Purification and characterization of *sn*-1-stearoyl-2-arachidonoylglycerol kinase from pig testes

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1-Stearoyl-2-arachidonoylglycerol (SAG) kinase was identified in the particulate fraction of pig testes. This activity was enriched by hydroxyapatite and blue dye chromatography. The enzyme was selective for polyunsaturated diradylglycerol species and activity was not modulated by other diradylglycerol species or sphingomyelin metabolites. Further purification resulted in the isolation of 55 and 50 kDa proteins that corresponded with SAG kinase activity. These results support the view that the phosphorylation of polyunsaturated diradylglycerol is regulated by structural determinants in the molecule.

INTRODUCTION

The generation of second messengers from precursor molecules is one of the earliest changes in cells stimulated with hormones and growth factors. *sn*-1,2-Diradylglycerol (DAG) is one such second messenger and is an activator of protein kinase C [1]. Because protein kinase C activity has been implicated in controlling many biological processes such as differentiation, secretion and mitosis, DAG plays a key regulatory role within the cell. Furthermore DAG is an essential component of phospholipids. Intracellular DAG concentration is increased rapidly after receptor-stimulated PtdIns(4,5)P_2 hydrolysis in a reaction that also increases cytosolic Ins(1,4,5)P_3 concentration. The function of Ins(1,4,5)P_3 is to release calcium from intracellular stores and together with the phosphoinositide-derived DAG they activate certain protein kinase C isoforms. PtdIns(4,5)P_2 hydrolysis, increased Ins(1,4,5)P_3 concentration and calcium release are transient inside cells, whereas after receptor stimulation the levels of intracellular DAG remain elevated for at least 60 min [2–4]. An important characteristic of a second messenger is its specific removal and in the case of DAG, this involves the ATP-dependent conversion to phosphatidate (PA) catalysed by a diacylglycerol kinase. This PA is then metabolized to phosphatidylinositol to complete the 'PI cycle'. Thus the second, prolonged, phase of DAG production is probably derived from receptor-stimulated hydrolysis of non-inositide-containing phospholipids, such as phosphatidylcholine [3–7]. The generation of DAG from phosphatidylcholine requires the phospholipase D-catalysed production of PA followed by dephosphorylation to DAG catalysed by phosphatidate phosphohydrolase.

DAG kinase activity has been reported in A431 cells [8], endothelial cells [9], HL60 cells [10], Jurkat T-cells [11–13], Swiss 3T3 cells [14,15], NIH3T3 and Ras-transformed fibroblasts [16]. DAG kinase activities have been characterized in rat brain [17,18], baboon brain [19], *Drosophila* heads [20] and bovine testes [21]. Mammalian DAG kinase activities have been found to be cytosolic [16,18,22], membrane-associated or membrane-bound [19,21–23], cytoskeletal [8] or nuclear [24]. The first DAG kinase to be purified, characterized and cloned was an 80 kDa protein from pig lymphocyte cytosol [25]. A second cytosolic DAG kinase isoform has been identified in rat brain [17,18] and in NIH3T3 cells [16]. A broadly common feature of cytosolic DAG kinases reported so far is that they show little overall selectivity for any of the naturally occurring diradylglycerols [14–16,18] and are also able to phosphorylate synthetic saturated short-chain diradylglycerols [16]. Furthermore it has also been reported that the 80 kDa cytosolic DAG kinase phosphorylates monoacylglycerol to produce lysophosphatidate [18,22]. In addition, sphingosine has been shown to activate [11,12,25], and ceramide to inhibit [10], soluble DAG kinase activity *in vitro*.

An immunologically and electrophoretically identical isoform of the 80 kDa DAG kinase has been detected in particulate fractions [22]. This may be explained by the report that the 80 kDa DAG kinase translocates to the membrane [16,23,26] or cytoskeleton of cells [8], in response to epidermal growth factor stimulation [26]. It has also been shown that protein kinase C isoforms phosphorylate DAG kinase *in vitro* [26,27].

Analysis of the molecular species of DAG that are elevated after receptor stimulation has shown that the levels of polyunsaturated DAG species, such as 1-stearoyl-2-arachidonoylglycerol (SAG), 1-stearoyl-2-eicosatrienoylglycerol and 1-stearoyl-2-eicosapentaenoylglycerol, were raised at early time points but quickly returned to basal levels. The levels of saturated and monounsaturated DAG species, such as 1-palmitoyl-2-oleoylglycerol and dioleoylglycerol (DOLG), were elevated at longer times after stimulation [5–7]. Analysis of the acyl chain structure of phospholipids has shown that polyunsaturated DAG species are characteristically found in polyphosphoinositides and to a smaller extent phosphatidylcholine [7]. Thus the presence of a DAG kinase that is selective for polyunsaturated DAG species has been inferred [14,15]. Analysis of the phosphorylation of SAG in fibroblasts, both *in vitro* and *in vivo*, has shown that this polyunsaturated DAG was predominantly converted to PA and phosphoinositides by an ATP-dependent activity that was almost exclusively membrane-bound [14,15,28]. Conversely, a saturated diradylglycerol, 1-stearoyl-2-myristoylglycerol, has been shown to be predominantly metabolized to triacylglycerol and phosphatidylcholine [28]. It seems likely that the SAG kinase found in membrane fractions is the enzyme that metabolizes the DAG species that are derived from PtdIns(4,5)P_2 and is relevant in

Abbreviations used: DAG, *sn*-1,2-diradylglycerol; PA, phosphatidate; DOLG, dioleoylglycerol; SAG, 1-stearoyl-2-arachidonoylglycerol; DTT, dithiothreitol.

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cellular signalling, and that this enzyme is distinct from the previous reports of cytosolic or membrane-associated 80 kDa DAG kinase isoforms. The tissue distribution of DAG kinases has shown that when compared with brain, the particulate fraction of testes contained a considerably higher proportion of SAG-selective kinase activity over non-selective DAG kinases [19]. Here we report the characterization and purification of an SAG kinase from the particulate fraction of porcine testes.

MATERIALS AND METHODS

Materials

Ceramic hydroxyapatite (80 μm) and protein assay reagent were from Bio-Rad Laboratories. Cibabcron Blue 3GA agarose, thiolpropyl-Sepharose, PMSF and all diacylglycerol analogues except SAG were from Sigma. SAG was from Avanti Polar lipids. Phosphatidylserine was from Lipid Products. *Escherichia coli* DAG kinase, octyl glucoside and dithiothreitol (DTT) were from Calbiochem Novabiochem. α-Dodecyl-ω-hydroxypoly-(oxyethane-1,2-diol) (Thesit) and ATP were from Boehringer Mannheim. [γ-^{32}P]ATP was from Amersham. All solvents, including ethanediol, were from Fisons. Strataclean resin was from Stratagene. All other reagents were from Sigma.

Tissue preparation

Pig testes were collected from a local abattoir on ice, decapsulated, sectioned and snap-frozen in liquid nitrogen before storage at -70 °C. The following procedures were performed on ice or at 4 °C. Tissue sections of approx. 40 g were defrosted in 100 ml of 50 mM Tris/HCl (pH 8.0)/300 mM sucrose/1 mM DTT supplemented with PMSF. Once the tissue had defrosted it was minced with scissors, processed in a glass–Teflon homogenizer for six passes and sieved. The particulate material was centrifuged at 100000 ***g*** for 45 min at 4 °C. The resultant pellet was resuspended in 50 ml of 50 mM Tris/HCl (pH 8.0)/300 mM sucrose/1 mM DTT/PMSF/2.5 % (w/v) sodium cholate for 30 min. The suspension was centrifuged at 100000 ***g*** for 30 min to remove detergent-insoluble material.

Purification of SAG kinase

Ceramic hydroxyapatite (10 g) was washed several times with water before equilibration in 50 mM Tris/HCl (pH 8.0)/1 mM $MgCl_2$/100 mM $ZnCl_2$/1 mM DTT/25 mM KH_2PO_4/10 % (v/v) ethanediol/0.2 % Thesit (buffer A) and packed in an XK column (2.5 cm × 5 cm) and connected to an FPLC system (Pharmacia). NaCl was added to the detergent extract to a final concentration of 1 M and this was pumped on to the column at 2.5 ml/min. The unbound material was collected as one fraction and the column was washed with 4 vol. of buffer A, which was collected with the unbound material. Bound protein was eluted with a nonlinear gradient of buffer B (buffer A plus 500 mM KH_2PO_4) over 450 ml at 2.5 ml/min, and 15 ml fractions were collected. High-specific-activity fractions from peak II were applied to a Cibacron Blue 3GA agarose column previously equilibrated in buffer A (1 cm × 5 cm) at 1 ml/min. Unbound material was collected as one fraction. Bound material was eluted from the column at 0.75 ml/min with a nonlinear gradient of 50 mM Tris/HCl (pH 8.0)/1 mM $MgCl_2$/100 mM $ZnCl_2$/1 mM DTT/750 mM KH_2PO_4/30 % (v/v) ethanediol/0.5 % Thesit (buffer C). The total gradient volume was 120 ml and 4 ml fractions were collected.

Thiolpropyl-Sepharose was washed with water and packed in a column (2.6 cm × 2 cm). The column was connected to an FPLC system and equilibrated with 50 mM Tris/HCl (pH 8.0)/1 mM $MgCl_2$/100 mM $ZnCl_2$/1 M NaCl/10 % (v/v) ethanediol/0.2 % Thesit. Fractions containing enzyme activity were pooled and applied to the thiolpropyl-Sepharose column at 2 ml/min. Unbound material was collected as one fraction. Bound material was eluted with 50 mM Tris/HCl (pH 8.0)/1 mM $MgCl_2$/100 μM $ZnCl_2$/50 mM DTT/1 M NaCl/10 % (v/v) ethanediol/0.2 % Thesit.

Fractions containing enzyme activity were pooled and applied to a hydroxyapatite column (Calbiochem; HPLC grade, 0.5 cm × 1 cm) connected to a SMART system (Pharmacia) previously equilibrated in buffer A at 500 μl/min. Unbound material was collected as one fraction. Bound material was eluted from the column at 500 μl/min with a linear gradient of 500 mM KH_2PO_4 in buffer A, in a total volume of 5 ml; 400 μl fractions were collected. SAG kinase activity was further purified by cation exchange on the SMART system. The cation-exchange column was equilibrated in buffer containing ethanediol and Thesit, and bound material was eluted with a linear gradient of KCl (0–1 M) in buffer A.

Determination of total protein concentration

Total protein was determined with the Bio-Rad protein assay kit assay, with BSA as standard.

SDS/PAGE analysis

Proteins were analysed by SDS/PAGE [10 % (w/v) gel]. Samples were prepared for SDS/PAGE by a procedure that removes solutes by using Strataclean resin. Proteinaceous solutions were mixed with resin and centrifuged at 14000 ***g***; the resin was washed twice with water and then boiled with SDS/PAGE sample buffer before loading in the gel well. Gels were stained with a diamine silver [29].

Determination of SAG kinase activity

Reactions were performed in 100 μl and the final concentrations in the assays were: 1 mM SAG, 4.4 mM phosphatidylserine, 70 mM octyl glucoside and 1 mM ATP with 1 μCi of [γ-^{32}P]ATP (final specific radioactivity in the assay of 10 Ci/mol), 5 mM $MgCl_2$, 20 mM NaF, 1 mM DTT and 50 mM Tris/HCl, pH 8.0. Reactions were started by the addition of ATP. After incubation at 37 °C for 30 min, reactions were terminated by the addition of chloroform/methanol/conc. HCl (15:300:5, by vol.). Phases were split by the addition of chloroform and water and the lower phase was dried *in vacuo*. Products and reactants were separated by TLC (silica G60, 5 cm × 20 cm) in chloroform/methanol/acetic acid (26:6:3, by vol.). Radioactivity was detected and quantified by phosphorimage analysis (Molecular Dynamics; Scanner SI). The amount of ^{32}P-labelled phosphatidate is quantified in phosphorimage units for chromatographic profiles. Kinetic parameters were determined with the Enzfitter program.

Argentation TLC

For argentation TLC, 10 cm × 20 cm silica plates (silica F60) were dried at 110 °C for 30 min, dipped for 5 min in 5 % (w/v) silver nitrate and oven-dried at 110 °C for 1 h. Samples were applied and the plates were developed in a saturated atmosphere of chloroform/methanol/acetic acid (40:4:4, by vol.). The R_F value for dioleoyl phosphatidate was 0.5 and for 1-stearoyl-2-arachidonoyl phosphatidate was 0.38. Radioactivity was detected and quantified by phosphorimage analysis.

RESULTS

Purification of SAG kinase activity

SAG kinase activity in the particulate fraction of pig testes could not be extracted with NaCl up to a final concentration of 2 M. Several detergents were able to release SAG kinase activity from this fraction (results not shown); of these, 2.5 % (w/v) sodium cholate was found to be the most effective. Extraction of enzyme activity was concentration-dependent and quantitative. SAG kinase activity was not detected in the detergent-insoluble fraction. It was not practicable to use sodium cholate for chromatography because of its charged nature and relatively high critical micellar concentration; thus 0.2 % (w/v) Thesit was chosen to maintain enzyme activity through subsequent purification procedures. This detergent does not absorb at 280 nm and has proved useful in the purification and subsequent sequencing of other membrane proteins [30].

Approx. 20 % of the total SAG kinase activity in the detergent extract did not bind to the hydroxyapatite column. The specific activity of this unbound activity was less than half that of the starting material (result not shown). Figure 1(a) shows the elution profile of protein and SAG kinase activity from the hydroxyapatite column. Two peaks of enzyme activity were detected, of which the first peak (HPT I) coincided with the major peak of eluted protein. This represented 25 % of total enzyme activity bound to the column and had a specific activity four times that of the starting material (as shown in Table 1). The second peak of SAG kinase activity (HPT II) bound to hydroxyapatite with high affinity. This SAG kinase activity was enriched approx. 50-fold in the pooled fractions and up to 80-fold in the peak fraction when compared with the starting material. These results for hydroxyapatite chromatography are representative of more than 10 preparations. Overall SAG kinase activity recovery was greater than 100 %.

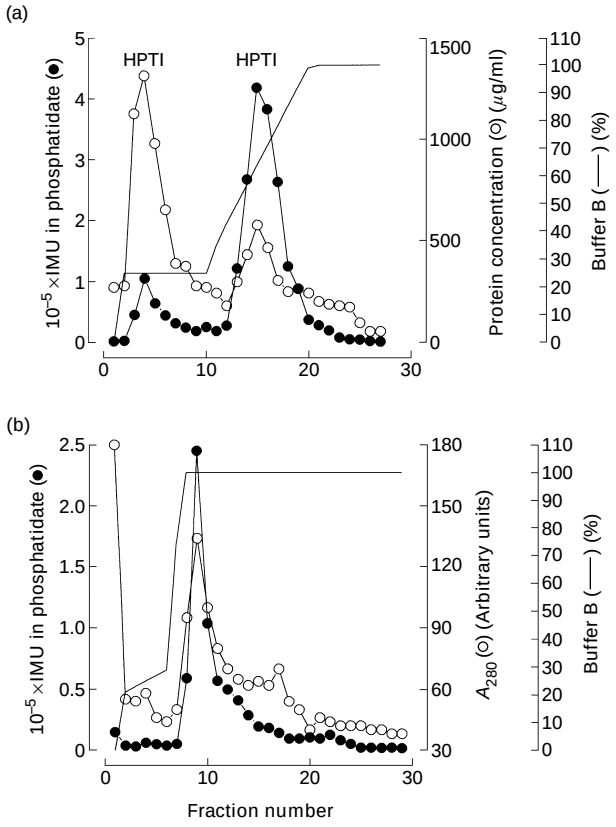

Figure 1 Chromatographic separation of SAG kinase activity

Detergent-extracted membranes from the particulate fraction of pig testes was loaded on the ceramic hydroxyapatite column connected to the FPLC. Bound protein was eluted with a nonlinear gradient of potassium phosphate in Thesit and ethanediol (**a**). SAG kinase activity is plotted as phosphorimage units (IMU) in phosphatidate. (**b**) The high-specific-activity SAG kinase activity was loaded on a Cibacron Blue 3GA agarose column and bound material was eluted with a nonlinear gradient of potassium phosphate, ethanediol and Thesit. SAG kinase activity and protein were determined in the fractions.

Table 1 Initial purification of SAG kinase

The results shown are representative of several purifications at this scale. SAG kinase activity was determined with 1 mM SAG and 1 mM ATP. Routinely the peak fractions of peak II from the hydroxyapatite column were 80–90-fold enriched in SAG kinase activity. The peak fraction from the Cibacron Blue column was 1000-fold enriched in enzyme activity.

Stage	Specific activity (pmol/min per mg)	Total protein (mg)	Total activity (pmol/min)	Fold purification	Recovery (%)
Membranes	0.045	875	36.6	1	100
HPT I	0.0026	18	5.0	5.8	14
HPT II (pool)	2.26	34	76	52	70
CBB (pool)	21	2.4	46	484	61

Cibacron Blue 3GA dye has been used in the purification of kinases and phosphatases; it was noted that SAG kinase activity bound most tightly to this dye, although it also bound to green and red dyes (results not shown). When active fractions from the hydroxyapatite column were applied to this column, up to 75 % of protein did not bind, whereas up to 80 % of SAG kinase activity did. Figure 1(b) shows the elution profile of protein and SAG kinase activity. Bound SAG kinase activity could not be eluted with KCl up to 2.5 M (results not shown). The resolution of the SAG kinase activity on this column was dependent on the nonlinear gradient of 30 % (v/v) ethanediol and 0.5 % Thesit. It therefore seems likely that the mode of interaction between the SAG kinase and the resin was in part due to hydrophobicity. This type of mixed interaction has also been reported for yeast hexokinase [31]. At this stage enzyme activity could be stored at −70 °C. Active fractions from the Cibacron Blue column were effectively desalted by covalent chromatography with thiolpropyl-Sepharose. This column bound 100 % of the SAG kinase activity and protein (assessed by SDS/PAGE and silver staining). Enzyme activity was eluted as a single peak under isocratic conditions with buffer C containing 50 mM DTT. The presence of excess DTT precluded the determination of protein concentration in these samples.

Analysis of the lipid substrate specificity of partly purified SAG kinase activity

Various studies have highlighted the possibility that diradylglycerols containing saturated or unsaturated fatty acids are selectively metabolized, whereas sphingomyelin metabolites might modulate the activity of DAG kinase isoforms [10–12,25]. Thus the substrate selectivity of partly purified pig SAG kinase activity was assessed. In parallel incubations *E. coli* DAG kinase was used as a control for the phosphorylation of the lipid species. This enzyme is catalytically active at pH 8.0, although it

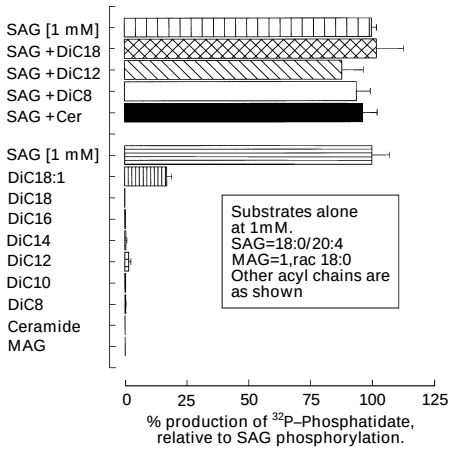

Figure 2 Substrate selectivity of the partly purified lipid kinase

SAG kinase was purified by hydroxyapatite and Cibacron Blue chromatography. The lipids were presented in the phosphatidylserine/octylglucoside mixed micelle alone at 1 mM or with 1 mM SAG. The amount of phosphatidate produced was quantified by phosphorimage analysis. The results from triplicate determinations are presented as percentages of the image units present in stearoylarachidonoyl phosphatidate (±S.D.). The results are representative of two other experiments.

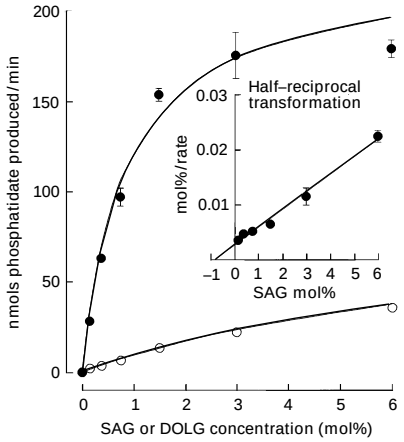

Figure 3 Substrate–velocity relationship for SAG and DOLG

The substrate–velocity relationship for SAG and DOLG was determined over the concentration range shown. The ATP concentration was 2 mM. Kinetic parameters were determined with the Enzfitter program. The results presented are means ± S.D. for triplicate determination. For SAG phosphorylation similar results were obtained in three experiments and for DOLG a similar result was obtained in one other experiment.

Table 2 The effect of dioleoylglycerol on SAG phosphorylation

DOLG (1 mM) was mixed with SAG (1 mM) and phosphorylated and the products were separated by argentation TLC and quantified by phosphorimage analysis. The results are presented as percentages of the image units obtained in 1-stearoyl-2-arachidonoyl phosphatidate (± range) from duplicate determinations, and similar results were obtained in two other experiments.

Diglyceride	Phosphorylation relative to SAG (%)
SAG	100 ± 13
SAG + DOLG	87 ± 8
DOLG	14 ± 1
DOLG + SAG	6.8 ± 0

is used in the DAG mass assay at pH 6.6 [32]. The partly purified mammalian lipid kinase was presented with the specified lipid at 1 mM in a phosphatidylserine/octyl glucoside micelle. The results in Figure 2 are shown relative to SAG phosphorylation. The mammalian lipid kinase did not phosphorylate any of the DAG species with disaturated fatty acid side chains. These DAG species were phosphorylated equally well by the bacterial enzyme under the same assay conditions (results not shown). Further, these lipids were neither inhibitors nor activators of the SAG phosphorylation catalysed by the SAG kinase. In all the preparations tested, a small amount of di-C_{12} was reproducibly converted to the corresponding phosphatidate. This was never greater than 1% of total SAG phosphorylation.

1-Monostearoyl-*rac*-glycerol (MAG in Figure 2) was not phosphorylated by the pig testicular kinase, although it was phosphorylated in parallel incubations by the *E. coli* DAG kinase to produce lysophosphatidate (results not shown). It has been reported that the 80 kDa pig brain cytosolic DAG kinase and rat brain cytosolic DAG kinase phosphorylated both monoacylglycerol and DAG [18,20]. Thus this pig testicular enzyme has a catalytic requirement for both fatty acid chains to be present, and the fatty acid in the *sn*-2 position must be unsaturated. These results are in good agreement with the reports of other particulate DAG kinase activities such as the arachidonoyl-selective DAG kinase from testes [19,21], but are in contrast with reports of cytosolic DAG kinases [16,18,22]. Ceramide has been reported to inhibit the activity of a cytosolic DAG kinase [10]. However, 1 mM ceramide was neither a substrate, activator nor inhibitor of the pig testis particulate SAG kinase isolated here. Sphingosine was not phosphorylated by the pig SAG kinase; however, this metabolite was poorly phosphorylated by the bacterial DAG kinase (results not shown).

DOLG was phosphorylated by the partly purified preparation of SAG kinase. This DAG has one double bond in each chain and the relative conversion of this species to phosphatidate was approx. 15% of SAG phosphorylation, as shown in Figure 2 and Table 2. In detergent extracts of pig testes, DOLG phosphorylation was approx. 33% of SAG phosphorylation. The ability of DOLG to modulate SAG kinase activity was investigated using argentation TLC, which separated both of the phosphatidates on the basis of degree of saturation. The results in Table 2 show that dioleoylglycerol (1 mM) had a small effect on the phosphorylation of SAG (1 mM), whereas the presence of 1 mM SAG inhibited the phosphorylation of 1 mM DOLG by approx. 50%. The substrate–velocity relationships for the phosphorylation of both these substrates by the pig SAG kinase are shown in Figure 3. The apparent K_m for SAG was 0.85 mol% [equivalent to 0.56 ± 0.09 mM SAG (mean ± S.D.), representative of three other experiments]. The apparent K_m for DOLG was 95 mol% [55 ± 5 mM DOLG (mean ± S.D.); a similar result was obtained in a further experiment]. Thus the differential effects of these diradylglycerols on the relative conversions to phosphatidates can be explained by the relative affinity of the enzyme for the substrates. The K_m for SAG phosphorylation reported here is in good agreement with the reported values of 0.35 mM for the bovine testicular arachidonoyl-selective DAG kinase [21] and 0.54 mM for the membrane-associated DAG kinase from *Drosophila* heads [20]. 1-Stearoyl-2-arachidonoyl phosphatidate

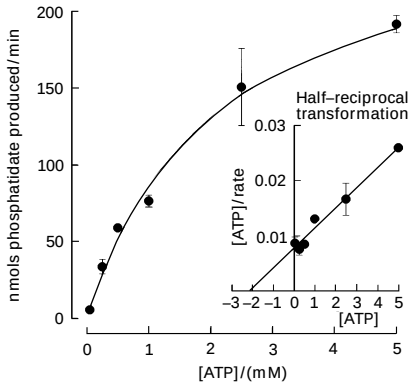

Figure 4 Substrate–velocity relationship for ATP

The substrate–velocity relationship for ATP was determined over the concentration range shown. The SAG concentration was 1 mM. Kinetic parameters were determined with the Enzfitter program. The results are means ± S.D. for triplicate determinations, and similar results were obtained in two other experiments.

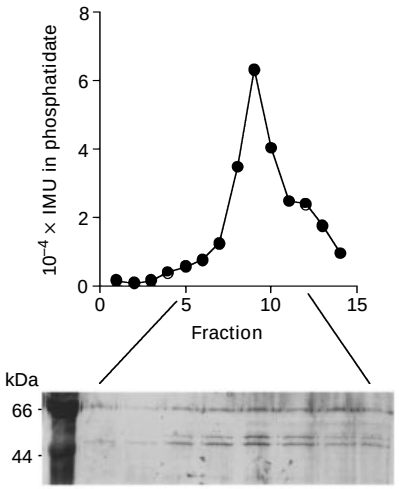

Figure 5 Further purification of SAG kinase

After hydroxyapatite and Cibacron Blue agarose chromatography, SAG kinase was desalted and then concentrated before cation-exchange chromatography. The column was eluted with a linear gradient of NaCl (1 M in buffer) and SAG kinase activity determined in the fractions. Active fractions were analysed by SDS/PAGE and stained with diamine silver.

was not an inhibitor of SAG phosphorylation up to a concentration of 0.5 mM (results not shown).

The substrate–velocity relationship between the partly purified SAG kinase activity and ATP was investigated. The results in Figure 4 show that the enzyme obeyed simple Michaelis–Menten-type kinetics with an apparent K_m for $MgATP^{2-}$ of approx. 2 mM. This result is considerably different from the K_m of 88 μM reported for the bovine testicular arachidonoyldiacylglycerol-glycerol kinase [21]. However, the pig testicular SAG kinase was able to use both ATP and GTP in the catalytic reaction (results not shown), a result also reported for the bovine enzyme [21].

Further analytical purification of SAG kinase

Enzyme activity was further concentrated by hydroxyapatite chromatography on the SMART system. After hydroxyapatite chromatography, SAG kinase activity was subjected to cation exchange on a Mono S column, also on the SMART system. The elution profile of SAG kinase activity from the column is shown in Figure 5. The peak of activity was analysed by SDS/PAGE. Owing to the small amounts of protein and the presence of potassium phosphate, detergent and ethanediol in the sample, which interfered with aspects of SDS/PAGE and silver staining, a method was developed to isolate protein before electrophoresis. This process involved the capture of the protein with Strataclean resin followed by several washes and in-gel electroelution of the proteins. The protocol was shown to give a better than 95% recovery of 50 ng (1 μg/ml) BSA from a solution containing detergent, ethanediol and potassium phosphate (results not shown). The results in Figure 5 show that two protein bands were present in fractions that contained enzyme activity as eluted from the Mono S column. The upper band, which closely correlated with enzyme activity, had an apparent molecular mass of approx. 55 kDa; the lower band had a molecular mass of approx. 50 kDa. From the silver-stained gel the estimated total protein recovered in fractions 8–12 was 100 ng, which gives a specific activity in these fractions of 142 pmol/min per mg, an enrichment of approx. 3000-fold and a recovery of 0.5%. The appearance of a protein band at 55 kDa is close to the molecular mass of the bovine testicular arachidonoylglycerol kinase, which was reported to be 58 kDa [21]. The 50 kDa band might represent a non-catalytically active fragment of the 55 kDa band; this breakdown of the SAG kinase, when highly purified, might account for the poor recoveries over the final stages of purification.

DISCUSSION

We have isolated the SAG kinase from pig testicular membranes. It is important to note that the enzyme was purified from the membrane fraction of pig testes and as such it is localized to the hydrophobic compartment of cells, where DAG is found and functions. The hydrophobic nature of this protein is underscored by the need for detergent and ethanediol to maintain reasonable stability; however, despite this, at stages of high purity considerable losses of activity were encountered. The isolation of a kinase that catalyses only the phosphorylation of polyunsaturated diacylglycerols provides the mechanism by which the cell can terminate the lipid half of the phosphoinositide signal pathway. In stimulated cells the predominant polyunsaturated DAG species are 1-stearoyl-2-arachidonoylglycerol, 1-stearoyl-2-eicosatrienoylglycerol and 1-stearoyl-2-pentaenoylglycerol [7]. Although the enzyme isolated here was purified on the basis of SAG phosphorylation it seems likely that the other polyunsaturated species would also be substrates. Dioleoylglycerol is one of the predominant monounsaturated DAG species in cells, and after stimulation the levels of SAG increase to those of DOLG. Thus the phosphorylation of SAG, even in the presence of DOLG reported here, illustrates the molecular selectivity of this SAG kinase.

The first mammalian DAG kinase to be isolated and cloned was the 80 kDa protein from pig lymphocytes; this cDNA has been designated DGKα [25]. Sequence analysis of the cDNA identified two EF hands motifs for calcium binding, two cysteine-rich zinc finger motifs and three potentially unique nucleotide-binding sites. Zinc finger motifs were originally described as mediating protein–nucleotide interactions; however, because cysteine-rich zinc finger-like sequences are repeated in tandem in protein kinase C isoforms [1], neuronal chimaerin [33] and DAG kinase [25], it has been proposed that this motif mediates a protein–lipid interaction [33]. Subsequently, the human hom-

ologue of DGKα has been identified and analysis of its expression has shown that it is expressed only in lymphocytes and oligodendrocytes [34]. Cloning strategies based on oligonucleotides derived from DGKα have isolated two related DAG kinase genes (termed DGKβ and γ but originally termed DGK II and III [35,36]). These clones are larger than DGKα and are expressed predominantly in the particulate fractions of neuronal tissues but not testes. DGKβ and γ isoforms also have two EF hands, two zinc fingers and a kinase domain and, like DGKα, show little selectivity for polyunsaturated DAG species [36]. Thus the pig testicular diradylglycerol kinase purified here is not closely related to any of these DAG kinase isoforms.

During the completion of this research an arachidonoyl-selective diacylglycerol kinase was isolated and characterized from the particulate fraction of bovine testes [21]. The protein had a molecular mass of 58 kDa by both SDS/PAGE and gel filtration. It seems likely that the pig SAG kinase is closely related to this protein. Although the strategy used here was different from that used in the purification of the bovine testicular enzyme, both proteins seemed to bind to cation- and anion-exchange columns. Further, our measurements of the substrate selectivity, affinity for polyunsaturated diradylglycerols and molecular mass of the pig SAG kinase are very similar to those reported previously [21].

Recently, three novel DAG kinase genes have been isolated with cDNA cloning strategies. The DGKδ gene was identified from a testis library and encodes a 130 kDa particulate protein that showed no DAG selectivity [37]. The other new DGK genes were isolated from human endothelial cells and were termed hDGKζ and hDGKϵ [38,39]. The hDGKζ gene encoded for a protein of approx. 103 kDa that showed no selectivity for naturally occurring long-chain diradylglycerols [39]. However, analysis of the hDGKϵ cDNA showed that this sequence encoded for a 64 kDa protein that contained a DAG-binding site distinct from that found in the other DAG kinases. Overexpression of this cDNA in COS cells resulted in markedly increased arachidonoyl-selective DAG kinase activity in the cytosolic fraction [38]. hDGKϵ was expressed in testes but not in brain or lymphocytes. It seems likely that the pig SAG kinase activity isolated here and the bovine testicular SAG kinase [21] are homologues of the cloned human DGKϵ. However, it is not clear why the native activities were particulate whereas the expressed cloned protein was predominantly cytosolic [38]. It is therefore possible that the enzyme isolated here is distinct from DGKϵ. The isolation of SAG kinase from testicular tissues will allow its use in the manipulation of polyunsaturated DAG levels *in vivo*. Such experiments can be used to study the exact roles of DAG and phosphatidate in a diverse range of biological processes.

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