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Immunoquantitation of 80 kDa diacylglycerol kinase in pig and human lymphocytes and several other cells

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80 kDa diacylglycerol kinase (DGK) was immunoquantitated in cell homogenates and subcellular fractions. It was extremely abundant in the cytosol of various lymphocytes and comprised, in the highest case, more than 0.2% of the total soluble protein in T cell-enriched pig splenocytes. The lymphocyte membrane contained less than 10% of the total cellular DGK protein. The content of 80 kDa DGK in the human T cell leukemic cell line, Jurkat (360 ng/mg homogenate protein), was similar to those in pig and human peripheral blood lymphocytes. In contrast, the enzyme level was very low in the human promyeloblastic cell line, HL-60 (< 10 ng/mg homogenate protein), and was undetectable in human polymorphonuclear leukocytes. These findings indicate that the content of 80 kDa DGK is markedly variable depending on the type of cells, even though all these cells are known to accumulate phosphatidate rapidly upon cell stimulation.

Diacylglycerol kinase; Immunoquantitation; Lymphocyte; (Human, Jurkat cell, HL-60 cell, Polymorphonuclear leukocyte)

1. INTRODUCTION

Diacylglycerol kinase (DGK) is responsible for rapid accumulation of phosphatidate in agonist-stimulated cells [1,2]. It also plays an important role in metabolic control by initiating the resynthesis of phosphatidylinositols and regulating the level of diacylglycerol, a second messenger [3–5]. Phosphatidate and diacylglycerol are also precursors of the other phospholipids. Since DGK performs such important functions, it is conceivable that its activity is strictly controlled in stimulated and normal cells. The only DGK so far purified from animal sources is that from pig brain that has an apparent molecular mass of 80 kDa [6–8]. However, we have recently shown that pig tissues contain at least two other DGK isozymes that are immunologically distinct from the 80 kDa enzyme [9]. Moreover, it has also been shown that 80 kDa DGK occurs only in brain and lymphoid tissues and is almost absent in cells such as platelets and hepatocytes [9]. The multiplicity of DGK has also been reported in Swiss 3T3 cells [10,11]. The possibility therefore arises that different DGK isozymes function in different cell types under different control mechanisms. To elucidate the physiological significance of DGK, therefore, it is desirable to quantitate each DGK isozyme in different types of cells.

Here, we report the quantitation of 80 kDa DGK in human and pig lymphocytes and several other cells. For this purpose, we used immunoquantitation for two reasons. Firstly, only the 80 kDa isozyme can be measured by this method. Secondly, by using this procedure, it is possible to avoid difficulties associated with DGK activity assays. The difficulties have been discussed in detail for *E. coli* DGK [12,13]. Although the mixed micellar method has been developed for the bacterial enzyme [12,13] and recently applied to the fibroblast enzyme [10,11], we have found that octylglucoside used in this method strongly inhibited purified 80 kDa DGK (unpublished). Fur-

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Abbreviations: DGK, diacylglycerol kinase; PBL, peripheral blood lymphocytes; PMN, polymorphonuclear leucocytes.

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thermore, the occurrence of an endogenous inhibitor in rat brain has been reported [14].

2. MATERIALS AND METHODS

2.1. *Anti-80 kDa DGK antibody and human cell lines*

The preparation and characterization of rabbit antibody to pig brain 80 kDa DGK have been described [8,9]. The human T cell leukemic cell line, Jurkat, and the human promyeloblastic cell line, HL-60, were donated by Dr T. Uede of Sapporo Medical College. The cells were cultivated under 5% CO_2/95% air in RPMI 1640 medium (Nissui, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA).

2.2. *Isolation of cells*

Pig splenocytes and thymocytes were isolated, after hypotonic hemolysis if necessary, by lymphocyte M (Cedarlane, Canada) gradient centrifugation as in [15]. Splenocyte were fractionated into T cell- and B cell-enriched populations by a nylon fiber column [15]. Peripheral blood lymphocytes (PBL) and polymorphonuclear leucocytes (PMN) were isolated from fresh human blood by dextran sedimentation, hypotonic hemolysis, and Ficoll-Hypaque (Pharmacia, Sweden) centrifugation [15].

2.3. *Cell homogenization and fractionation*

Cells to be examined were suspended in 25 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 2 mM EDTA, 0.5 mM DTT, 10 μM ATP, and 1 mM phenylmethylsulfonyl fluoride. The suspension was briefly sonicated at 0°C and centrifuged at $200 \times g$ for 5 min to remove cell debris, and the resultant supernatant was used as cell homogenate. The homogenate was fractionated into the cytosolic and membrane fractions by centrifugation at $100000 \times g$ for 1 h. The membrane pellet was washed once with the same buffer. For immunoblot analysis, each preparation was boiled for 3 min in Laemmli sample buffer [16].

2.4. *Purification of DGK*

80 kDa DGK was purified from pig thymus cytosolic fraction by the same method as described for pig brain [6,7]. The purified enzyme was more than 90% pure judging from scanning densitometry of SDS-polyacrylamide gels that had been stained with Coomassie brilliant blue.

2.5. *Quantitation of 80 kDa DGK by immunoblotting*

Immunoblotting was carried out as in [9]. Briefly, cell proteins were separated by SDS-polyacrylamide gel electrophoresis [16], followed by electrophoretic transfer (in a Bio-Rad apparatus) to a nitrocellulose membrane (0.45 μm, Schleicher and Schuell, FRG). The membrane was treated with anti-80 kDa DGK immunoglobulin G (50 μg/ml) for 2 h, and then incubated with ^{125}I-protein A (0.5 μg, 14–20 μCi/20 ml, ICN) for 1 h. After extensive washing, the immunoreactive bands were detected by autoradiography (Fuji X-ray film) at −80°C. Quantitation of 80 kDa DGK was performed by means of densitometric tracing of the radioactive bands in a densitometer (Asuka model OZ-802, Japan). At least two different amounts of purified 80 kDa DGK (20–70 ng) and crude cell homogenate (10–250 μg protein) were analyzed on the same gel to quantify the enzyme.

2.6. *Other methods*

Immunoprecipitation of soluble DGK [9], assay of DGK activity [6] and protein determination [17] were carried out as described. Bovine serum albumin was used as a standard for protein determination.

3. RESULTS AND DISCUSSION

Fig.1 shows the results of immunoquantitation of purified pig thymus 80 kDa DGK in the presence and absence of crude pig thymus cytosol. The immunoreactive signal determined by densitometric tracing of the autoradiograph increased linearly as the amount of purified enzyme applied was increased. The slope of the calibration curve was not significantly altered by the addition of crude cytosol, indicating that this method is applicable to DGK quantitation in crude homogenates. As shown in fig.2, the antibody used could recognize 80 kDa DGK not only in pig thymus but also in pig and human lymphocytes. The cross-reactivity of the antibody with the human enzyme was confirmed by the finding that DGK activity in the cytosolic fractions from human cells could be immunoprecipitated by the antibody (fig.3). The antibody precipitated more than 90% of the activity from PBL and Jurkat, but only limited precipitation (40%) was achieved from HL-60, probably because of the presence of non-immunoreactive DGK isozyme(s) in the last-mentioned cell line. As can be seen in fig.2, the 80 kDa DGK content was markedly variable depending on cell types. In human cells, HL-60 contained the 80 kDa isozyme to a much lesser extent than lymphocytes, and no immunoreactive band was detected for PMN even when large amounts (up to 250 μg) of homogenate protein were applied.

The results of immunoquantitation of 80 kDa DGK in different pig and human cell homogenates, cytosols and membrane fractions are summarized in fig.4. The most remarkable finding was that in all the lymphocyte preparations examined the enzyme was highly enriched in the soluble (cytosolic) fraction as compared to the membrane fraction. Since the cytosolic fraction contained 50–60% of the total homogenate protein, it can be calculated that more than 90% of cellular 80 kDa

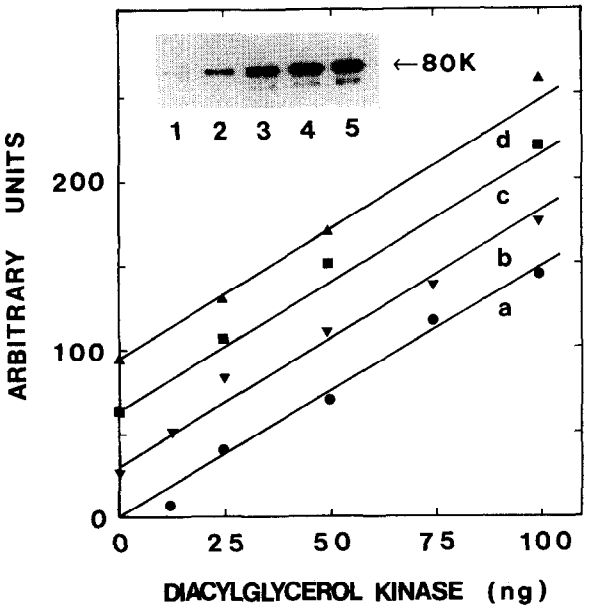

Fig.1. Immunoquantitation of 80 kDa diacylglycerol kinase. Different amounts (12.5–100 ng protein) of the enzyme purified from pig thymus cytosol were analyzed by immunoblotting using rabbit antibody and ^{125}I-protein A. The enzyme was quantitated from integrated areas of the absorbance determined by densitometric tracing of the radioautograph. The quantitation was carried out without (●) or with added thymus cytosolic fraction [(▼) 5.3, (■) 10.5, (▲) 15.8 μg cytosolic protein]. Inset: autoradiograph of immunoblot of the purified enzyme [lanes: (1) 12.5, (2) 25, (3) 50, (4) 75, (5) 100 ng protein].

DGK was soluble in these cells. Although we determined the enzyme level in various regions of pig brain as reported in [9], only the data obtained with the whole brain and pituitary are shown in fig.4. Various brain regions contained comparable amounts of the enzyme (80–130 ng/mg homogenate protein) except for pituitary and pineal glands (both 10 ng/mg). Unlike the case for lymphocytes, no marked enrichment of the enzyme in the cytosol was observed for all the brain regions analyzed. DGK has so far been reported to be located in various membrane fractions in addition to the cytosolic fraction [5,8,10,11,18–21]. It remains to be ascertained whether the marked enrichment of DGK in the cytosol is restricted to lymphocytes.

The 80 kDa isozyme requires phospholipids as activators [6,22], and its substrate, diacylglycerol, is believed to be generated in the membrane of

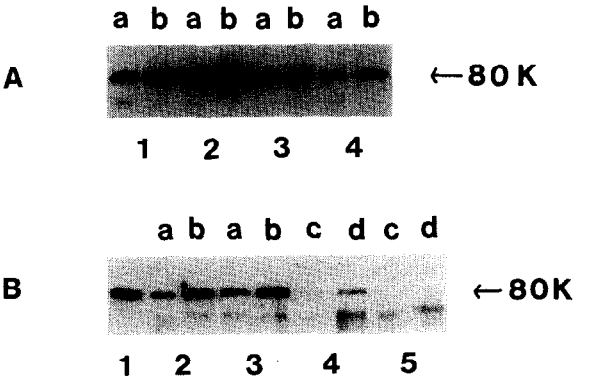

Fig.2. Immunoblot analysis of 80 kDa diacylglycerol kinase in pig and human cell homogenates. Autoradiographs of pig (A) and human (B) cell homogenates analyzed by immunoblotting are presented. (A) Lanes: (1) thymocytes, (2) T cell-enriched splenocytes, (3) B cell-enriched splenocytes, (4) peripheral blood lymphocytes. Lanes a and b represent 25 and 50 μg, respectively, of homogenate protein. (B) Lanes: (1) purified 80 kDa enzyme (20 ng protein) as the standard, (2) peripheral blood lymphocytes, (3) Jurkat, (4) HL-60, (5) polymorphonuclear leukocytes. The amounts of homogenate protein analyzed were: (a) 20, (b) 40, (c) 100, (d) 200 μg.

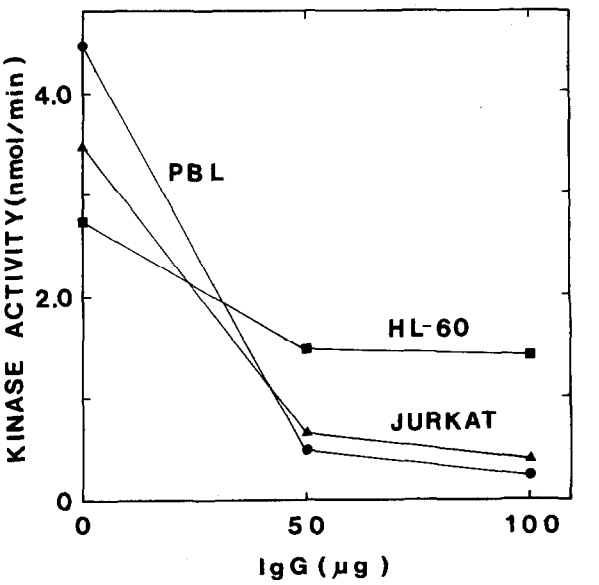

Fig.3. Immunoprecipitation of cytosolic diacylglycerol kinase from human cells. The cytosolic fractions from Jurkat (▲, 32 μg protein), HL-60 (■, 74 μg), and peripheral blood lymphocytes (PBL, ●, 14 μg) were incubated successively with rabbit antibody and Pansorbin (Calbiochem, USA). Immunoprecipitates were removed by centrifugation, and the enzyme activity remaining in the supernatant was determined.

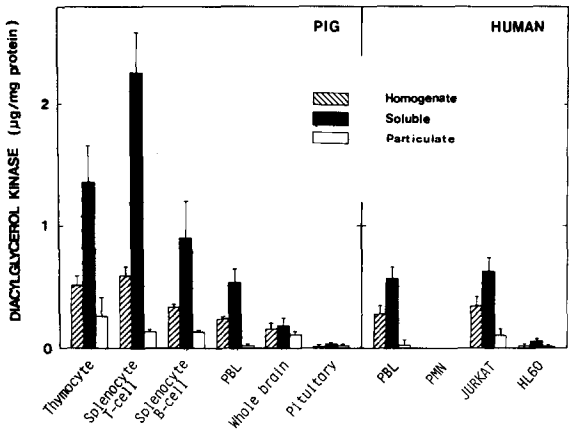

Fig.4. Quantitative estimation of 80 kDa diacylglycerol kinase in pig and human cell homogenates, cytosolic fractions and membranes. The content of the 80 kDa enzyme was estimated by densitometric tracing of autoradiographs after immunoblotting. The values (means ± SD) were obtained from 4–13 experimental determinations. PBL, peripheral blood lymphocytes; PMN, polymorphonuclear leukocytes.

agonist-stimulated cells. This raises the possibility that cytosolic DGK is an inactive (or less active) reservoir form. This notion is supported by the observation that the translocation of DGK activity from the cytosol to the membranes occurs in several types of cells [22–24]. We have further found that phosphorylation of soluble 80 kDa DGK by protein kinase C caused its binding to phosphatidylserine vesicles [25]. However, it remains to be clarified whether the 80 kDa enzyme or other DGK isozymes are involved in the translocation phenomenon.

The reason why lymphocytes contain such high levels of the 80 kDa enzyme, which represents more than 0.2% of total soluble protein in the case of pig splenic T cells, is not yet known. It is, however, likely that this particular DGK species plays an important role in immunological reactions. The considerable variation noted in the enzyme content among different types of lymphocytes suggests that 80 kDa DGK is involved in (a) function(s) specific to certain types of lymphocytes. The enzyme content in the cytosol of HL-60 (56 ± 16 ng/mg protein) is only one-tenth those determined for Jurkat (660 ± 140 ng/mg) and human PBL (580 ± 94 ng/mg). As mentioned above, the immunoprecipitation experiments suggest the occurrence in HL-60 cells of non-immunoreactive DGK species in addition to the 80 kDa enzyme. The apparent absence of the immunoreactive enzyme in human PMN is interesting in view of the presence of 80 kDa DGK in undifferentiated HL-60 cells.

The anti-pig 80 kDa DGK antibody used here has previously been shown to exhibit only limited cross-reactivity toward DGKs from other animal species. In fact, we could confirm that DGKs from rat and mouse thymus did not react with the antibody (not shown). Fortunately, however, the antibody could recognize human 80 kDa DGK, suggesting that the pig and human enzymes possess very similar antigenic properties.

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