Distribution of distinct arachidonoyl-specific and non-specific isoenzymes of diacylglycerol kinase in baboon (*Papio cynocephalus*) tissues

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We investigated the diacylglycerol kinase species present in several baboon tissues using the substrates *sn*-1-stearoyl-2-arachidonoyl diacylglycerol and *sn*-1,2-didecanoyl diacylglycerol. Chromatography of octyl glucoside extracts of the baboon (*Papio cynocephalus papio*) tissues on hydroxyapatite columns revealed the presence of three diacylglycerol kinase species with different substrate preferences. One species markedly 'preferred' the substrate *sn*-1-stearoyl-2-arachidonoylglycerol, the two other species preferred *sn*-1,2-didecanoylglycerol. Measurement of the activity of the baboon brain diacylglycerol kinases toward diacylglycerols with a range of different fatty acid chains revealed a strict preference of the arachidonoyl diacylglycerol kinase for *sn*-1-acyl-2-arachidonoyl diacylglycerol, whereas the other enzymes showed no preference toward several long-chain-fatty-acid-containing diacylglycerols. The arachidonoyl diacylglycerol kinase was particularly abundant in brain and testis, whereas liver was practically devoid of this enzyme. The arachidonoyl diacylglycerol kinase from baboon brain was found to be predominantly associated with the particulate fraction and exhibited an apparent molecular mass of 130 kDa.

INTRODUCTION

Diacylglycerol kinases catalyse the phosphorylation of *sn*-1,2-diacylglycerol (DG) and thereby form phosphatidic acid (PA). In stimulated cells that show a phosphoinositide response they are believed to contribute to the phosphoinositide cycle by initiating the resynthesis of phosphatidylinositol (see [1,2] for review). They may also attenuate the activation of protein kinase C [3,4], and the possibility exists that a DG kinase might regulate the levels of DG and PA that are available for the biosynthesis of phospholipids in unstimulated cells.

Soluble DG kinases from mammalian sources have been purified to homogeneity or near-homogeneity from pig brain [5], bovine brain [6], rat brain [7] and rat liver [8]. Molecular masses of 80, 72 and 120 kDa were reported for the DG kinases from pig brain, bovine brain and rat liver respectively. An 80 kDa DG kinase that cross-reacts with antibodies to the pig brain enzyme is also present in pig thymus [9] and certain human lymphocytes [10]. Pig thymus cytosol contains an additional immunologically distinct DG kinase species of 280 kDa, whereas platelets exhibit a unique 120 kDa species [9].

A detailed functional comparison of the different soluble DG kinases has yet to be done. Studies of the substrate specificity of the purified DG kinase from

bovine brain showed little specificity for DGs that differed in their fatty acid composition [6], but no comparable data for the other DG kinases are available. Such data are needed because the phosphoinositides of animal cells predominantly consist of the *sn*-1-stearoyl-2-arachidonoyl species. Thus, if a DG kinase were to contribute to the phosphoinositide cycle, one might expect that it would show a preference for arachidonic acid-containing DGs. Recent studies in our laboratory are of interest in this regard because they demonstrated that the membranes of Swiss 3T3 cells contain a DG kinase activity that has this property [11,12]. The arachidonoyl-DG kinase activity was sufficient to account for the increase in PA observed in 3T3 cell membranes during stimulation by platelet-derived growth factor. Furthermore, the formation of PA and phosphatidylinositol from the cell-permeable DG, *sn*-1,2-didecanoylglycerol (diC₁₀), was decreased during platelet-derived-growth-factor stimulation, whereas that of PA and phosphatidylinositol from endogenous DG was increased, suggesting that an arachidonoyl-DG kinase participates in the phosphatidylinositol cycle in 3T3 cells [11].

Little is known about the enzyme responsible for the observed arachidonoyl-DG kinase activity. In the present studies, we took a first step toward its characterization by addressing the following questions: (1) How does the enzyme activity distribute among animal tissues? (2) Can

Abbreviations used: DG, diacylglycerol; 18:0/20:4 DG, *sn*-1-stearoyl-2-arachidonoylglycerol; 16:0/20:4 DG, *sn*-1-palmitoyl-2-arachidonoylglycerol; 14:0/20:4 DG, *sn*-1-myristoyl-2-arachidonoylglycerol; 20:4/18:0 DG, *sn*-1-arachidonoyl-2-stearoylglycerol; 18:0/18:1 DG, *sn*-1-stearoyl-2-oleoylglycerol; 18:0/18:2 DG, *sn*-1-stearoyl-2-linoleoylglycerol; 18:0/22:6 DG, *sn*-1-stearoyl-2-docosahexaenoylglycerol; 18:1/18:1 DG, *sn*-1,2-dioleoylglycerol; O-16:1/20:4 DRG, 1-hexadec-1'-enyl-2-arachidonoyl diradylglycerol; diC₁₀, *sn*-1,2-didecanoylglycerol; PA, phosphatidic acid; PA₁₀, didecanoyl phosphatidic acid; arachidonoyl-DG kinase, DG kinase previously termed 'M-DG kinase' [12], that 'prefers' arachidonoyl-DG as substrate; LDH, lactate dehydrogenase; DTT, dithiothreitol.

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it be chromatographically separated from other DG kinases? (3) Which additional features of diradylglycerols does it recognize? (4) What is its molecular mass?

EXPERIMENTAL

Materials

[γ - 32 P]ATP (3000 Ci/mmol) and [*glycerol*-1,2,3- 3 H]*sn*-2-arachidonoylmonoacylglycerol (37.0 Ci/mmol) were purchased from NEN; docosahexaenoic acid was from NuCheck; *sn*-1-palmitoyl-2-arachidonoyl phosphatidylcholine, *sn*-1-myristoyl-2-arachidonoyl phosphatidylcholine, distearoyl phosphatidylcholine, diarachidonoyl phosphatidylcholine, bovine heart phosphatidylcholine, 18:1/18:1 DG, diC₁₀ and pig brain phosphatidylserine were from Avanti; *sn*-1-stearoyl-2-oleoyl phosphatidylcholine, 18:0/18:2 DG and PA₁₀ were from Serdary; 18:0/20:4 DG, egg PA, β -octyl glucoside, leupeptin, pepstatin, soybean trypsin inhibitor (type 1-S), phospholipase A2 (*Naja naja* venom) and phospholipase C (*Bacillus cereus*) were from Sigma; sodium deoxycholate was from Calbiochem; Triton X-100 was from Baker; hydroxyapatite (Bio-Gel HT) was from Bio-Rad; pre-coated cellulose plates (0.1 mm) for t.l.c. of PA species were from EM-Science; Sephacryl S-300 (high resolution) and standard proteins for gel filtration were from Pharmacia LKB Biotechnology.

Hexadec-1-enyl-2-arachidonoylglycerophosphocholine was synthesized from bovine heart phosphatidylcholine as described [13], except that the hexadec-1-enylglycerophosphocholine was isolated by reverse-phase h.p.l.c. [14]. *O*-16:1/20:4 DRG was prepared by phospholipase C digestion of hexadec-1-enyl-2-arachidonoylglycerophosphocholine [15]. *sn*-1-Arachidonoyl-2-stearoyl phosphatidylcholine and *sn*-1-stearoyl-2-docosahexaenoyl phosphatidylcholine were synthesized by acylation of the corresponding 1-lysophosphatidylcholine with the proper anhydride by the method of Mangroo & Gerber [16] with the following modifications: in order to minimize possible isomerization of the lysophosphatidylcholines, non-esterified fatty acids were removed by washing the lysophosphatidylcholines six times with anhydrous diethyl ether [17]; the reacylated phosphatidylcholines were purified by column chromatography on silica gel, followed by reverse-phase h.p.l.c. on a C₁₈ column [18].

14:0/20:4 DG, 16:0/20:4 DG, 18:0/18:1 DG, 18:0/22:6 DG and 20:4/18:0 DG were prepared by phospholipase C digestion of the corresponding phosphatidylcholines as described [15]. The DGs prepared from phosphatidylcholines and the purchased 18:1/18:1 DG and 18:0/18:2 DG were repurified by t.l.c. on silica-gel G plates (Analabs) that had been sprayed with borate. The solvent systems used were ether/hexane (2:23, v/v) followed by ether/hexane (2:3, v/v) in the same dimension [19], in an atmosphere of argon. The 1,2-DGs were eluted from the scraped silica with chloroform. Concentrations of the DG solutions were established by measurements of glycerol concentrations after alkaline hydrolysis as follows: the chloroform of the DG solutions was evaporated under nitrogen; the lipids were hydrolysed with 0.25 ml of KOH [1 M in 95% (v/v) ethanol] at 70 °C for 30 min; the solutions were neutralized with 0.85 ml of HClO₄ (0.35 M in water); two phases were formed by addition of 0.5 ml

of chloroform, and the liberated glycerol was measured in the aqueous phase as described [20], except that the concentration of NADH in the assay mixture was 0.25 mM, and the glycerol concentrations were calculated from a standard curve established with solutions of known glycerol concentrations.

Preparation of baboon tissue homogenates

Fresh tissues were collected from male baboons anaesthetized with halothane. The tissues were homogenized with a Polytron (Brinkman Institute) homogenizer in 5 ml of ice-cold buffer A [20 mM-Mops (pH 7.2)/0.25 M-sucrose/1 mM-dithiothreitol (DTT)/1 mM-EGTA/pepstatin/leupeptin (1 μ g/ml each)/soybean trypsin inhibitor (50 μ g/ml)]/g of tissue. DG kinase levels were measured in the fresh homogenates. The homogenates were stored at -70 °C before being chromatographed on hydroxyapatite. DG kinase activity of the tissue homogenates was stable to one round of freeze-thawing.

Preparation of particulate and supernatant fractions from baboon brain

Homogenized baboon brain was centrifuged at 100 000 g for 1 h at 4 °C. The pellets were washed with 5 ml of buffer A/g of original tissue. The 100 000 g supernatants were pooled and filtered through a 0.45 μ m-pore-size filter. The washed pellets were resuspended in buffer A at 12–15 mg of protein/ml. Particulate and supernatant fractions were stored at -70 °C.

Hypo-osmotic lysis of brain membranes

The brain membrane suspension was mixed with glycerol to 3.9 M final concentration, incubated for 20 min on ice, then diluted with 9 vol of ice-cold water containing 1 mM-DTT, 1 mM-EGTA, and a mixture of proteinase inhibitors (1 μ g each of pepstatin and leupeptin/ml, and 50 μ g of soybean trypsin inhibitor/ml). The mixture was homogenized in a Dounce tissue grinder, incubated on ice for 30 min and centrifuged for 1 h at 100 000 g. The pellet was resuspended in buffer B [20 mM-Mops (pH 7.2)/1 mM-DTT/1 mM-EGTA/pepstatin/leupeptin (1 μ g/ml each)/soybean trypsin inhibitor (50 μ g/ml)] and submitted to another round of glycerol swelling/hypo-osmotic lysis.

Detergent extraction of DG kinase activity

In contrast with Swiss 3T3 cells [12], complete solubilization of DG kinase activity was not achieved by the addition of octyl glucoside alone, but also required the presence of 0.3 M-KCl. Tissue homogenates and membrane suspensions were therefore extracted for 30 min on ice with 2% octyl glucoside/0.3 M-KCl (in buffer B) at 7–12 mg of protein/ml, then centrifuged for 1 h at 100 000 g. After this treatment, the high-speed supernatants contained 90–100% of the DG kinase activity of the tissue homogenates, except for the supernatant from muscle which contained 70% of this activity.

Successive extractions of DG kinase activity from brain membranes with salt and detergent

Particulates from baboon brain were successively extracted with 0.3 M-KCl, then 2% octyl glucoside, and finally 0.3 M-KCl together with 2% octyl glucoside. The

extractions were all performed for 30 min on ice in buffer B containing 20% (v/v) glycerol. The glycerol was required to stabilize the activity of the arachidonoyl-DG kinase. After each extraction, soluble and particulate fractions were separated by centrifugation for 1 h at 100 000 *g*.

Chromatography on hydroxyapatite

Octyl glucoside/KCl extracts of tissue homogenates or brain membranes were loaded on to hydroxyapatite columns that had been pre-equilibrated in buffer B containing 1% octyl glucoside and 0.3 M-KCl. An amount of extract containing 100–300 mg of total protein was loaded on to a 1 cm × 12 cm hydroxyapatite column at 7 ml/h. After loading the sample the column was washed with 5 ml of 1% octyl glucoside/0.3 M-KCl in Buffer B. It was then successively treated with 5 ml of 0.15 M-potassium phosphate, pH 7.2, containing 1% octyl glucoside, 1 mM-DTT, and the above-mentioned proteinase inhibitors; a 120 ml linear gradient of potassium phosphate, pH 7.2, from 0.15 M to 0.60 M in presence of 1% octyl glucoside, 1 mM-DTT and the proteinase inhibitors; and finally 20 ml of 0.60 M gradient buffer. Fractions (2 ml each) were collected, and DG kinase activity was assayed in portions of every second fraction. Chromatography was performed entirely at 4 °C.

Dialysis of the DG kinases

Aliquots of peaks A, B and C derived from hydroxyapatite chromatography of baboon brain were dialysed extensively against 0.1 M-Tris (pH 7.5)/1 mM-DTT/pepstatin/leupeptin (1 µg/ml each) to remove the octyl glucoside present in the fractions. Dialysis was performed at 4 °C in membrane tubing (Spectrapor) of 12 000–14 000 *M_r* cut-off. At the end of the dialysis period the dialysis bags were opened and the dialysed proteins were mixed with 0.1 vol. of 20 mM-deoxycholate, 10% Triton X-100 or 10% octyl glucoside while still inside the dialysis tubing. The detergent-containing solutions were carefully brought in contact with the entire surface area of the membrane tubing, then pipetted out with transfer plastic pipettes into Microfuge tubes kept on ice. Recoveries of DG kinase activity in the dialysed samples were 100, 50 and 70% for aliquots from peaks A, B and C respectively.

Gel filtration of the arachidonoyl-DG kinase

An aliquot of peak C derived from hydroxyapatite chromatography of baboon brain was subjected to gel filtration on a column (1.6 cm × 85 cm) of Sephacryl S-300 that had been preequilibrated with buffer B containing 1% octyl glucoside, 0.3 M-KCl and 10% (v/v) glycerol. The elution was done at a flow rate of 16 ml/h. The activity of the arachidonoyl-DG kinase was assayed in the effluent and, on the basis of this, the enzyme's molecular mass was estimated from a linear plot of log(molecular mass) versus elution volume established with standard proteins (thyroglobulin, catalase, albumin and ribonuclease A) chromatographed under exactly the same conditions. The molecular mass of the octyl glucoside micelles in the chromatographic conditions was calculated from the elution volume of micelles that had been labelled with a trace of [³H]glycerol-labelled 2-arachidonoyl-*sn*-glycerol of sp. radioactivity 37.0 Ci/mmol.

Assay of DG kinase activity

Several assays were used to measure the activity of DG kinase.

(1) An assay carried out in the presence of octyl glucoside and both diC₁₀ and 18:0/20:4 DG, modified from MacDonald *et al.* [11]. This assay was carried out for 2 min at room temperature in the presence of 60 mM-Mops (pH 7.2)/0.86 mM-DTT/18 mM-MgCl₂/73 mM-octyl glucoside/2.9 mM-diC₁₀/2 mM-18:0/20:4 DG/3.3 mM-phosphatidylserine/0.52 mM-[γ-³²P]ATP (900–2500 c.p.m./pmol). Each assay mixture contained 5–30 µg of protein from the tissue homogenates or the brain fractions. Other assay constituents were as described in [11]. Under these conditions the rates of incorporation of ³²P from [γ-³²P]ATP into both PA₁₀ and PA were linear with respect to protein concentration. The products, long-chain PA and PA₁₀, were extracted and separated by t.l.c. on cellulose plates developed with the upper phase of an ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (90:50:20:30, by vol.) mixture as described in [11]. The PA and PA₁₀ were detected by Nile Blue staining and autoradiography (1–2 h exposure of Kodak XAR 2 film at –70 °C with an intensifying screen) and the corresponding bands were excised and counted for radioactivity in 10 ml of scintillation liquid (Ecolume; ICN).

(2) An assay carried out in the presence of octyl glucoside and single substrates at a concentration of 3.8 mM. Other assay constituents were as described for assay (1). Separate experiments done with several DG species, and the separated DG kinases, showed that the substrates were present at saturating concentrations under these conditions.

(3) An assay carried out in the presence of Triton X-100 instead of octyl glucoside and single substrates. The assay was carried out for 2 min at room temperature in the presence of 1% Triton X-100, 1.2 mM-DG and 1.1 mM-PS. Other assay constituents were as described for assay (1).

(4) An assay carried out in the presence of deoxycholate as described by Kanoh & Ohno [8]. Briefly, the assay was carried out for 10 min at 30 °C in the presence of 0.1 M-Tris, pH 8.0, 20 mM-NaF, 10 mM-MgCl₂, 2 mM-deoxycholate, 0.5 mM-DG and 2 mM-[γ-³²P]ATP (900 c.p.m./pmol).

The PA products of assays (2), (3) and (4) were extracted and chromatographed on thin-layer plates as described for assay (1). The Figure legends indicate the assay that was used for the particular experiments.

Other methods

Protein concentrations were measured by the method of Lowry *et al.* [21], with serum albumin as standard. LDH measurements were performed as described by Reeves & Fimognari [22].

RESULTS

Distribution of DG kinase activity among tissues

In order to select a tissue to address the questions stated in the Introduction, we used two different approaches to screen baboon tissues for their DG kinase activities. First, we used a mixed-micellar assay to study DG kinase activities in tissue homogenates. Secondly, we

Fig. 1. DG kinase activities in baboon tissues

Tissue homogenates from a 15 kg adult male baboon (*P. c. papio*) were assayed for DG kinase activity toward a mixture of 18:0/20:4 DG (■) and diC₁₀ (▨) as described in the Experimental section (assay 1). The results are expressed in pmol PA or PA₁₀ formed/min per mg of protein. Each value is the mean result for three assays. Similar results (not shown) were obtained in tissues from an adult male of another subspecies of baboon, *P. c. anubis*.

used a chromatographic technique to separate DG kinase species present in octyl glucoside extracts of the baboon tissues.

In the mixed-micellar assay, previously used for 3T3 cells [11], two substrates were present simultaneously at an equimolar concentration: 18:0/20:4 DG, a preferred substrate of the 3T3-cell arachidonoyl-DG kinase, and diC₁₀, a substrate used efficiently by the non-specific DG kinase activity in the cytosol of 3T3 cells [12]. The use of both substrates in the same assay guaranteed accurate comparisons of the DG kinase activities under identical conditions. The PAs formed by the phosphorylation of the two substrates differed in the length of their acyl chains. This difference allowed their separation by t.l.c. [11] and led to the choice of diC₁₀ as one of the substrates.

With this assay, large variations in the levels of DG kinase activities were observed among the fresh baboon tissues tested (Fig. 1). DG kinase activity toward 18:0/20:4 DG was highest by far in brain and testis. It was considerably lower in spleen and very low in heart, kidney, liver and muscle. Most tissues showed similar levels of enzyme activity toward the second substrate, diC₁₀. The enzyme(s) in testis, however, markedly preferred 18:0/20:4 DG to diC₁₀. In this respect, the testis DG kinase activity resembled that of Swiss-3T3-cell membranes [12]. The relative distribution of enzyme activity in brain, liver and spleen of a male rhesus monkey (*Macaca mulatta*) was similar to that observed in baboon tissues, and a high, preferential, enzyme activity toward 18:0/20:4 DG was also observed in bovine testis and rat testis (results not shown). It is therefore likely that similar variations in DG kinase activity can occur in other species.

Distribution of DG kinase species among baboon tissues

To screen baboon tissues by the chromatographic approach, we developed a method for separating several

Fig. 2. Separation of three baboon brain DG kinase species with different substrate preference

An octyl glucoside/KCl extract of baboon (*P. c. papio*) brain containing 100 mg of protein was chromatographed on a hydroxyapatite column and the DG kinases were eluted with a potassium phosphate gradient as described in the Experimental section. The DG kinase activity of the effluent was measured (assay 1) with a mixture of 18:0/20:4 DG (●) and diC₁₀ (□). Peaks A, B and C appeared at 0.15, 0.30 and 0.48 M-potassium phosphate respectively. Recovery of DG kinase activity in the hydroxyapatite fractions was 41% (18:0/20:4 DG activity) and 43% (diC₁₀ activity). Similar results were obtained during the chromatography of brain extracts from another baboon, *P. c. anubis* (results not shown).

DG kinases. Chromatography on hydroxyapatite resolved three DG kinases from octyl glucoside extracts of baboon brain (Fig. 2). Clear differences were observed in the substrate preference of the DG kinases. The ratio of activities toward 18:0/20:4 DG and diC₁₀ in the integrated peaks were 0.31, 0.27 and 6.1 for peaks A, B and C respectively. Therefore peak C contained a DG kinase that could be tentatively identified as an arachidonoyl-specific DG kinase. Subsequent detailed studies on the substrate preference of the DG kinases confirmed that the enzyme in peak C was an arachidonoyl-DG kinase (see below). Distribution of the recovered activity toward 18:0/20:4 DG among peaks A, B and C was 6, 19 and 75% respectively, indicating that most of the baboon brain activity toward 18:0/20:4 DG was contributed by the arachidonoyl-DG kinase. Peaks that were eluted in similar positions and showed similar preference toward DGs were also observed during the chromatography of brain extracts from a pigtail monkey, *Macaca nemestrina* (result not shown). Similar DG kinase species are therefore likely to be present in the brains of other species.

The three DG kinase species were also present in the other baboon tissues that we screened. However, both the relative distribution and the levels of the enzyme species varied significantly among the tissues (Fig. 3). Most noticeably, testis contained almost exclusively the arachidonoyl-DG kinase (peak C) in large amounts; brain contained large amounts of both the arachidonoyl-DG kinase and the DG kinase in peak B; spleen and kidney contained predominantly the arachidonoyl-DG kinase, albeit in small amounts, and liver showed

Fig. 3. Distribution of DG kinase species in baboon tissues

Octyl glucoside/KCl extracts of baboon tissues were chromatographed on hydroxyapatite as described for Fig. 2. A, B and C represent integrated areas of DG kinase activity toward 18:0/20:4 DG (■) and diC₁₀ (▨) of peaks A, B and C respectively and are comparable with those shown in Fig. 2. The data were normalized for a 100 mg protein load on each hydroxyapatite column. Recoveries of the fresh tissue homogenate DG kinase activity toward 18:0/20:4 DG in the hydroxyapatite fractions were: for brain, 41 %; kidney, 84 %; liver, 60 %; muscle, 76 %; spleen, 23 %; and testis, 28 %. Note that the scales of the DG kinase activities differ for each diagram.

strikingly low levels of this enzyme. The activity toward 18:0/20:4 DG contributed by the arachidonoyl-DG kinase was 92, 91, 75, 73, 69 and 22 % of the total recovered activity from testis, spleen, brain, kidney, muscle and liver respectively. Therefore, in all the baboon tissues tested except liver, an arachidonoyl-DG kinase was responsible for most of the activity toward 18:0/20:4 DG. The enzymes from the different tissues were eluted from hydroxyapatite with the same potassium phosphate concentration, indicating that they were similar. However, the relative activity of the arachidonoyl-DG kinases toward 18:0/20:4 DG and diC₁₀ varied; it was high for brain, kidney, spleen and testis (6.1–13.8), low for liver and muscle (3.3 and 1.8 respectively). The lower ratios found for the latter tissues may be less accurate, owing to the difficulty in measuring very low levels of arachidonoyl-DG kinase. However, we cannot exclude the possibility that the arachidonoyl-DG kinases of these tissues may exhibit different properties. We also do not know if the peaks of DG kinase activity contained single enzymes.

Recoveries of fresh-tissue-homogenate DG kinase activities were low in testis, spleen and brain (see the legend to Fig. 3). The basis for the low recoveries in testis, spleen and brain was not clear, but approx. 75 % of the DG kinase activity toward both substrates was lost when spleen or testis homogenates containing octyl glucoside were maintained at 4 °C for 24 h (spleen) or 48 h (testis). Therefore the unfractionated DG kinases of testis and spleen were unstable. Instability of DG kinase has been reported previously [5–8,12]. The distribution of the DG kinase species among the baboon tissues tested may have been influenced by the recovery of the enzymes. However, the recoveries of the DG kinase activities toward 18:0/20:4 DG and diC₁₀ were similar within each tissue, suggesting that no selective loss occurred. Furthermore, we recently found that the presence of glycerol greatly improved the recovery of testis DG kinase activities from hydroxyapatite (R. Lemaitre

Fig. 4. Substrate specificities of the DG kinase species

An octyl glucoside/KCl extract of baboon (*P. c. anubis*) brain homogenate was chromatographed on hydroxyapatite as shown in Fig. 2. The DG kinase activity in 10 μ l aliquots of 2 ml fractions from the middle of peak A (a), peak B (b) and peak C (c) were measured with DG species with different fatty acid chains and with an alkenylacylglycerol, in separate assays, as described in the Experimental section (assay 2). Each value is the mean for three assays. Similar results were obtained in a separate experiment (results not shown).

& J. Glomset, unpublished work). Under these conditions, the relative distribution of the testis DG kinases was that shown in Fig. 3.

Properties of the brain arachidonoyl-DG kinase

We selected brain tissue for further study because it

contained relatively high levels of all the DG kinases. We studied in detail the substrate preference of the arachidonoyl-DG kinase, measured the distribution of the DG kinases among particulate and supernatant fractions and determined the molecular mass of the arachidonoyl-DG kinase by gel filtration.

Substrate specificity. To establish the substrate preference of the arachidonoyl-DG kinase, we measured the enzyme's activity toward *sn*-1,2-DGs that contained various fatty acids. The arachidonoyl-DG kinase exhibited a strong preference for DGs containing arachidonic acid in the *sn*-2 position (Fig. 4c). The activity of the enzyme toward 18:0/20:4 DG was similar to that toward 16:0/20:4 DG and 14:0/20:4 DG and was 4–10-fold higher than the activity toward DGs containing monoenoic or dienoic fatty acids in the *sn*-2 position. Similar observations were made for the arachidonoyl-DG kinase in 3T3-cell membranes [12]. In addition, the brain arachidonoyl-DG kinase did not efficiently utilize 18:0/22:6 DG or 20:4/18:0 DG. Furthermore, the arachidonoyl-DG kinase showed diminished activity toward *O*-16:1/20:4 DRG, a diradylglycerol containing arachidonic acid in the *sn*-2 position and hexadec-1-ene linked by an ether bond in the *sn*-1 position. Therefore the arachidonoyl-DG kinase has a preference for an ester bond in the *sn*-1 position of the glycerol as well as a preference for an arachidonoyl moiety in the *sn*-2 position. In contrast, no substrate preference was found for the other DG kinases (Figs. 4a and 4b). In particular, the non-specific DG kinases utilized the substrate, diC₁₀, to a similar or greater extent than long-chain DGs. Furthermore, although the activities of the non-specific DG kinases toward diC₁₀ and 18:0/20:4 DG were similar when the substrates were presented separately, diC₁₀ was used preferentially by the enzymes when both substrates were presented together and competed for phosphorylation (Figs. 5a and 5b).

We investigated the ability of different assay systems to detect the substrate preference of the arachidonoyl-DG kinase. To this effect, the utilization of 18:0/20:4 DG and 18:1/18:1 DG by the brain DG kinases separated on hydroxyapatite was compared using three different assay systems: (a) the octyl glucoside assay used throughout the present study, (b) a deoxycholate-containing assay mixture, initially introduced by Kanoh &

Fig. 5. Comparison of the utilization of 18:0/20:4 DG and diC₁₀ by the DG kinases

The DG kinase activity in 10 µl aliquots from peaks A, B and C (Fig. 2) was measured with 18:0/20:4 DG (■) and diC₁₀ (▨), presented separately (a) or together (b), as outlined in the Experimental section (assay 2 and assay 1 respectively). Each value is the mean for three assays.

Ohno [8] and since widely used by other investigators and (c) a Triton X-100-containing assay mixture similar to the octyl glucoside assay mixture, but where octyl glucoside was substituted with Triton X-100 (Table 1). The activity of the arachidonoyl-DG kinase was strongly decreased in the deoxycholate assay as compared with that in the octyl glucoside assay. Furthermore, the enzyme exhibited no substrate preference in the deoxycholate assay (Table 1). The substrate preference also was not observed when the deoxycholate assay was performed for 2 min at room temperature in the presence of PS, conditions that resemble those of the octyl

Table 1. Comparison of the utilization of 18:0/20:4 DG and 18:1/18:1 DG by the brain DG kinases in different assay systems

An octyl glucoside/KCl extract of baboon (*P. c. anubis*) brain homogenate was chromatographed on hydroxyapatite as described for Fig. 2. Samples of 0.5 ml from peaks A, B and C were dialysed to remove the octyl glucoside present, then resuspended in octyl glucoside, deoxycholate or Triton X-100 as described in the Experimental section. DG kinase activity in the respective samples was measured in the presence of octyl glucoside, deoxycholate or Triton X-100 (assays 2, 4 and 3 of the Experimental section respectively). Results are expressed in nmol of PA formed/min per sample of the hydroxyapatite fractions. Each value is the mean result for two assays. Similar results were obtained in a separate experiment (not shown).

		PA formed (nmol/min per sample)					
Assay...		Octyl glucoside		Deoxycholate		Triton X-100	
Peak	Substrate...	18:0/20:4 DG	18:1/18:1 DG	18:0/20:4 DG	18:1/18:1 DG	18:0/20:4 DG	18:1/18:1 DG
A		1.30	1.08	0.64	0.72	0.21	0.20
B		0.22	0.18	0.38	0.36	0.02	0.02
C		1.89	0.54	0.15	0.11	0.91	0.23

Table 2. Partition of DG kinase activities and LDH activity in brain supernatant and particulate fractions

Baboon (*P. c. papio*) brain particulate fraction and 100000 *g* supernatant were prepared from a brain homogenate and the particulates were lysed hypo-osmotically as described in the Experimental section; the initial supernatant, the lysis supernatant and the lysed particulate fraction were analysed for their LDH activity and their DG kinase activity toward a mixture of 18:0/20:4 DG and diC₁₀ (assay 1). Results are expressed as percentages of total enzyme activities in the homogenate. Note that in this experiment we used the brain homogenate shown in Fig. 1 and that similar results were obtained with the brain homogenate of another subspecies of baboon, *P. c. anubis* (results not shown).

Enzymic activity	Fraction...	Activity (% of that in homogenate)		
		Initial 100000 <i>g</i> supernatant	Supernatant from lysis	Lysed particulate fraction
LDH		67	28	5
18:0/20:4 DG kinase		15	20	60
DiC ₁₀ kinase		30	30	40

glucoside assay. The activity of the arachidonoyl-DG kinase in the Triton X-100 assay was half that in the octylglucoside assay. However, in contrast with the deoxycholate assay, the enzyme exhibited a 4-fold preference for 18:0/20:4 DG as compared with 18:1/18:1

DG in both the octyl glucoside assay and the Triton X-100 assay. The non-specific DG kinase from peak A showed about twice as much activity and that from peak B about half as much activity in the octyl glucoside assay as in the deoxycholate assay. Both enzymes showed decreased activities in the Triton X-100 assay. No substrate preference was observed for the non-specific DG kinases in any of the assay systems.

Membrane association. We explored the possibility that the DG kinase species might differ in their membrane association by investigating their distribution between baboon brain particulates and 100000 *g* supernatant, prepared as described in the Experimental section. Then, because the particulates still contained LDH activity, they were subjected to hypo-osmotic lysis, and both a lysed particulate fraction and a lysis supernatant were

Fig. 6. Partitioning of the DG kinase species of baboon brain between particulate and supernatant fractions

An octyl glucoside/KCl extract of baboon (*P. c. anubis*) brain particulate fraction and a brain supernatant fraction, which had been supplemented with octyl glucoside and KCl to corresponding final concentrations, were separately chromatographed on hydroxyapatite as described for Fig. 2. The results are presented as in Fig. 3. The DG kinase activities were normalized to reflect the relative amounts in particulate and supernatant fractions. Recovery of DG kinase activity toward both substrates was approx. 50% for both the particulate and supernatant fractions. Similar results were obtained with particulate and supernatant fractions from a male macaque monkey (*Macaca nemestrina*) (results not shown).

Fig. 7. Gel filtration of the arachidonoyl-DG kinase

An octyl glucoside/KCl extract of baboon (*P. c. anubis*) brain homogenate was chromatographed on hydroxyapatite as described for Fig. 2. A 2 ml fraction from the middle of peak C was chromatographed on a Sephacryl-300 column as described in the Experimental section. The DG kinase of the effluent was measured with 18:0/20:4 DG (assay 2). The arrow indicates the elution volume of octyl glucoside micelles, measured as described in the Experimental section. Recovery of DG kinase activity in the column fractions was 35%. The column was calibrated with thyroglobulin, catalase, albumin and ribonuclease A.

prepared. The original 100 000 g supernatant, the lysis supernatant and the lysed particulate fraction were analysed for their contents of LDH activity and DG kinase activity toward 18:0/20:4 DG and diC₁₀ (Table 2). The distribution of the DG kinase activities was very different from that of the soluble enzyme, LDH. A substantial fraction of each DG kinase activity was found in the lysed particulate fraction and was clearly membrane-associated. However, the fraction of the activity toward 18:0/20:4 DG was higher than that of the activity toward diC₁₀. Similar results were obtained with a brain homogenate prepared from a juvenile male baboon *P. c. anubis*.

The distribution of the DG kinases among the brain particulate and supernatant fractions was further investigated by chromatography on hydroxyapatite. Most of the arachidonoyl-DG kinase was again found in the particulate fraction. However, the other DG kinases were distributed more evenly (Fig. 6). The hydroxyapatite chromatography pattern of the particulate lysis supernatant resembled that of the original supernatant (results not shown).

Finally, the solubility of the brain DG kinase species eluted from hydroxyapatite was tested by diluting the octyl glucoside to a concentration of 0.25%, which is below the critical micellar concentration. After dilution and centrifugation at 100 000 g, 95, 75 and 15% of the DG kinase activity in peaks A, B and C respectively remained in solution.

We conclude from these data that (1) each of the three DG kinases of baboon brain partitions between particulate and supernatant fractions, (2) most of the arachidonoyl-DG kinase is associated with the particulate fraction and remains soluble only when the concentration of detergent exceeds the critical micellar concentration, and (3) the non-specific DG kinases are predominantly associated with supernatant fractions.

Apparent molecular mass of the arachidonoyl-DG kinase. The brain arachidonoyl-DG kinase was eluted from a Sephacryl S-300 column as a single peak with an apparent molecular mass of approx. 150 kDa (Fig. 7). The molecular mass of octyl glucoside micelles in the same buffer system was found to be 20 kDa. The arachidonoyl-DG kinase was only soluble in octyl glucoside concentrations above the critical micellar concentration (see above), indicating that it was associated with the detergent micelles. Consequently, the molecular mass of the enzyme corrected for that of one micelle is probably no greater than 130 kDa. If the enzyme contained additional bound molecules or was present in a polymeric form, its true molecular mass might be considerably lower. The enzyme could not be chromatographed under dissociating conditions because of its instability. Therefore a more precise determination of the enzyme's molecular mass will have to await its purification to homogeneity.

DISCUSSION

We have developed a chromatographic method that separates an arachidonoyl-DG kinase from non-specific DG kinases. Using this method, we observed large amounts of arachidonoyl-DG kinase in brain and testis, and strikingly low amounts in liver. Subsequent studies focused on the brain arachidonoyl-DG kinase. The brain

enzyme showed a strict preference for *sn*-1-acyl-2-arachidonoyl DG, partitioned mostly with particulate fractions and exhibited an apparent molecular mass of 130 kDa.

To our knowledge this is the first report of a chromatographic separation of DG kinase species that differ in their substrate preference, as well as the first demonstration of the existence of an arachidonoyl-DG kinase in animal tissues. Yamada & Kanoh [9] showed the existence of three soluble DG kinase species in pig tissues. Two were found in brain and thymus and another one in platelets. We cannot correlate the DG kinase species observed in baboon tissues with the ones present in pig tissues because of major differences between the two studies. In particular, different chromatographic techniques were used to separate the enzymes; the separation in our studies was done in the presence of octyl glucoside; our studies included both soluble and membrane-extracted DG kinases; the pig enzymes were not studied for their substrate preference. It should be noted that the specificity of the brain arachidonoyl-DG kinase cannot be detected in assays containing deoxycholate. The mechanism by which deoxycholate inhibits phosphorylation of the preferred substrate of the enzyme is not known. In contrast with the arachidonoyl-DG kinase, the non-specific DG kinases from brain show similar activities and no substrate preference in the presence of deoxycholate or octyl glucoside. The selective inhibition of the arachidonoyl-DG kinase activity by deoxycholate may provide an explanation for the lack of detection of this enzyme by other investigators.

Brain arachidonoyl-DG kinase was found mostly associated with particulate fractions. The enzyme required both 0.3 M-KCl and 2% octyl glucoside to be extracted efficiently from membranes. In addition, the enzyme was largely insoluble in the absence of detergent micelles, indicating that it is a hydrophobic protein. These properties suggest that the arachidonoyl-DG kinase is a membrane protein. However, small but reproducible amounts of arachidonoyl-DG kinase were also found in brain supernatant fractions. It is possible that the enzyme exists both as a membrane protein and as a soluble complex or that it translocates from one compartment to another. We also cannot exclude the possibility that the arachidonoyl-DG kinase in the supernatant was a proteolytic fragment of the membrane enzyme, though a 'cocktail' of proteinase inhibitors were present throughout the preparation of brain fractions. The observation of smaller species during the gel filtration of the arachidonoyl-DG kinase from brain homogenate may be relevant to this issue.

The non-specific DG kinases were also found in both supernatant and particulate fractions. Unlike the arachidonoyl-DG kinase, however, they partitioned preferentially into supernatant fractions and were mostly soluble in the absence of detergent micelles. The behaviour of the non-specific DG kinases may resemble that of the soluble 80 kDa DG kinase of pig brain which is found in both soluble and membrane compartments [23]. In addition, translocation of soluble DG kinases has been observed in other systems [7,24,25].

The brain arachidonoyl-DG kinase showed a strict preference for *sn*-1-acyl-2-arachidonoyl DG. The arachidonic acid chain could not be substituted by mono- or di-unsaturated fatty acid chains, as was previously observed with the 3T3-cell arachidonoyl-DG kinase activity.

In addition, the arachidonic acid could not be substituted by docosahexaenoic acid or be esterified to the *sn*-1 position, nor could an *sn*-1 alkenyl ether be substituted for an *sn*-1-acyl ester. It is therefore likely that the binding site of the arachidonoyl-DG kinase recognizes several structural components of *sn*-1-acyl-2-arachidonoyl DGs in addition to the arachidonoyl chain. It should be pointed out that our findings about the enzyme's specificity are limited to mixed-micellar-assay systems. In order to assess the biological relevance of the present observations it will be necessary to determine whether the arachidonoyl-DG kinase retains its specificity when incorporated in well-defined liposomes that contain a mixture of substrates and phosphatidylserine.

The significance of the multiplicity of DG kinase species remains to be established. An interesting question will be to determine whether several DG kinase species coexist in the same cells. This question will be best addressed when the arachidonoyl-DG kinase has been purified and specific antibodies and mRNA probes become available. The functions of the individual DG kinase species are also unknown. The specificity of the arachidonoyl-DG kinase for *sn*-1-acyl-2-arachidonoyl-DG is compatible with the possibility that the enzyme is responsible for the phosphorylation of the DG that is released during the breakdown of phosphoinositides. Indeed, evidence has been presented that an arachidonoyl-DG kinase in 3T3 cells participates in the phosphatidylinositol cycle [11]. Whether the arachidonoyl-DG kinase in baboon tissues has a role analogous to the corresponding enzyme in 3T3 cells is not known. One of the isoforms of protein kinase C in brain, type III protein kinase C, is preferentially activated by 18:0/20:4 DG [26]. Therefore the arachidonoyl-DG kinase may play a role in attenuating the activation of type III protein kinase C in this tissue.

Additional questions are raised by the observation of large variations in the level of the arachidonoyl-DG kinase among baboon tissues. In particular, what is the significance of the very low levels of enzyme in liver and the high levels in brain and testis? Is the small amount of arachidonoyl-DG kinase in liver sufficient to participate in the phosphatidylinositol cycle or does another DG kinase fulfil this role in this tissue? Does the arachidonoyl-DG kinase have an additional specialized function in brain and testis?

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