative activity taking the control with no inhibitor as 100% activity. The assays were performed in triplicates and the results are presented as the mean ± the standard deviation of the mean. Lysates from mock transfected insect cells were used as negative controls.

3. Results

Because of the specific binding of DGKε to an arachidonoyl group, there was a particular interest to evaluate the behavior of 2-AG with this isoform of DGK. The substrate specificity and kinetic constants for DGKε has been recently reported [13]. Using the preferred substrate of DGKε, SAG, as a positive control, the rate of phosphorylation of 2-AG was only 6.35% ± 0.15% that of SAG. Thus, 2-AG essentially is a very poor substrate for DGKε. We also evaluated 2-OG as a substrate of this isoform of DGK, but the rate of phosphorylation was even lower than for 2-AG, reflecting the specificity of DGKε for arachidonoyl-containing lipids.

A possible contribution to the slow rate of phosphorylation of these monoglycerides is that their partitioning between aqueous and micellar phases favors water solubility. However, this is not a major

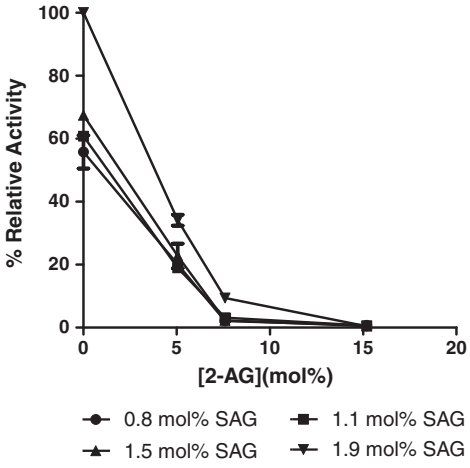

Fig. 2. Effect of varying concentration of 2-AG on inhibition of DGKε. Lipid films were created with 0.76, 1.14, 1.52 and 1.90 mol% SAG as substrate and 0, 5.1, 7.6 and 15.2 mol % 2-AG as inhibitor and used in a mixed micelle activity assay. The data are presented as percent relative activity taking the activity of 1.9 mol% SAG with 0 mol% 2-AG as 100%. The data points are mean ± SEM, $n = 3$.