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Different effects of sphingosine, R59022 and anionic amphiphiles on two diacylglycerol kinase isozymes purified from porcine thymus cytosol

Fumio Sakane, Keiko Yamada and Hideo Kanoh

Department of Biochemistry, Sapporo Medical College, Sapporo 060, Japan

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Porcine thymus cytosol contains two immunologically distinct forms of diacylglycerol kinase (DGK) [Yamada, K. and Kanoh, H. (1988) Biochem. J. 255, 601–608]. These 2 DGK species, having apparent molecular masses of 80 and 150 kDa, were purified from the thymus cytosol. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the 150-kDa DGK gave 2 polypeptide bands of 50 and 75 kDa, whereas the 80-kDa DGK yielded a single protein band. The 80-kDa DGK was markedly activated by 10–20 μM sphingosine as well as by the known anionic activators such as phosphatidylserine and deoxycholate. In contrast, the 150-kDa DGK was fully active in the absence of the anionic activators and was strongly inhibited by sphingosine (IC_{50}, 20 μM). The putative DGK inhibitor R59022 inhibited the 80-kDa DGK (IC_{50}, 10 μM), but had little effect on the 150-kDa form. It is therefore clear that in the thymus cytosol there are at least 2 DGK isozymes operating under different control mechanisms.

Diacylglycerol kinase; Isozyme; Purification; Sphingosine; R59022; Thymus; (Pig)

1. INTRODUCTION

In protein kinase C-mediated signal transduction [1,2] diacylglycerol kinase (DGK) is thought to play an important role in regulating the intracellular level of diacylglycerol, an activator of protein kinase C. In fact, the putative DGK inhibitor R59022 [3–9] and diacylglycerol analogues [10,11] have been reported to potentiate cellular protein kinase C activity probably by elevating the diacylglycerol level. Since phosphatidate, the product of DGK reaction, is also of biological importance [12–14], knowledge concerning DGK is essential for the elucidation of the mechanism of signal transduction. However, only limited information is available concerning DGK because of the difficulty associated with its assay [15,16] and purification.

We have purified a DGK having a molecular mass of 80 kDa (80-kDa DGK) from pig brain [17,18]. By using rabbit antibodies to 80-kDa DGK [19] as a tool, we have found that the occurrence of 80-kDa DGK is restricted to brain and lymphocytes [20] and that several DGK species, which do not react with antibodies to 80-kDa DGK, are present in pig and human cells [19–21]. It is, therefore, likely that the agonist-induced accumulation of phosphatidate observed in a wide range of cells [22] is due to DGK isozymes other than the 80-kDa DGK.

In the present study, we have purified the 80-kDa DGK and another DGK having a molecular mass of 150 kDa from porcine thymus cytosol. Examination of the purified enzymes showed that the 2 DGK isozymes are affected by various activators and inhibitors in markedly different manners. Fur-

Correspondence address: H. Kanoh, Department of Biochemistry, Sapporo Medical College, West-17, South-1, Sapporo 060, Japan

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thermore, sphingosine, which has been widely used as an inhibitor of protein kinase C [23–28], has been found to activate the 80-kDa DGK directly.

2. MATERIALS AND METHODS

2.1. *Purification of DGK isozymes*

We have previously shown that pig thymus cytosol is rich in DGK activity (15–20 nmol product/min per mg protein), 15–20% of which is resistant to heat treatment (41°C, 5 min) [20]. Heat-labile DGK, which is immunologically identical with the 80-kDa enzyme purified from pig brain [17,18], could be purified by the same method as reported for the pig brain enzyme [17,18]. In brief, pig thymus (400 g) was homogenized with 1.2 l buffer A (25 mM Tris-HCl, pH 7.4,/0.25 M sucrose/1 mM EDTA/0.5 mM DTT/10 μM ATP/10 μg/ml leupeptin) containing 50 mM NaCl. The cytosol fraction obtained from the homogenate was divided into 2 equal portions, and each portion was applied to a DE-52 (Whatman) column (5 × 13 cm) equilibrated with the same buffer. After washing the 2 columns with the same buffer, the heat-labile activity was eluted with buffer A containing 0.1 M NaCl (see fig.1). The enzyme thus eluted was further purified by ammonium sulfate fractionation (40–60% saturation), Sephadex G-150 gel filtration, ATP-agarose column chromatography, and hydroxyapatite column chromatography. The specific activity of the final preparation was 15 μmol product/min per mg protein.

After elution of the heat-labile activity from the DE-52 columns, heat-stable DGK activity could be eluted with buffer A containing 0.2 M NaCl (fig.1). The active fractions were pooled (400 ml) and stirred for 2 h with 70 ml heparin-Sepharose (Pharmacia) equilibrated with buffer A containing 0.2 M NaCl. The Sepharose beads were washed with 250 ml of the same buffer and packed to a column. After washing the column with 1 l of the same buffer, elution was conducted with a linear NaCl gradient (0.2–1.0 M) in buffer A. Heat-stable DGK activity was thereby eluted at about 0.7 M NaCl. The active fractions were pooled and brought to 40% saturation with respect to ammonium sulfate. After stirring for 30 min, the precipitate was collected by centrifugation (8000 × g for 20 min), dissolved in 10 ml buffer A containing 0.2 M NaCl, and applied to a Sephadex G-150 column (2.6 × 80 cm) equilibrated with the same buffer. The activity appeared at an elution volume corresponding to M_r 150000. The pooled active fractions were diluted 4-fold with buffer A and applied to an ATP-agarose column under the same conditions as described for the 80-kDa DGK [17]. The eluted enzyme was dialyzed against buffer A containing 50 mM NaCl and applied to a Mono Q HR 5/5 column (Pharmacia) connected to an FPLC system (Pharmacia). The enzyme formed a sharp peak at 180 mM NaCl when eluted from the Mono Q column by a linear NaCl gradient (50–350 mM) in 20 ml buffer A. The enzyme thus obtained was very labile and could not be processed further, but could be stored at −80°C in the presence of bovine serum albumin (1 mg/ml). The specific activity of the final preparation was difficult to assess because of rapid inactivation, but was usually in the range of 8–9 μmol product/min per mg protein. This represented 2500–3000-fold purification as estimated from the heat-stable DGK activity present in the starting cytosol.

2.2. *DGK activity assay*

The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EGTA, 1 mM DTT, 20 mM NaF, 2 mM [γ-^{32}P]ATP (ICN, 10000–20000 cpm/nmol), 10 mM $MgCl_2$, 0.5 mM 1,2-diolein (Sigma) and enzyme (usually 10 μl) in a final volume of 100 μl. When necessary, the mixture also received deoxycholate, phosphatidylserine (Avanti), or sphingosine (Sigma). Chloroform solutions of these 2 compounds were evaporated to dryness under N_2 and dispersed into 10 mM Tris-HCl (pH 7.4)/0.15 M NaCl by sonication. Prior to the start of the reaction, diolein was dispersed into the reaction mixture lacking ATP and enzyme by sonication. The reaction was started by adding ATP and enzyme and run for 3 min at 30°C. The formation of phosphatidate was determined by the butanol extraction method [17]. In experiments using octylglucoside, the activity was measured by thin-layer chromatography as described in [29]. The DGK inhibitor R59022 was kindly supplied by Dr D. de Chaffoy de Courcelles of Janssen Company (Belgium) and used as suspension in dimethyl sulfoxide. The final dimethyl sulfoxide concentration was less than 1% (v/v) and did not affect the enzyme activity. During the enzyme purification, the activities of 2 DGKs were assayed in the presence of 0.5 mM deoxycholate, though this detergent was later found to be inhibitory to the heat-stable kinase.

2.3. *Other methods*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described in [20] using a 7.5% separating gel. Molecular weight markers were from Sigma. Immunoblotting and heat treatments of DGKs were carried out as described in [20].

3. RESULTS

Porcine thymus cytosol contains 2 DGK species, which can be distinguished from each other with respect to reactivity with antibodies to pig brain 80-kDa DGK and heat stability [20]. As shown in fig.1, these 2 DGK species could be clearly separated from each other by stepwise elution from a DE-52 column. The activity eluted with 0.1 M NaCl was completely inactivated by treatment at 41°C for 5 min, whereas more than 90% of the activity eluted with 0.2 M NaCl was resistant to this treatment. We could purify these 2 DGKs as described in section 2.1. Although we previously reported that the heat-stable enzyme in crude cytosol had an apparent M_r of 280000 [20], gel filtration of the partially purified preparation indicated that its molecular mass is 150 kDa. In this study, therefore, the heat-stable kinase will be called 150-kDa DGK.

As shown in fig.2, upon SDS-PAGE, the 80-kDa (heat-labile) DGK yielded a major protein band at 83 kDa together with a faint band at 70 kDa. As noted previously [20], both the major and faint

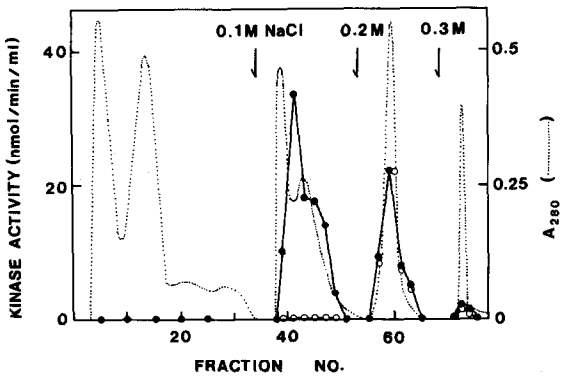

Fig.1. Separation of DGK isozymes by DE-52 column chromatography. Pig thymus cytosol (5 ml, 9.74 mg protein/ml) was applied to a column of DE-52 (2 × 5 cm) and 3-ml fractions were collected. After washing the column with buffer A containing 50 mM NaCl, the enzyme was eluted stepwise as indicated in the figure. (●—●) Total enzyme activity; (○—○) the activity remaining after heat treatment at 41°C for 5 min.

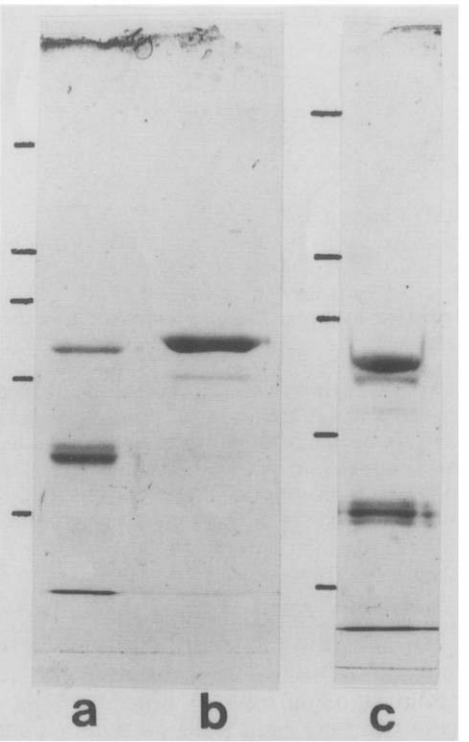

Fig.2. SDS-PAGE of purified DGK. The final preparations of heat-stable (lane a, 3 μg protein) and heat-labile (lane b, 5 μg) species of DGK were analyzed under reducing conditions. Lane c is the electrophoresis of the mixture of the two enzyme species. The bars indicate the position of molecular weight markers: from the top, myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase b (97.4 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa). Gels were stained with Coomassie brilliant blue.

bands reacted with antibodies to the 80-kDa DGK in immunoblot analysis (not shown). Furthermore, storage of the enzyme at −20°C for longer time tended to increase the 70-kDa band. It is therefore likely that the 70-kDa band was a degradation product of the 83-kDa enzyme. On the other hand, the 150-kDa (heat-stable) DGK gave 2 bands at 75 and 50 kDa, and this pattern was unchanged when analyzed under non-reducing conditions. Although the 2 bands were co-eluted from the Mono Q column together with the enzyme activity, we could not confirm whether the 150-kDa enzyme was composed of 2 different subunits, because the enzyme was very unstable at this purification stage. However, the enzyme preparation did not contain a polypeptide that is identical with the 80-kDa enzyme (fig.2). Moreover, upon immunoblot analysis neither the 75- nor 50-kDa band reacted with anti-80-kDa DGK antibodies (not shown). It was thus clear that the thymus cytosol contains 2 different DGK isozymes.

Recently, the mixed micelle method using octylglucoside has been developed for assay of *Escherichia coli* DGK [15,16] and applied to the fibroblast enzyme [29]. In our hands, however, octylglucoside was extremely inhibitory to the purified 80-kDa DGK at any concentration tested, though the 150-kDa DGK was somewhat more resistant to the detergent (data not shown). The presence of relatively high concentrations of diacylglycerol and phosphatidylserine, as used in this assay method [15,16,29], protected the 150-kDa enzyme, but not the 80-kDa enzyme, from the detergent-induced inhibition to a limited extent (data not shown). Since our purpose was to compare the enzymatic properties of the 2 DGK isozymes, we decided not to use the mixed micelle method for assay. Instead, we used sonicated diacylglycerol suspension as substrate in the following experiments.

As reported for pig brain 80-kDa DGK [17], the 80-kDa kinase purified from pig thymus exhibited a very low activity in the absence of anionic activators such as phosphatidylserine and deoxycholate (fig.3). In marked contrast, the 150-kDa

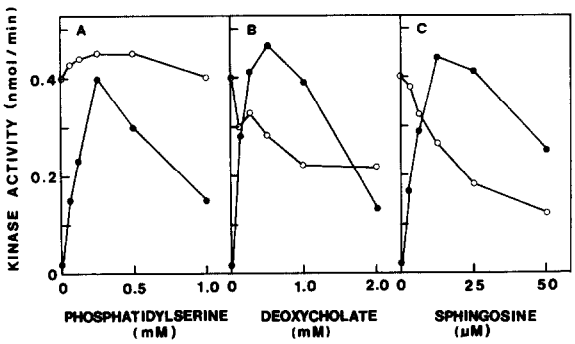

Fig.3. The activity of 2 DGKs in the presence of added lipids. The activities of the 80-kDa (●—●, 30 ng of protein) and the 150-kDa (○—○, 50 ng) diacylglycerol kinases were assayed in the presence of phosphatidylserine (A), or deoxycholate (B), or sphingosine (C) as indicated in the figure. Two species of DGK were assayed in parallel in the same experiment.

kinase was fully active without the activators. In fact, deoxycholate, but not phosphatidylserine, was rather inhibitory. An unexpected finding was that sphingosine, a long carbon chain base, activated the 80-kDa DGK to extents similar to those attainable by the anionic activators (fig.3c). This base was, however, a potent inhibitor of the 150-kDa DGK, causing 50% inhibition at 20 μM. This concentration is similar to or lower than that used to inhibit cellular protein kinase C activity [23–28].

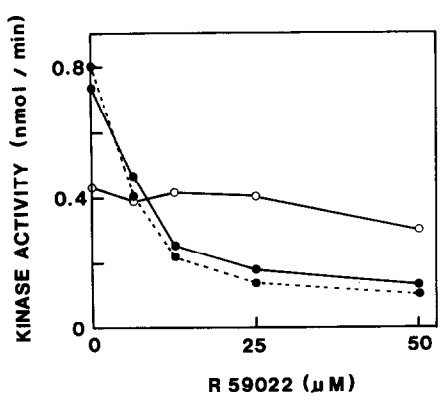

Fig.4. Effects of R59022 on 2 DGK isozymes. The effects of various concentrations of R59022 were tested in the reaction mixture containing 0.2 mM phosphatidylserine and 0.5 mM diolein. (●—●) 80-kDa DGK (30 ng of protein); (○—○) 150-kDa DGK (100 ng). The 80-kDa DGK was assayed also in the presence of 20 μM sphingosine instead of phosphatidylserine (dotted line).

The DGK inhibitor R59022 has been reported to inhibit DGK activity of human erythrocyte and platelet membranes [3], but its effect on purified DGKs has not yet been examined. As shown in fig.4, R59022 inhibited the purified 80-kDa DGK in the presence of phosphatidylserine or sphingosine with an IC_{50} value of about 10 μM, a concentration comparative to that used to inhibit DGK activity of intact cells [3–9]. However, R59022 failed to inhibit the 150-kDa DGK significantly at concentrations lower than 25 μM. At 100 μM this compound inhibited the 150-kDa enzyme by about 40% (not shown), indicating that IC_{50} is higher than 100 μM in this case.

4. DISCUSSION

In this study, in addition to heat-labile 80-kDa DGK, a heat-stable DGK having an apparent molecular mass of 150 kDa was purified from porcine thymus cytosol. Although the chemical nature of the 150-kDa enzyme remains to be elucidated, this species is immunologically distinct from the 80-kDa DGK. Furthermore, these two DGK isozymes differ markedly in their responses to various activators and inhibitors, suggesting that they are modulated by entirely different mechanisms. The 2 DGK isozymes were inhibited to different extents by octylglucoside used in the mixed micelle assay method [15,16,29]. This assay method may be applied to the 150-kDa enzyme, but appeared to be totally inapplicable to the 80-kDa DGK because of strong inhibition caused by this detergent. When applying this assay method, it appears to be necessary to check whether the enzyme preparation contains octylglucoside-sensitive DGK species.

The activity of DGK, including that from *E. coli* membranes [15,16], has been customarily assayed in the presence of anionic activators such as acidic phospholipids and deoxycholate. The results of the present study, however, indicate that sphingosine, a cationic lipid, markedly activates the 80-kDa DGK. The 150-kDa enzyme, on the other hand, is strongly inhibited by sphingosine. Sphingosine is generally thought to be an inhibitor of protein kinase C [23] and has been widely used to assess the function of protein kinase C in intact cells [24–28]. However, it is likely that some of the reported effects of sphingosine are at least partly due to ac-

tivation or inhibition of different species of DGK. In fact, our unpublished results indicate that sphingosine causes marked accumulation of phosphatidate in a human leukemic cell line (Jurkat), which has been reported to be rich in 80-kDa DGK [21]. This novel action of sphingosine should be carefully studied in different cells containing different DGK isozymes.

R59022 has been developed as a DGK inhibitor. It does inhibit 80-kDa DGK, but does not affect the activity of 150-kDa DGK. So far the effect of this inhibitor has been most actively investigated in such cells as platelets [3-5] and neutrophils [6-9], but our previous studies have shown that these cells apparently lack the R59022-sensitive 80-kDa enzyme [20,21]. It is not known whether these cells contain unidentified DGK species that are R59022-sensitive and immunologically distinct from the 80-kDa enzyme.

The occurrence of DGK isozymes having different specificities toward molecular species of diacylglycerol has been described in murine fibroblasts [29]. We do not know whether the DGK isozymes purified in this study exhibit any specificity toward diacylglycerol species. Detailed enzymological studies are required to determine their specificities and also to elucidate the mechanisms of their activation and inhibition by various effectors. We have shown that the 80-kDa DGK is predominantly cytosolic and highly enriched in pig and human lymphocytes [20,21]. The distribution of the 150-kDa enzyme in different types of cells and its intracellular localization would be an interesting subject of future studies.

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