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Occurrence of immunoreactive 80 kDa and non-immunoreactive diacylglycerol kinases in different pig tissues

Keiko YAMADA and Hideo KANOH*
Department of Biochemistry, Sapporo Medical College, West-17, South-1, Sapporo 060, Japan

We surveyed diacylglycerol kinase in different pig tissues by using rabbit antibody immunospecific to the brain 80 kDa enzyme [Kanoh, Iwata, Ono & Suzuki (1986) J. Biol. Chem. **261**, 5597–5602]. Among the other tissues examined, the immunoreactive 80 kDa enzyme was found only in the thymus and, to a much lesser extent, in the spleen, although this enzyme species was widely distributed in a variety of brain regions. Other tissues such as platelets, kidney, heart and liver contained little, if any, immunoreactive enzymes. Gel filtration of cytosolic enzymes from several tissues revealed the presence of three major activity peaks, apparently corresponding to 280, 120 and 80 kDa. Thymus and spleen contained the immunoreactive 80 kDa species together with non-immunoreactive 280 kDa enzyme. In the case of platelets, the kinase consisted almost exclusively of non-immunoreactive 120 kDa species with some 280 kDa enzyme. In an attempt to characterize the different kinase forms, the thymus enzyme was chosen for further studies because of its high activity. No immunoreactive proteins were detected in Western-blot analysis when the 280 kDa enzyme was solvent-extracted, proteinase-treated or preincubated in the presence of Ca^{2+}. In comparison with the 80 kDa species, the 280 kDa enzyme was much more heat-stable and less dependent on deoxycholate in the assay mixture. Although the purification of different forms of the kinase is required to confirm the presence of isoenzymes, the results show that there exist several immunologically distinct diacylglycerol kinase species.

INTRODUCTION

In the early stage of cell-surface receptor-linked stimulation of a variety of cells, diacylglycerol kinase is known to cause a rapid accumulation of phosphatidic acid by phosphorylating diacylglycerol released from membrane phosphoinositides (for reviews see refs. [1–3]). As with other lipid kinases operating in the stimulated cells [4–7], the mechanism of activation and physiological function of diacylglycerol kinase are not well understood. Detailed study of this lipid kinase is required for understanding the signal transduction mechanism, since recent reports from several laboratories [8–10] have shown that the enzyme competes with protein kinase C [2] for the utilization of diacylglycerol, a ubiquitous intracellular second messenger, thus acting as an attenuator of this multifunctional protein kinase. It is also worthy of note that the product of the kinase action, phosphatidic acid or lysophosphatidic acid [11], has been shown to act as a potent agonist in a number of cell systems [12–17], presumably by acting as a Ca^{2+} ionophore or by binding to its membrane receptors.

Diacylglycerol kinase appears to have similar properties to protein kinase C [2] with respect to utilization of diacylglycerol, phosphatidylserine-dependency [9,18,19] and its apparent translocation from the cytosol to membranes [19,20]. In spite of its central role in diacylglycerol metabolism in stimulated cells, relatively little is known of the protein chemical nature of the kinase. We previously suceeded in purifying diacylglycerol kinase from pig brain [18,21], and revealed the dependency of its action on phosphatidylcholine and phosphatidylserine [18,21]. In our subsequent work employing rabbit antibody against the purified enzyme [11], a multiplicity of forms of the kinase protein was suggested from the observation that the antibody only partially precipitated the brain enzyme, which exhibited no cross-reactivity with the liver kinase. In accordance with this result, Besterman *et al.* [19] described co-purification of multiple proteins when rat brain kinase was recovered from a phosphatidylserine affinity column. In the case of phosphoinositide-specific phospholipase C, which is activated before diacylglycerol kinase in stimulated cells, several isoenzymes have been characterized with respect to their protein chemical nature and enzymological properties [22–26]. However, purification of diacylglycerol kinase has so far been described only for pig brain 80 kDa enzyme [11,21], and little information is available on the presence of the kinase isoforms. The purpose of the present investigation is to demonstrate the multiplicity of diacylglycerol kinase, and also to test for the occurrence of the 80 kDa brain enzyme in other tissues. We describe the existence of several diacylglycerol kinase isoforms differing in their apparent molecular sizes and antigenicities.

EXPERIMENTAL

Materials

The preparation and characterization of rabbit antibody against pig brain diacylglycerol kinase have been described previously [11]. Heat-killed formalin-fixed *Staphylococcus aureus* cells (strain Cowan) (Pansorbin) were bought from Hoechst Japan, Tokyo, Japan. Pig anti-(rabbit IgG) antibody and horseradish peroxidase–rabbit anti-peroxidase complex were obtained from Dako Immunochemicals, Copenhagen, Denmark. Molecular-mass markers for SDS/polyacrylamide-gel electrophoresis were obtained from Bio-Rad Labora-

* To whom correspondence should be addressed.

tories, Richmond, CA, U.S.A. In gel filtration the standard proteins used were catalase (232000 Da), human γ-globulin (155000 Da), bovine serum albumin (66000 Da) and ovalbumin (45000 Da), purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. L-Tosylphenylalanylchloromethane- ('TPCK')-treated trypsin (type XIII) and V8 proteinase (*S. aureus*) were bought from Sigma Chemical Co. and ICN Immunobiologicals, Lisle, IL, U.S.A., respectively. Commercial sources of other chemicals were as follows: [γ-^{32}P]ATP was from ICN Radiochemicals, Irvine, CA, U.S.A.; ATP (disodium salt), DL-dithiothreitol and 1,2-diolein were from Sigma Chemical Co.; phenylmethanesulphonyl fluoride was from Chemical Dynamics Corp., South Plainfield, NJ, U.S.A.; Sephacryl S-300 was from Pharmacia, Uppsala, Sweden; acrylamide and SDS were from Nakarai Chemicals, Kyoto, Japan.

Enzyme preparation

Fresh brain, liver, spleen, heart, thymus and kidney were collected from pigs in the municipal slaughterhouse, and quickly frozen on solid CO_2. The thymus was identified by its bilobate structure and by microscopic examination of haematoxylin/eosin-stained tissue sections. Various brain regions were macroscopically dissected. Tissues were stored at −80 °C until use.

Platelets were prepared by following the procedure described for human blood [27]. Briefly, the blood was treated with 0.15 vol. of acid/citrate/dextrose solution (85 mM-trisodium citrate/111 mM-glucose/71 mM-citric acid) to prevent coagulation. The blood was first centrifuged at 200 *g* for 20 min at room temperature to obtain the platelet-rich plasma. Platelets were collected by centrifugation at 800 *g* for 5 min. After being washed with phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/1.5 mM-KH_2PO_4/8.7 mM-Na_2HPO_4 buffer, pH 7.4), the pellets were stored at −80 °C.

The tissues were thawed, rinsed with 0.9% NaCl and homogenized in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.) in buffer A (25 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose, 50 mM-NaCl, 1 mM-EDTA, 2 mM-EGTA, 0.5 mM-dithiothreitol, 10 μM-ATP, 0.1 mM-phenylmethanesulphonyl fluoride and 0.02% NaN_3). All operations were done at 0–4 °C. The homogenates were centrifuged at 200 *g* for 10 min, and the supernatant obtained at this step was used for enzyme assay and immunoblot analysis. To obtain cytosolic enzyme, the homogenates were further centrifuged at 8000 *g* for 20 min, followed by a centrifugation at 100000 *g* for 1 h.

Assay of diacylglycerol kinase activity

Incubation was for 3 min at 30 °C in 125 μl of reaction mixture containing 1 mM-deoxycholate and 2 mM-[γ-^{32}P]-ATP (5000–10000 c.p.m./nmol) exactly as described previously [18]. Less than 15 μg of homogenate or cytosolic proteins was assayed to maintain linearity of the reaction with respect to the amount of enzyme and incubation time.

Immunoblotting

Various tissue homogenates or cytosolic fractions were boiled for 3 min in Laemmli's sample buffer [28] and subjected to SDS/7.5%-polyacrylamide-gel electrophoresis [28]. The proteins were then electrophoretically transferred to a nitrocellulose membrane (0.45 μm pore size; Bio-Rad Laboratories) by using a Bio-Rad apparatus. Proteins transferred to the membranes were immunostained as follows: incubated for 2 h in 5% (w/v) skim milk (Snow Brand Milk Products Co., Tokyo, Japan) in phosphate-buffered saline to block non-specific binding sites, incubated for 2 h with immune IgG (25 μg/ml), incubated for 1 h with pig anti-(rabbit IgG) antibody at 1:50 dilution, and incubated for 20 min with peroxidase–anti-peroxidase complex at 1:50 dilution. All antibody dilutions were made in phosphate-buffered saline containing 1% skim milk, and incubations were conducted at room temperature. In between incubations, the membranes were extensively washed with phosphate-buffered saline containing 0.05% Tween-20. Immunoreactive proteins were detected by using 4-chloro-1-naphthol as peroxidase substrate. The control staining used pre-immune IgG at the same concentration. The protein markers were simultaneously transferred and the membranes were stained with 0.1% Amido Black in 25% (v/v) propan-2-ol containing 10% (v/v) acetic acid.

Immunoprecipitation of diacylglycerol kinase

Various amounts of immune IgG were incubated for 1 h on ice with cytosolic kinase or enzymes taken from gel-filtration chromatography. Buffer A containing 1 mg of bovine serum albumin/ml was used in a final volume of 100 μl. Next, 50 μl of Pansorbin in phosphate-buffered saline was added, and the incubation was continued for 1 h in an ice bath. The mixture was then centrifuged at 1000 *g* for 2 min, and the enzyme activity remaining in the supernatant was determined. Control incubation used pre-immune IgG, which did not affect the enzyme activity.

Gel filtration of cytosolic enzymes

Samples (1–4 ml) of the cytosolic kinase from several tissues were subjected to gel filtration on a column (1.8 cm × 82 cm) of Sephacryl S-300 equilibrated with buffer A. The elution was done at a flow rate of 15 ml/h, and 2 ml fractions were collected.

Limited proteolysis and heat treatment of thymus diacylglycerol kinase

The enzymes separated by gel filtration (0.7–1.0 mg of protein/ml) were incubated in buffer A in the presence of trypsin (trypsin/protein ratio 1:200, w/w) or V8 proteinase (ratio 1:50, w/w). The incubation was conducted for periods ranging from 0 to 20 min at 0 °C or higher temperatures. The proteinase digestion was terminated by adding $\frac{1}{4}$ vol. of 5-fold-concentrated Laemmli's sample buffer [28] and immediately boiling for 3 min. The 0 min treatment used boiled proteinases. Samples were subjected to SDS/10%-polyacrylamide-gel electrophoresis followed by electrophoretic transfer to nitrocellulose paper for immunoblot analysis.

In the case of heat treatment, the enzyme solutions were adjusted to 500 μg of protein/ml in buffer A, and placed in a water bath at 41 °C. At the indicated time intervals samples were taken and chilled in an ice bath.

RESULTS

Immunochemical studies on diacylglycerol kinase from different tissues

As an initial study, the kinase activity of various tissue homogenates was measured under the assay conditions

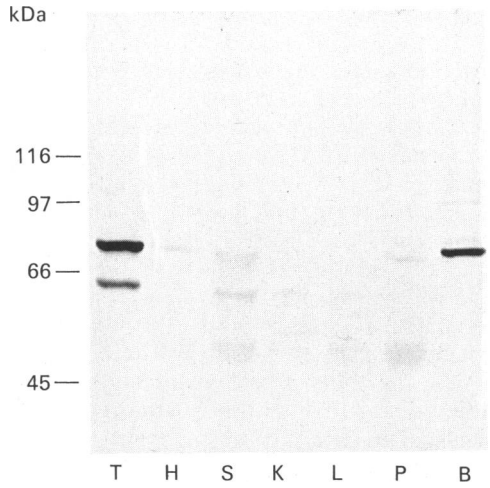

Fig. 1. Immunoblot of diacylglycerol kinase in various pig tissues

Tissue homogenates (60 μg of protein each) were analysed by SDS/7.5%-polyacrylamide-gel electrophoresis followed by electrophoretic transfer to nitrocellulose paper. Immunostaining of the transferred proteins was done by using rabbit antibody against pig brain 80 kDa enzyme as described in the text. The tissues are: T, thymus; H, heart; S, spleen; K, kidney; L, liver; P, platelets; B, brain.

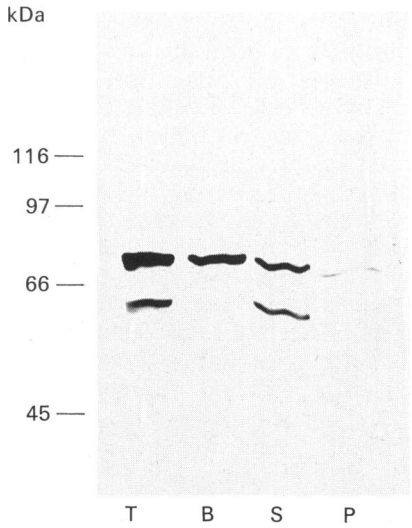

Fig. 2. Immunoblot of several tissue homogenates containing an equivalent kinase activity

Immunoblot was done with tissue homogenates containing an equivalent kinase activity (0.6 nmol/min) as described in Fig. 1 legend. The tissues are: T, thymus (17 μg of protein); B, brain (55 μg); S, spleen (90 μg); P, platelets (240 μg).

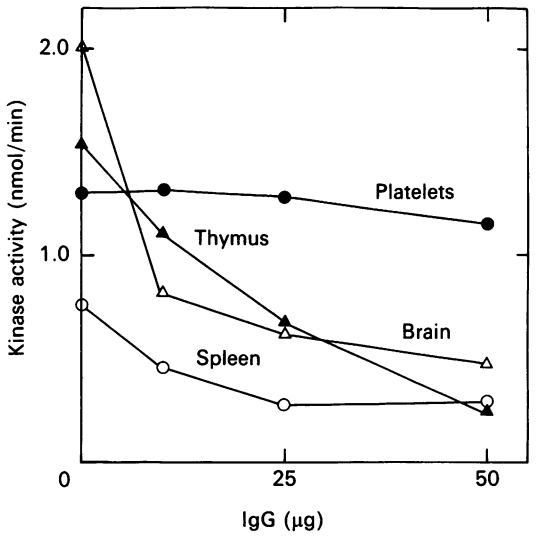

Fig. 3. Immunoprecipitation of cytosolic diacylglycerol kinase from several tissues

The cytosolic fractions from platelets (●, 560 μg of protein), thymus (▲, 45 μg), spleen (○, 135 μg) and brain (△, 170 μg) were successively incubated with rabbit antibody and Pansorbin as described in the text. The use of higher concentrations of immune IgG than those given did not cause further precipitation of enzyme activity. Pre-immune IgG tested for the thymus and brain enzymes did not affect the activity (results not shown).

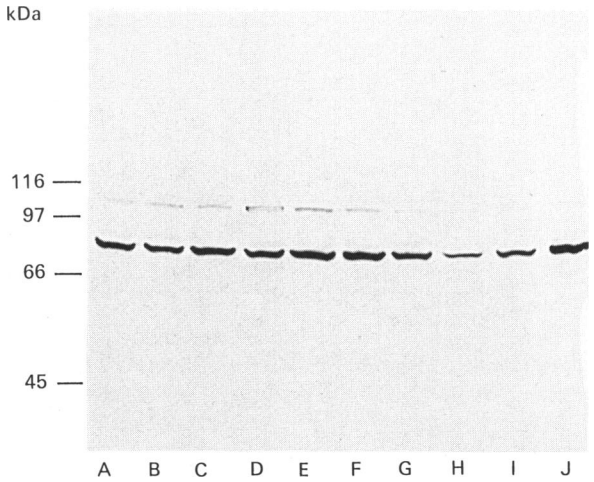

Fig. 4. Immunoblot of diacylglycerol kinase from different pig brain regions

The homogenates of various brain regions (150 μg of protein each) were analysed as described in Fig. 1 legend. The staining other than 80 kDa protein was also detected to a variable extent with pre-immune IgG. Lane A, whole brain; lane B, grey matter; lane C, white matter; lane D, cerebellum; lane E, middle brain; lane F, pons; lane G, thalamus; lane H, pituitary; lane I, pineal; lane J, medulla oblongata.

established for pig brain enzyme. Although detailed studies on assay conditions for different tissues were not performed, the observed activity was used as an approximate guide for performing immunochemical analysis. In three or four independent enzyme assays the thymus homogenates always gave the highest activity (25–40 nmol/min per mg of protein), which was 3–4-fold higher than that of brain (6–10 nmol/min per mg). The spleen homogenates (3–4 nmol/min per mg) gave an activity about half that of brain, and very low activities were obtained with heart (0.3–0.7 nmol/min per mg), kidney (0.6–1.0 nmol/min per mg), platelets (1–2 nmol/min per mg) and liver (0.2–0.5 nmol/min per mg). The

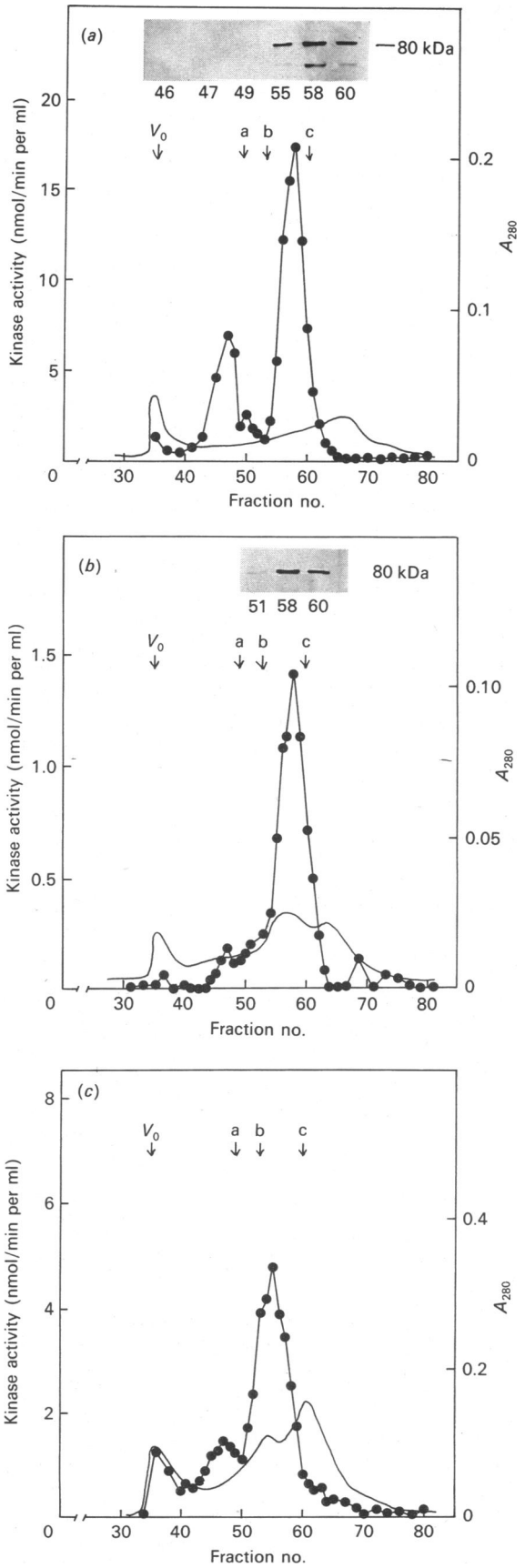

Fig. 5. Elution of diacylglycerol kinase from a Sephacryl S-300 column

The chromatography was performed as described in the text with the cytosolic enzymes from thymus (9.7 mg of protein, *a*), brain (9.6 mg, *b*) and platelets (60 mg, *c*). ——, A_{280}; ●—●, diacylglycerol kinase activity. The recoveries of the applied enzyme activities are 70% for thymus kinase, 25% for brain kinase and 62% for platelet kinase. The results of the immunoblot analysis of the collected fractions (50 μl each) are presented in panels (*a*) and (*b*). The elution positions of Blue Dextran (V_0), catalase (a), human γ-globulin (b) and bovine serum albumin (c) are indicated.

specific enzyme activity of the cytosolic enzymes was similar to that obtained with the tissue homogenates. In all tissues the kinase activity was distributed in both soluble and particulate fractions.

A fixed amount of homogenate protein was analysed by an immunoblot with the antibody against pig brain 80 kDa enzyme [11]. A very similar result was obtained with cytosolic fractions (not shown). As shown in Fig. 1, the immunoreactive 80 kDa kinase was exceptionally enriched in the thymus as well as in the brain. Other tissues, however, did not give significantly immunoreactive proteins. The faintly stained bands in these other tissues, except for spleen, were also detected with pre-immune IgG, probably representing non-specific staining. In the case of thymus an immunoreactive 60 kDa protein was also detected and this protein was not stained with pre-immune IgG. In spite of several efforts (see below), we could not characterize the 60 kDa protein further.

The apparent lack of immunoreactive proteins in several tissues may be due to a low content of enzyme protein or due to the presence of non-immunoreactive kinase species. To clarify this, homogenates containing an equivalent kinase activity were analysed in the next experiment (Fig. 2). Tissues other than those listed could not be studied because of the very low enzyme activity. The spleen was also found to contain the immunoreactive 80 kDa and 60 kDa proteins as detected in the thymus, whereas platelets gave essentially no immunoreactive proteins. In these studies the staining of the thymus 80 kDa protein was always more marked than those of spleen and brain, and the two immunoreactive proteins of the spleen migrated somewhat faster than those of the other two tissues. In order to confirm that the platelet kinase did not cross-react with the antibody, the cytosolic enzyme from these tissues was immunoprecipitated (Fig. 3). In accordance with our previous results [11], the antibody only partially precipitated (65–75%) the brain enzyme. It should be noted that the antibody achieved almost complete precipitation of partially purified brain cytosolic enzyme [11]. A considerable part of the kinase from the thymus and spleen was also precipitated. In repeated experiments non-precipitable kinase activity in the thymus and spleen represented 20–35% and 30–50% respectively of the total activity. On the other hand, the platelet enzyme failed to react with the antibody, indicating that the platelet kinase is immunologically distinct from the 80 kDa enzyme present in the lymphoid tissues and brain.

Since the occurrence of the 80 kDa kinase appeared to be rather limited among the tissues examined, we thought it necessary to investigate the regional distribution of the immunoreactive enzyme species in the brain, which is composed of many different cell types. As shown in Fig. 4, all brain regions examined contained the 80 kDa

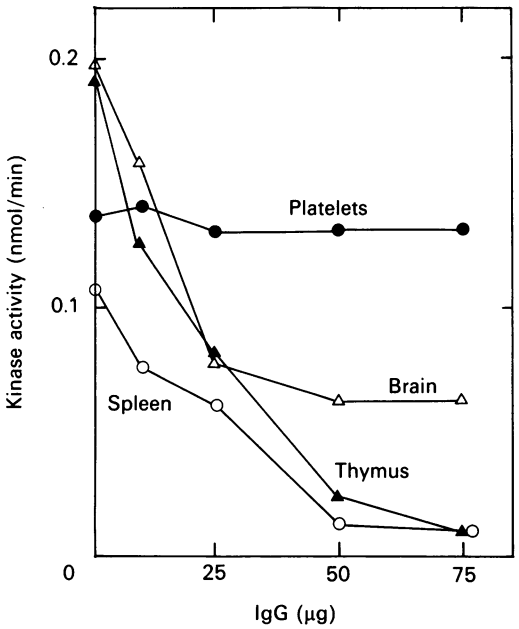

Fig. 6. Immunoprecipitation of the major kinase fractions separated by gel filtration

The major kinase fractions corresponding to the apparent molecular masses of 80 kDa for the brain (△, 30 μg of protein), thymus (▲, 10 μg) and spleen (○, 67 μg) and of 120 kDa for platelets (●, 135 μg) were immunoprecipitated as described in the text.

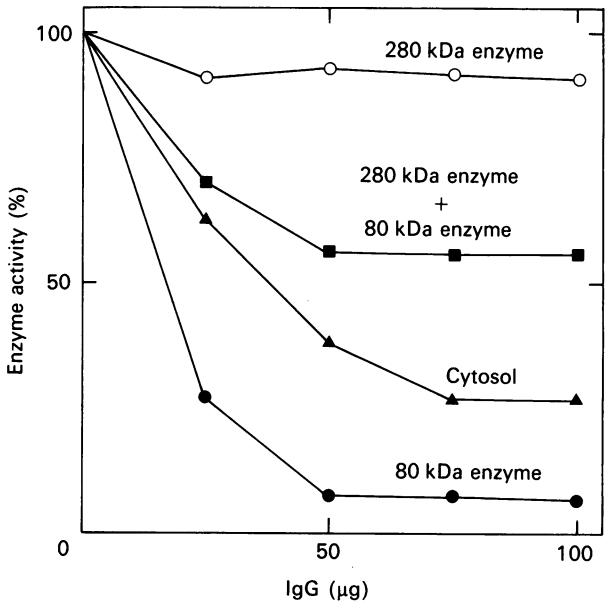

Fig. 7. Immunoprecipitation of the two thymus kinase forms separated by gel filtration

The two diacylglycerol kinase species separated by Sephacryl S-300 chromatography were treated with the antibody independently or in a mixed condition. The enzyme preparations used are as follows: 80 kDa enzyme (●, 53 μg of protein, 1.82 nmol/min of activity); 280 kDa enzyme (○, 45 μg, 2.2 nmol/min); the mixed enzymes (■, 1.1 nmol/min of 280 kDa enzyme plus 0.91 nmol/min of 80 kDa enzyme). The original thymus cytosol (▲, 90 μg, 2.65 nmol/min) was also tested. The results are presented as the relative enzyme activity remaining in the supernatant after immunoprecipitation.

enzyme. The relatively faint staining of pineal and pituitary could account for the lower enzyme activity in these regions. It should be noted that the 60 kDa protein detected in the lymphoid tissues was absent from any of the brain regions. In these experiments the staining of 105 kDa bands occurred to a variable extent in all brain regions even with the pre-immune IgG (see also Fig. 1).

Gel filtration of cytosolic enzymes

In an attempt to elucidate the heterogeneity of diacylglycerol kinase, the cytosolic enzyme from thymus was directly fractionated by gel filtration (Fig. 5*a*). This tissue showed a major activity peak corresponding to an apparent molecular mass of 80 kDa, and this peak contained the immunoreactive 80 kDa and 60 kDa proteins, which, for unknown reasons, were not separated from each other. Further, the thymus contained, unexpectedly, another activity peak at 280 kDa, and this fraction did not give immunoreactive bands in an immunoblot. Essentially, the same gel-filtration pattern was observed with the spleen cytosol (results not shown). This unexpected finding led us to analyse further the brain (Fig. 5*b*) and platelet (Fig. 5*c*) cytosolic enzymes. The brain kinase yielded a major activity peak at the 80 kDa position. The minor peak at 280 kDa was also reproducibly observed with a variable enzymic activity. In repeated experiments the 280 kDa fraction comprised 5–15% and 20–40% of the recovered activities from the brain and thymus respectively. The 280 kDa kinase appeared to be more pronounced in the lymphoid tissues. However, we could not definitely prove this because the crude enzymes were not stable, resulting in low recovery of enzyme activities. Interestingly, the platelet kinase did not give the 80 kDa peak, but instead showed a major peak at 120 kDa with a minor 280 kDa shoulder. As shown in Fig. 6, immunoprecipitation experiments employing enzymes separated by gel filtration confirmed that the platelet kinase could not react with the antibody, whereas the 80 kDa kinase from the spleen and thymus was almost completely precipitated. The results also show that the brain 80 kDa fraction still contained immunoreactive and non-immunoreactive components as observed with the crude cytosol (Fig. 3).

Studies on soluble diacylglycerol kinase from thymus

The results so far described indicate that there are several immunologically distinct diacylglycerol kinases. The tissue-characteristic occurrence of different enzyme forms, in particular 120 kDa species in the platelet, may support the concept of the presence of diacylglycerol kinase isoenzymes. In an attempt to substantiate the results obtained with the crude enzymes, we selected for further studies the two kinase forms obtained by gel filtration of thymus cytosol. We also attempted to find the conditions where the high-molecular-mass form might be converted into a lower-molecular-mass form, or vice versa.

In the first experiment, the 280 kDa and 80 kDa fractions were mixed and treated with the antibody (Fig. 7). The 280 kDa enzyme was not significantly precipitated, in agreement with the immunoblot results (Fig. 5*a*). In this mixing experiment the antibody precipitated

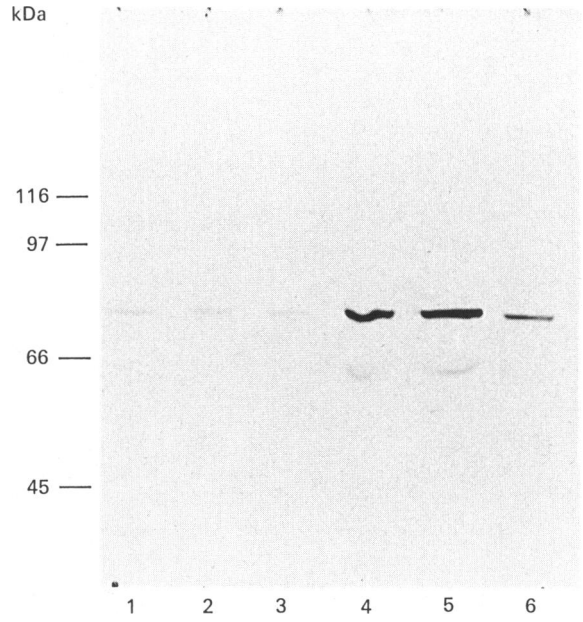

Fig. 8. Immunoblot of the two forms of thymus diacylglycerol kinase after preincubation

The two kinase species taken from gel filtration had comparable specific activities (25 and 30 nmol/min per mg of protein for 80 kDa and 280 kDa kinases respectively). The 280 kDa enzyme (lanes 1–3) and 80 kDa enzyme (lanes 4–6) (both at 700 μg of protein/ml) were incubated in the absence (lanes 2 and 5) and in the presence (lanes 3 and 6) of 10 mM-$CaCl_2$ for 1 h at 37 °C. Lanes 1 and 4 contain non-treated enzymes. Each lane contains 50 μg of protein. The faint bands at 80 kDa in lanes 1–3 are due to an incomplete separation of the two enzymes, which occasionally occurred in repeated experiments.

the portion of enzyme activity that could account for the added 80 kDa enzyme, thus ruling out a possible interference of immunological reaction occurring in the 280 kDa fraction. Further, the non-precipitable kinase activity in the original cytosol amounted to 35 %, which coincided with the relative amount of 280 kDa activity in this particular enzyme preparation as determined by gel filtration.

We next checked the effects of endogenous proteinases on the kinase proteins. The original cytosol (not shown) or the separated 280 kDa and 80 kDa enzymes (Fig. 8) were preincubated in the presence and in the absence of Ca^{2+} before immunoblot analysis. The pattern of the immunoblot was not affected by the preincubations, although incubation with Ca^{2+} caused loss of the 80 kDa protein. The results suggest that there would be no significant interconversion *in vitro* of the two kinase species, and that the immunoreactive 60 kDa protein was not readily formed from other species. Lipid extraction of the 280 kDa fraction also failed to give immunoreactive proteins (results not shown).

We treated the two forms of the kinase with trypsin (Fig. 9) and V8 proteinase (results not shown) under different conditions with various proteinase concentrations and incubation temperatures. The 80 kDa enzyme was extremely sensitive to trypsin treatment, and by a milder treatment conducted at 0 °C we noted formation of a 38 kDa immunoreactive fragment. V8 proteinase also degraded the 80 kDa enzyme, but failed to produce

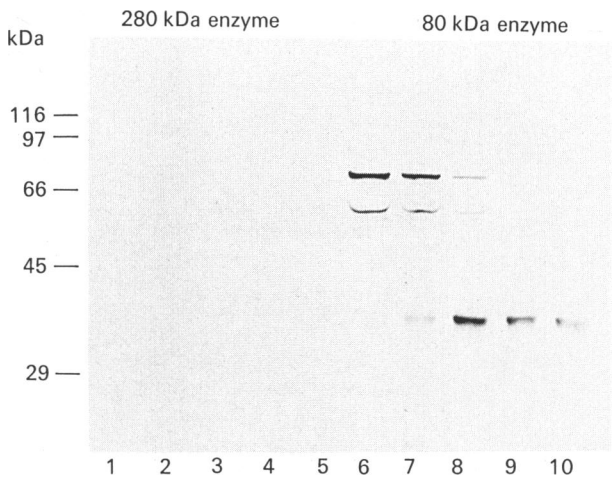

Fig. 9. Limited proteolysis with trypsin of the two forms of thymus diacylglycerol kinase

The 280 kDa enzyme (lanes 1–5) and 80 kDa enzyme (lanes 6–10) preparations, which were used in the experiments given in Fig. 8, were incubated with 3.5 μg of trypsin at 0 °C for 0 min (lanes 1 and 6), 2 min (lanes 2 and 7), 5 min (lanes 3 and 8), 10 min (lanes 4 and 9) and 20 min (lanes 5 and 10). After incubation, the enzymes were boiled in Laemmli's sample buffer and analysed by SDS/10 %-polyacrylamide-gel electrophoresis followed by transfer to nitrocellulose paper. Immunostaining was done as described in the text. Each lane contains 50 μg of protein.

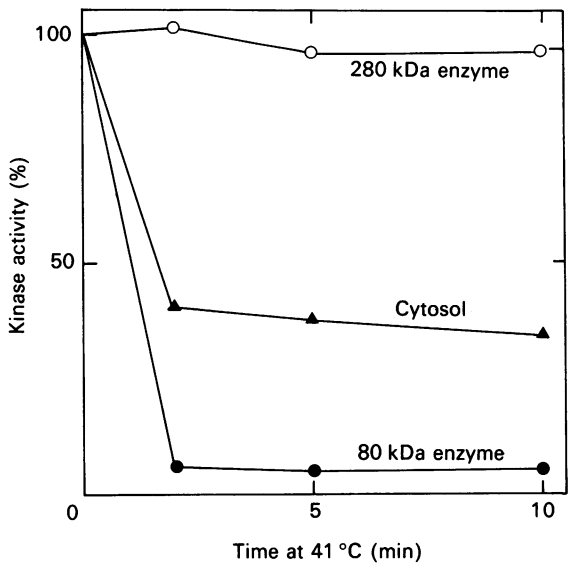

Fig. 10. Thermal inactivation of thymus diacylglycerol kinase

The 280 kDa enzyme (○) and 80 kDa enzyme (●) fractions and the original cytosol (▲) were incubated at 41 °C for various lengths of time at the same protein concentration (500 μg/ml). At the indicated time intervals samples were taken and cooled in ice for more than 10 min. The enzyme activity was then measured as described in the text.

immunoreactive fragments. Throughout these experiments the 280 kDa fraction failed to yield immunoreactive proteins, suggesting that this enzyme form may be structurally distinct from the 80 kDa kinase.

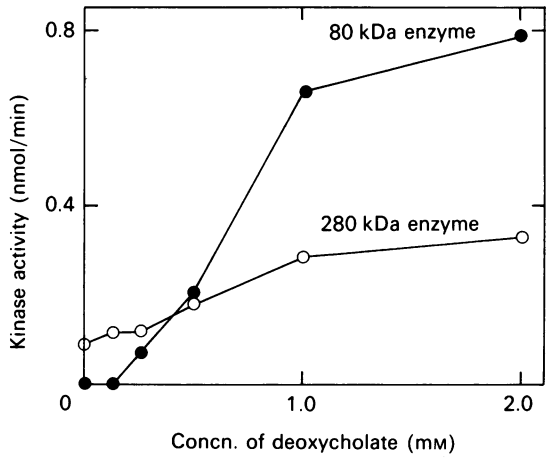

Fig. 11. Effect of deoxycholate on the activities of the two forms of thymus diacylglycerol kinase

The 80 kDa enzyme (●, 22 μg of protein) and 280 kDa enzyme (○, 14 μg) were incubated in the standard assay conditions with various concentrations of deoxycholate. Diacylglycerol was dispersed by sonication in the reaction mixture without enzyme and deoxycholate.

We also found significant differences in the biochemical properties of the two thymus kinase species. When the original cytosol was heated at 41 °C (Fig. 10) the thymus kinase was shown to be composed of heat-stable and -unstable components. In parallel treatment of the separated enzymes, the heat-unstable component was represented by the 80 kDa enzyme, whereas no significant inactivation occurred in the case of the 280 kDa kinase. The loss of activity of the 80 kDa enzyme was not due to proteinase degradation, but due to heat-denaturation, since the immunoblot (results not shown) showed that staining of this protein was not affected even by a 10 min-long treatment. As shown in Fig. 11, the activity of the 80 kDa kinase was completely dependent on the added deoxycholate. The 280 kDa enzyme, on the other hand, showed a considerable activity in the absence of detergent.

The purification of different kinase forms and detailed studies of their protein chemical as well as enzymological properties would clarify the physiological implications of the present findings. However, our attempts to purify the enzymes other than 80 kDa kinase have not been successful.

DISCUSSION

The present work demonstrates that pig tissues contain several diacylglycerol kinase species, which are apparently different from the 80 kDa kinase with respect to their antigenicity and molecular sizes. Immunoreactivity of the kinase species was studied in their native forms (immunoprecipitation) and after their denaturation in the presence of SDS and 2-mercaptoethanol (immunoblot). In both cases a comparable amount of the immunoreactive 80 kDa enzyme was simultaneously analysed to ensure the reliability of the detection methods. We selected the thymus cytosolic 280 kDa enzyme as a representative of non-immunoreactive kinase species, and showed that no immunoreactive proteins could be released from this enzyme by solvent extraction, proteinase degradation and preincubation. Again the 80 kDa enzyme served as a control in these treatments. Since the purification of animal diacylglycerol kinase has been achieved only for pig brain 80 kDa enzyme [18,21], we do not know the protein chemical natures and structural relationships of the different kinase forms. The attempt to purify the thymus 280 kDa kinase was unsuccessful, largely because of its aggregation during the purification procedures. At the present stage of investigation we assume that such apparent lack of immunoreactivity of the kinase species may be caused by a covalent modification of the enzyme molecule or by a structural unrelatedness.

At present, basic information on the exact nature of the kinase heterogeneity is lacking, and detailed studies on enzymological properties of different kinase forms are needed to assess the physiological implication of the present finding. However, the presence of immunologically distinct enzyme forms in different tissues may be relevant to their specific functions. The 80 kDa enzyme previously purified from the brain exhibited a rather limited occurrence among the tissues, being abundant only in the thymus and much less in the spleen. This immunoreactive kinase showed a tissue distribution pattern similar to that of protein kinase C [29–31] with respect to the enrichment in the brain and lymphoid tissues and the relatively low content in the pituitary and pineal within the brain. It should be also noted that this type of kinase was associated with tissues exhibiting high enzyme activity. So far the role of diacylglycerol kinase has been mostly studied in the agonist-stimulated cells. However, in non-stimulated cells the action of this enzyme must be under strict control to allow the entry of diacylglycerol into biosynthetic routes leading to the formation of major glycerolipids. Therefore the physiological implication of the relatively high contents of 80 kDa kinase, in particular in the thymus, awaits further elucidation. Protein kinase C has been shown to be composed of several isoenzymes of similar molecular mass (approx. 80 kDa) with distinct immunological [32–34] and biochemical [32,35–37] properties. The present data obtained with the polyclonal antibody did not exclude the possibility that the immunoreactive 80 kDa kinase is also a mixture of closely related isoenzymes. The variable staining of tissue homogenates containing an equivalent enzyme activity (Fig. 2) might indicate the heterogeneity of the 80 kDa enzyme.

Among the tissues examined, the platelets were unique in containing non-immunoreactive 120 kDa enzyme with an apparent lack of 80 kDa species. Recently, the function of diacylglycerol kinase has been assessed by using an inhibitor [8,10,38,39] originally developed for the platelet enzyme [8]. It is yet to be tested whether the inhibitor is effective solely with the 120 kDa enzyme. We earlier reported [40] that highly purified rat liver kinase showed an apparent molecular mass of 120 kDa on gel filtration. This coincidence of the molecular size is interesting, but we could not analyse pig liver kinase owing to its very low enzyme activity.

The high-molecular-mass form (280 kDa) of diacylglycerol kinase was detected to a variable extent in all tissues examined by gel filtration. This species was markedly heat-stable in comparison with the 80 kDa enzyme, and appeared to be more abundant in the lymphoid tissues. The lymphoid tissues also contained an

immunoreactive 60 kDa protein, the significance of which could not be assessed in the present work.

Our work is the first to study by immunological means the tissue distribution of phospholipid-synthesizing enzymes. The results revealed a rather complicated heterogeneity of diacylglycerol kinase and point to the importance of further characterization of each enzyme form.

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