Partial purification of a diacylglycerol lipase from bovine aorta

Mark W. LEE and David L. SEVERSON*

MRC Signal Transduction Group, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

A diacylglycerol (DG) lipase has been purified from a soluble subcellular fraction of bovine aorta by $(NH_4)_2SO_4$ precipitation in the presence of 5.0% (w/v) Triton X-100, followed by chromatography on DEAE-Sephacel, heparin–Sepharose and octyl-Sepharose in the presence of either CHAPS or Triton X-100 detergents. Under basal conditions, the hydrolysis of a short-chain [^{3}H]dioctanoylglycerol ([^{3}H]diC$_8$) substrate was much greater than that of a long-chain 1-[1-^{14}C]palmitoyl-2-oleoyl-*sn*-glycerol (1-[^{14}C]POG) substrate. Lipase activity measured with 1-[^{14}C]POG was markedly enhanced by Triton X-100. In the presence of 0.1% Triton X-100, specific enzyme activities in the octyl-Sepharose fraction determined with 1-[^{14}C]POG or 1-stearoyl-2-[1-^{14}C]-arachidonoyl-*sn*-glycerol as substrates were the same as that measured with [^{3}H]diC$_8$. $MgCl_2$ (5 mM) or $CaCl_2$ (2 mM) also selectively stimulated lipase activity (up to 10–13-fold) measured with the long-chain (1-[^{14}C]POG) substrate only. The increase in relative specific activity in the octyl-Sepharose fraction was 60-fold and 155-fold, based on hydrolysis of [^{3}H]diC$_8$ and 1-[^{14}C]POG (+Triton X-100), respectively. Unlabelled diC$_8$ was a competitive inhibitor of 1-[^{14}C]POG hydrolysis, suggesting that a single lipase hydrolyses both the short-chain and long-chain DG substrates; selective stimulatory effects of non-ionic detergents and bivalent cations on the hydrolysis of 1-[^{14}C]POG may be due to effects on the physical properties of the substrate preparation. Monoacylglycerol lipase, DG kinase and cholesterol esterase activities could not be detected in the partially purified lipase preparation.

INTRODUCTION

Hydrolysis of membrane phospholipids generates diacylglycerol (DG) second messengers [1,2] that stimulate the activity of protein kinase C [3,4]. Attenuation of protein kinase C-mediated action will result from DG metabolism by DG kinase and/or the concerted action of DG and monoacylglycerol (MG) lipases [2,5]. DG catabolism by the lipase pathway in platelets [6,7], fetal membranes and decidua vera [8], neutrophils [9], rabbit aorta [10], rat brain microvessels [11] and dorsal root ganglion neurons [12] proceeds by the ordered cleavage of the acyl group in the *sn*-1 position of the glycerol backbone, followed by hydrolysis of the *sn*-2 acyl chain in the 2-MG intermediate. The lipase pathway can therefore release arachidonic acid from the *sn*-2 position of arachidonate-enriched DG, which can then be converted into other cellular mediators [5]. The DG lipase pathway is required for the prostanoid-dependent contraction of gastric smooth (longitudinal) muscle induced by epidermal growth factor [13].

In platelets, phosphorylation of DG by DG kinase represents the principal biochemical mechanism for signal termination [14]. However, DG lipase activity measured *in vitro* was greater than DG kinase activity in decidua vera [8], cardiac myocytes [15] and rabbit aorta [10]. Dioctanoylglycerol (diC$_8$), a cell-permeant DG analogue that activates protein kinase C in intact cells [16], has been used to monitor the flux of the DG through metabolic pathways in platelets [14] and other cells [17–19]. Metabolism of exogenous radiolabelled diC$_8$ by rabbit aortic smooth-muscle cells [17] and cultured A10 smooth-muscle cells [18] demonstrated that the DG lipase pathway was the predominant route for DG metabolism, consistent with measurements *in vitro* of DG lipase activity [10,18].

DG kinase has been purified to homogeneity from brain [20,21]. By comparison, most studies on DG lipase [6,10,22–24] have utilized crude subcellular fractions as an enzyme source because of limited success in purification attempts. Farooqui et al. [25,26] have described the partial purification of membrane-bound DG lipases from bovine brain. Given the significance of the DG lipase pathway for DG metabolism in aortic smooth-muscle cells, the objective of this investigation was to purify and characterize the enzyme from bovine aorta.

EXPERIMENTAL

Materials

[1-^{14}C]Oleic acid, glycerol [1-^{14}C]trioleate ([^{14}C]triolein), 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol (2-[^{14}C]SAG) and cholesterol [1-^{14}C]oleate were purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada). 1-[1-^{14}C]Palmitoyl-lysophosphatidylcholine was obtained from New England Nuclear Research Products (Boston, MA, U.S.A.). 1-[1-^{14}C]Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine were synthesized, and the corresponding diacylglycerols, 1-[1-^{14}C]palmitoyl-2-oleoyl-*sn*-glycerol (1-[^{14}C]POG) and 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol (2-[^{14}C]POG), were generated by treatment with phospholipase C as described by Hee-Cheong et al. [11]. [^{14}C]Diolein (approx. 77% 1,3 and 23% 1,2 isomers) with the radioactivity randomly distributed among the acyl positions was purchased from RoseChem (Los Angeles, CA, U.S.A.).

[^{3}H]Dioctanoylglycerol ([^{3}H]diC$_8$) was prepared by dephosphorylation of 1,2-dioctanoyl-[2-^{3}H]glycerol 3-phosphate, a custom-synthesis product from New England Nuclear Research Products, with alkaline phosphatase as described by Severson and Hee-Cheong [17]. 2-[1-^{14}C]Mono-olein was prepared by incubating [^{14}C]triolein with pancreative lipase [10]. Unlabelled lipids were purchased from Serdary Research Products (London,

Abbreviations used: DG, diacylglycerol; MG, monoacylglycerol; diC$_8$, dioctanoylglycerol; 2-[^{14}C]SAG, 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol; 1-[^{14}C]POG, 1-[1-^{14}C]palmitoyl-2-oleoyl-*sn*-glycerol; 2-[^{14}C]POG, 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol; [^{3}H]diC$_8$, dioctanoyl-[2-^{3}H]glycerol; S100, 100000 *g* supernatant fraction.

* To whom correspondence should be addressed.

Ontario, Canada). EcoLite scintillation fluid was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). Pre-coated glass t.l.c. plates (Sil G-25, 0.25 mm thickness; 20 cm × 20 cm) were obtained from Brinkmann Instruments (Oakville, Ontario, Canada). DEAE-Sephacel, heparin–Sepharose CL-6B, octyl-Sepharose CL-4B and conventional chromatography supplies were obtained from Pharmacia (Uppsala, Sweden). All biochemicals were of analytical grade or better.

Enzyme assays

DG lipase was assayed as previously described, with co-sonicates of radiolabelled DG and phosphatidylserine [17] as the substrate emulsion. Long-chain DGs ([^{14}C]diolein, 1-[^{14}C]POG and 2-[^{14}C]POG) in hexane and phosphatidylserine in chloroform were dried under N_2 and resuspended in potassium phosphate buffer, pH 7.0. The substrate mixtures were sonicated for 4 × 30 s bursts (75 W; BraunSonic 1510 sonicator) and assays were performed by incubating enzyme fractions with 0.1 ml of the substrate mixture in a final volume of 0.4 ml. Final assay concentrations were: 80 μM radiolabelled DG substrate (sp. radioactivity 2500–5000 d.p.m./nmol), 100 μM phosphatidylserine and 50 mM potassium phosphate (pH 7.0). After incubations for 20–60 min at 37 °C, reactions were terminated and the release of ^{14}C-labelled fatty acids was measured after liquid–liquid partitioning [11,27].

DG lipase activity was also determined with 1-[^{14}C]POG, 2-[^{14}C]SAG and [^{3}H]diC$_8$ substrates by a t.l.c. procedure. Assay parameters were the same as described above, but the reactions were terminated by addition of 2.0 ml of chloroform/methanol (2:1, v/v) and 0.1 ml of 5 M HCl. After addition of 0.3 ml of water, the samples were centrifuged and the upper phase was removed. The lower phase was dried under N_2, resuspended in chloroform/methanol (2:1, v/v), and applied to glass t.l.c. plates. After development in hexane/diethyl ether/acetic acid (80:20:1, by vol.) or heptane/diethyl ether/acetic acid (25:75:1, by vol.), the radioactivity in the ^{14}C-labelled non-esterified fatty acid (1-[^{14}C]POG substrate) and [^{14}C]MG or [^{3}H]MG bands (2-[^{14}C]SAG and [^{3}H]diC$_8$ substrates, respectively) was determined as described by Hee-Cheong et al. [11]. The latter solvent system generally provided better separation of the radiolabelled MG bands when quantification of released MG as an index of *sn*-1 hydrolysis was the primary goal. Results of the two assay methods (extraction and t.l.c.) were quite comparable for the long-chain substrates. Assays with 1-[^{14}C]POG (release of [^{14}C]palmitate) and [^{3}H]diC$_8$ (formation of [^{3}H]MG) substrates are specific for *sn*-1 hydrolysis; assays with 2-[^{14}C]SAG can measure hydrolysis of both the *sn*-1 (formation of [^{14}C]MG) and *sn*-2 (release of [^{14}C]arachidonate) positions. Previous studies with aortic MG lipase [28] have demonstrated that 1-[^{14}C]monoolein and 2-[^{14}C]mono-olein were hydrolysed at equal rates; therefore any acyl migration after *sn*-1 hydrolysis of 2-[^{14}C]SAG would not alter the determination of *sn*-2 lipase activity. Since the [^{14}C]diolein substrate consisted of a mixture of 1,2 and 1,3 isomers with radioactivity randomly distributed among all the acyl positions, lipase activity determined with this substrate cannot distinguish between hydrolysis of *sn*-1, *sn*-2 or *sn*-3 positions.

Simultaneous assays for DG lipase and kinase activities were performed as described by Severson and Hee-Cheong [17], utilizing [^{3}H]diC$_8$/phosphatidylserine and 1-[^{14}C]POG/phosphatidylserine co-sonicates in phosphate buffer. Assay conditions were as described above, except that 2 mM ATP and 10 mM $MgCl_2$ were added to the incubation. Assay mixtures were incubated at 37 °C for 20–45 min, and reaction products were extracted and subjected to a two-step t.l.c. procedure as described by Hee-Cheong et al. [11]. Lipase activity was determined from the formation of [^{3}H]MG or [^{14}C]palmitate; kinase activity was calculated from the formation of radiolabelled phosphatidic acid.

Assays for triacylglycerol and MG lipase activities were performed by either the extraction method or the t.l.c. procedure as described above, with [^{14}C]triolein/phosphatidylserine or 2-[^{14}C]mono-olein/phosphatidylserine co-sonicates as substrates, respectively. Cholesterol esterase activity was measured by the extraction method with a cholesterol [1-^{14}C]oleate/phosphatidylserine co-sonicate. Final assay concentrations of [^{14}C]monoolein, [^{14}C]triolein and cholesterol [1-^{14}C]oleate were also 80 μM.

Specific activities of all enzymes are routinely expressed as units/mg of protein, where 1 unit of activity was arbitrarily defined as the quantity of enzyme that resulted in the formation of 1 nmol of product in 1 h.

Preparation of subcellular fractions from bovine aorta

Frozen bovine aortae were purchased from Pel-Freez Biologicals (Rogers, AR, U.S.A.). A portion of bovine aorta was thawed at 4 °C in a solution of 1 mM EDTA, 1 mM dithiothreitol and 20 mM Hepes (pH 7.4; buffer A). After removal of the adventitia, the intima-media (approx. 30 g) was cut into small pieces and homogenized in 10 vol. of cold buffer A with a Polytron P-10 tissue homogenizer (2 × 30 s, rheostat setting of 7). The homogenate was then centrifuged at 1000 *g* for 20 min, and the supernatant was filtered through glass wool. The precipitate was re-suspended in buffer A, re-homogenized, and centrifuged again at 1000 *g* for 20 min. After filtration, the low-speed supernatants were combined and centrifuged at 100000 *g* for 60 min to obtain a high-speed supernatant fraction (S100). The pellet was resuspended in buffer A and homogenized in a Potter–Elvehjem homogenizer.

Partial purification of DG lipase from bovine aorta

A sample of the S100 fraction containing approx. 220 mg of protein was adjusted to 5.0% (w/v) Triton X-100. After dissolution of the detergent and further mixing for 30 min at 4 °C, the sample was subjected to $(NH_4)_2SO_4$ precipitation. At 4 °C in the presence of $(NH_4)_2SO_4$ (between 20 and 30% saturation), Triton X-100 partitions as a floating detergent phase, due to lowering of the detergent's cloud point [29]. Therefore the sample was adjusted from 0 to 20% saturation by addition of solid $(NH_4)_2SO_4$, equilibrated for 30 min at 4 °C, and centrifuged at 25000 *g* for 20 min. After centrifugation, the supernatant was adjusted to 30%-satd. $(NH_4)_2SO_4$ and equilibrated for 30 min as before. The 20–30%-satd.-$(NH_4)_2SO_4$ sample was centrifuged at 4 °C at 100000 *g* for 30 min in thin-walled polyallomer tubes (Beckman Instruments, Mississauga, Ontario, Canada), after which the infranatant was removed dropwise after puncture of the tubes. The detergent-enriched phase and the pellet remaining in the centrifuge tube were resuspended in up to 5 vol. of 1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 μM leupeptin/20 mM imidazole/HCl (pH 7.4; Buffer B). The suspension was centrifuged for an additional 10 min at 1000 *g* in order to remove insoluble material, and the soluble fraction was dialysed overnight against a 30-fold excess of Buffer B. The detergent 'extract' arising from the 20–30%-satd.-$(NH_4)_2SO_4$ step was used as the starting material for further purification. Assays of the remaining $(NH_4)_2SO_4$ fractions [0–20% pellet, 20–30% insoluble pellet, aqueous supernatant from 30% $(NH_4)_2SO_4$] indicated that most (70–75%) of the recovered DG lipase activity was found in the detergent-enriched phase.

Before ion-exchange chromatography, the dialysed Triton X-100 extract was adjusted to 7.5 mM CHAPS and gently stirred at 4 °C for 30 min. The sample was then loaded at a flow rate of 6 cm/h on to a DEAE-Sephacel column (1.6 cm × 20 cm; bed vol. 35 cm^3) equilibrated with 1 mM dithiothreitol/1 μM leupeptin/50 mM NaCl/10 % (v/v) glycerol/20 mM imidazole/HCl (pH 7.4). After sample loading, the column was washed at 15 cm/h with 1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 μM leupeptin/50 mM NaCl/10 % glycerol/7.5 mM CHAPS/20 mM imidazole/HCl (pH 7.4; Buffer C). DG lipase was eluted by an increasing linear NaCl gradient formed from equal volumes of Buffer C and the same buffer with 0.6 M NaCl. The A_{280} was monitored, and 3 ml fractions were collected during this step. Assays for DG lipase activity were performed in the presence of 0.1 % Triton X-100.

Fractions with DG lipase activity from DEAE-Sephacel were pooled and dialysed overnight against a 40-fold excess of 1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 μM leupeptin/15 % glycerol/20 mM Mops/KOH (pH 7.4). The dialysed sample was adjusted to 5 mM CHAPS and stirred for 20 min at 4 °C before loading at 15 cm/h on to a heparin–Sepharose column (1 cm × 10 cm; bed vol. 6.3 cm^3) previously equilibrated with 20 mM Mops/KOH (pH 7.4). The column was washed with 1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 μM leupeptin/50 mM NaCl/15 % glycerol/20 mM Mops/KOH (pH 7.4; Buffer D). DG lipase was eluted by an increasing linear NaCl gradient formed from equal volumes of Buffer D and the same buffer containing 0.6 M NaCl; 2 ml fractions were collected.

After heparin–Sepharose chromatography, samples containing DG lipase were pooled and adjusted to 300 mM NaCl. The sample was loaded directly on to an octyl-Sepharose column (1 cm × 10 cm; bed vol. 2.4 cm^3) at a flow rate of 7.6 cm/h, and 1.2 ml fractions were collected. After loading, the flow rate was increased to 15 cm/h and the column was washed sequentially with 5 bed vol. of 1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/300 mM NaCl/20 % glycerol/5 mM CHAPS/20 mM imidazole/HCl (pH 7.4), and 5 bed vol. of 1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/20 % glycerol/20 mM imidazole/HCl (pH 7.4; Buffer E). DG lipase was eluted from the column by an increasing linear Triton X-100 gradient formed from equal volumes of Buffer E and the same buffer containing 0.5 % Triton X-100.

After octyl–Sepharose chromatography, it was necessary to concentrate the samples containing DG lipase and adjust the concentration of Triton X-100. The detergent had significant influences on protein and enzyme assays, but its inclusion was imperative for lipase stabilization. Fractions containing DG lipase activity were perfused by hand over 0.5 ml of DEAE-Sephacel in a Pasteur pipette, and the flow-through was collected. The column was then washed with 1 mM EDTA/1 mM dithiothreitol/10 % glycerol/0.1 % Triton X-100/20 mM imidazole/HCl (pH 7.4; Buffer F), and the absorbance was monitored at 245 nm, where the absorbance of the Triton phenyl group is much less than at 280 nm [30]. The column was washed until the A_{245} returned to baseline, after which DG lipase was pulse-eluted with Buffer F containing 0.5 M NaCl. Samples containing significant amounts of protein on the basis of A_{245} were pooled and dialysed (in conjunction with samples from the DEAE-Sephacel and heparin–Sepharose pools adjusted to 0.1 % Triton X-100) against a 100-fold excess of Buffer F before assays. Samples stored in buffer solutions containing both 0.1 % Triton X-100 and 10 % glycerol retained DG lipase activity for a minimum of 8 weeks when stored at −80 °C. Protein concentrations were determined by the procedure of Spector [31], with BSA as standard and correcting for all buffer contributions.

RESULTS

Routinely, 60–70 % of total DG lipase activity was recovered in the S100 fraction, which also contained 85 % of total cellular protein. Inclusion of protease inhibitors (leupeptin, pepstatin) during homogenization did not significantly alter the distribution or recovery of lipase activity, nor did homogenization of fresh (not frozen) aorta. The enzyme was very stable in the crude S100 fraction; activity was retained for over 6 months when the S100 fraction was stored at −80 °C.

DG lipase in the S100 fraction was very hydrophobic and pre-aggregated. Routine $(NH_4)_2SO_4$ precipitation of DG lipase was not possible, as up to 30 % of enzyme activity precipitated before 20 % saturation. Therefore, phase partitioning [29] by $(NH_4)_2SO_4$ precipitation of the S100 fraction in the presence of 5.0 % Triton X-100 was utilized as the initial fractionation step; DG lipase readily partitioned into the detergent phase. Inclusion of detergent was also necessary for all subsequent purification procedures. Chromatography on DEAE-Sephacel and heparin–Sepharose was carried out in the presence of CHAPS, since Triton X-100 did not resolve lipase activity to the same degree. Fractionation of the S100 extract by $(NH_4)_2SO_4$ in the presence of CHAPS resulted in a poor recovery of enzyme activity.

Although the use of detergents was a pre-requisite for purification, their subsequent introduction into lipase assays markedly affected activity measurements. The effect of varying concentrations of Triton X-100 on the activity of DG lipase in the S100 fraction is shown in Figure 1(a). Under basal conditions (absence of detergent), the hydrolysis of [^{3}H]diC_8 was 8–10 times that of the long-chain DG substrate, 1-[^{14}C]POG. Addition of Triton X-100 resulted in a concentration-dependent inhibition of diC_8 hydrolysis. In assays with 1-[^{14}C]POG as substrate, DG lipase activity was inhibited at low detergent concentrations up to the critical micelle concentration (0.02 %, w/v, or 0.3 mM [32]), but higher concentrations produced a marked stimulation of enzyme activity (Figure 1a). As a result, the rate of hydrolysis of 1-[^{14}C]POG was equal to or greater than that of [^{3}H]diC_8 at detergent concentrations of 0.05–0.2 %.

Other detergents were also tested for their effects on S100 DG lipase activity determined with the long-chain 1-[^{14}C]POG substrate. Nonidet P-40 (0.1 %, w/v) produced the same degree of lipase stimulation as Triton X-100, whereas CHAPS and deoxy-

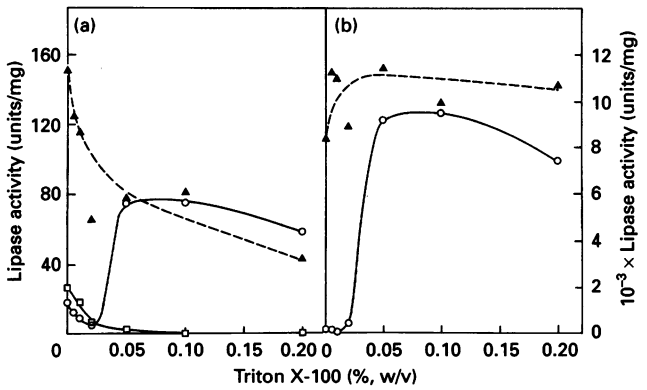

Figure 1 Influence of Triton X-100 on aortic DG lipase activity

DG lipase activity with 1-[^{14}C]POG (○) and [^{3}H]diC_8 (▲) as substrates, and MG lipase activity (□), were measured in the presence of the indicated concentrations of Triton X-100. Results are from a representative experiment. (**a**) S100 (crude) fraction; (**b**) octyl-Sepharose-purified preparation.

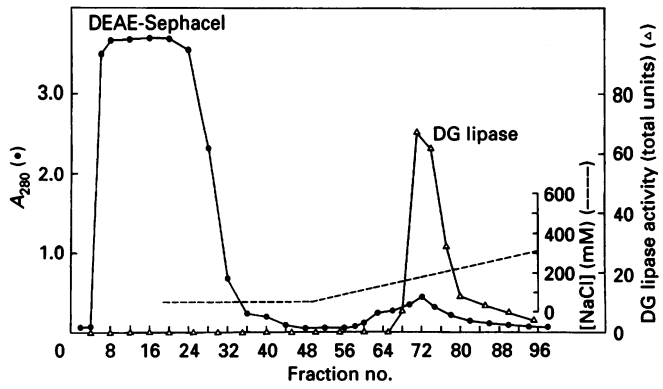

Figure 2 DEAE-Sephacel chromatography of aortic DG lipase

The detergent extract obtained from $(NH_4)_2SO_4$ precipitation in the presence of 5.0% Triton X-100 was applied to a DEAE-Sephacel column. DG lipase activity (△) was eluted by an increasing linear NaCl gradient (– – – –) after washing the column with buffer C and assayed as described in the Experimental section ([^{14}C]diolein substrate with 0.1% Triton X-100). Fraction volumes were 3.0 ml; the A_{280} is also shown (●). The prominent increase in A_{280} seen in early fractions was largely due to the elution of Triton X-100 during loading of the detergent-enriched $(NH_4)_2SO_4$ extract.

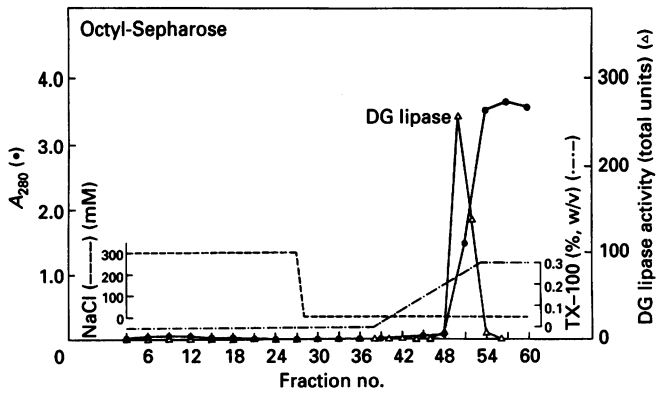

Figure 4 Octyl-Sepharose chromatography of aortic DG lipase

Fractions containing DG lipase activity from heparin–Sepharose were pooled, adjusted to 300 mM NaCl and loaded directly on to an octyl-Sepharose column at 4 °C. After washing the column sequentially with buffers containing 5 mM CHAPS and 300 mM NaCl (– – – –), DG lipase activity (△) was eluted by an increasing linear Triton X-100 gradient (TX-100; —·—·—) and assayed as described previously. Fraction volumes were 1.2 ml; the A_{280} (●) documenting the saturation of the column with Triton X-100 is also shown.

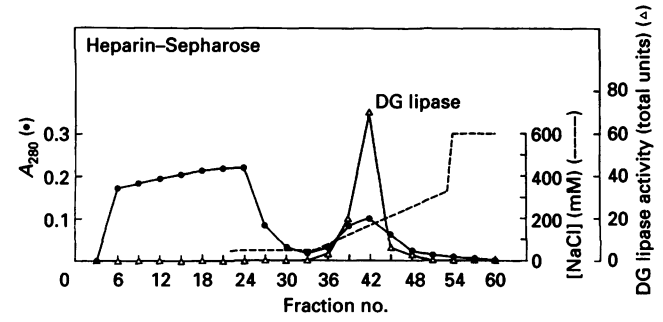

Figure 3 Heparin–Sepharose chromatography of aortic DG lipase

Fractions containing DG lipase activity from DEAE-Sephacel were pooled, dialysed and loaded on to a heparin–Sepharose column. DG lipase activity (△) was eluted by an increasing linear NaCl gradient (– – – –) and was assayed as described in the Experimental section ([^{14}C]diolein substrate with 0.1% Triton X-100). Fraction volumes were 2.0 ml; the A_{280} is also shown (●).

cholate were mildly and strongly inhibitory, respectively, over the wide range of concentrations.

MG lipase activity in the S100 fraction (determined with 2-[^{14}C]mono-olein in the absence of detergent) was 51.1 ± 1.6 units/mg (mean ± S.D.; $n = 3$), compared with 19.5 ± 7.8 units/mg ($n = 3$) for DG lipase (measured with 1-[^{14}C]POG). MG lipase activity was inhibited at all concentrations of Triton X-100 examined (Figure 1a). Hydrolysis of [^{14}C]diolein was also stimulated by the addition of Triton X-100; at 0.1% Triton X-100, S100 DG lipase activity was stimulated 5-fold, increasing from 5.1 ± 0.8 to 24.9 ± 2.6 units/mg ($n = 3$). Therefore, column fractions were assayed for DG lipase with [^{14}C]diolein in the presence of 0.1% Triton X-100 to augment the sensitivity of the assay. Inclusion of 0.1% Triton X-100 for assays of column fractions partially overcame the inhibitory effects of various buffer components such as CHAPS.

Representative profiles from DEAE-Sephacel, heparin–Sepharose and octyl-Sepharose chromatography are shown in Figures 2, 3 and 4 respectively. In each case, DG lipase activity was recovered as a single peak. Most of the DG lipase activity was eluted at 160–180 mM NaCl from the DEAE-Sephacel (Figure 2) and at 150–160 mM NaCl for the heparin–Sepharose columns (Figure 3). The lipase remained tightly bound to octyl-Sepharose even in the presence of 20% glycerol and in the absence of NaCl, and was eluted only when the column was perfused with Triton X-100 (Figure 4). Multiple bands were evident on silver-stained SDS/polyacrylamide gels of octyl-Sepharose fractions. Other techniques, including chromatography on thiopropyl-Sepharose and hydroxyapatite, or gel filtration, did not improve the overall purification. Consequently it was decided to characterize the properties of this partially purified lipase preparation.

The effect of Triton X-100 on DG lipase activity in the final octyl-Sepharose fraction is shown in Figure 1(b). The detergent produced a slight stimulation of [^{3}H]diC$_8$ hydrolysis by the partially purified enzyme, whereas S100 lipase activity was inhibited (Figure 1a). Triton X-100 stimulated the hydrolysis of 1-[^{14}C]POG by the octyl-Sepharose fraction (Figure 1b) with the same sensitivity as for the S100 fraction (Figure 1a), but the extent of the stimulation (45-fold increase in lipase activity) was much greater than that observed for the S100 fraction. Hydrolysis of 2-[^{14}C]SAG by the octyl-Sepharose fraction could not be detected in the absence of Triton X-100. Nonidet P-40 (0.1%) produced a 43-fold stimulation of 1-[^{14}C]POG hydrolysis, comparable with that seen with Triton X-100. The addition of 0.1% Tween 20 resulted in a 3-fold increase in lipase activity. Due to the differential effects of Triton X-100 on lipase activity determined with short- and long-chain DG substrates in the S100 starting fraction and partially purified fraction (Figure 1), the presence or absence of the detergent in assays with different substrates had a marked effect on the changes in enzyme specific activity at the various stages of the purification protocol.

Table 1 summarizes the results from a representative purification protocol for DG lipase activity, utilizing both short-chain ([^{3}H]diC$_8$) and long-chain (1-[^{14}C]POG and 2-[^{14}C]SAG in the presence of Triton X-100) substrates. Accurate determinations of DG lipase activity at the 20–30%-satd.-$(NH_4)_2SO_4$ step were not possible, because of the very high concentration of Triton X-100 in the detergent phase, which markedly influenced the lipase assays and protein measurements. A final 98.5-fold increase in

Table 1 Purification of DG lipase from bovine aorta

DG lipase activity in S100 and partially purified fractions was measured as described in the Experimental section; 0.1% Triton X-100 (TX-100) was included in assays with 1-[^{14}C]POG and 2-[^{14}C]SAG. Results are from a single representative purification protocol. Values for purification (fold) are given in parentheses.

Enzyme fraction	Protein (mg)	DG lipase (units/mg)		
		diC_8	1-[^{14}C]POG + TX-100	2-[^{14}C]SAG + TX-100
S100	210	170 (1.0)	72 (1.0)	69 (1.0)
DEAE-Sephacel	10.1	660 (3.9)	434 (6.0)	474 (6.9)
Heparin–Sepharose	1.8	2010 (11.8)	1530 (21.3)	1611 (23.4)
Octyl-Sepharose	0.086	16700 (98.5)	16300 (227.0)	18861 (274.0)

relative specific activity was obtained for the octyl-Sepharose sample when assayed with [^{3}H]diC_8 (Table 1). If Triton X-100 (0.1%) was present, DG lipase activity ([^{3}H]diC_8 hydrolysis) in the S100 and octyl-Sepharose fractions was 112 and 20270 units/mg respectively, giving a 181-fold increase in specific activity under these assay conditions. The rates of hydrolysis of the two long-chain substrates, 1-[^{14}C]POG and 2-[^{14}C]SAG, determined in the presence of Triton X-100, were very similar for each of the steps, and gave 227- and 274-fold increases in specific activity for the octyl-Sepharose fraction (Table 1). As noted previously with a different partially purified enzyme preparation (Figure 1), the hydrolysis rates for [^{3}H]diC_8 and 1-[^{14}C]POG plus Triton X-100 were very similar in the octyl-Sepharose fraction (Table 1). In four separate purification protocols, DG lipase activity was increased by 60.0 ± 22.7-fold in the final octyl-Sepharose fraction, with an overall yield of 4.9 ± 0.5%, based on diC_8 hydrolysis. By comparison, hydrolysis of the long-chain 1-[^{14}C]POG increased 155 ± 41.7-fold ($n = 4$) at the octyl-Sepharose step (assays containing Triton X-100). DG kinase activity was 4-fold and 15-fold greater than lipase activity in the S100 fraction when assayed with [^{3}H]diC_8 and 1-[^{14}C]POG respectively, but could not be detected after the first column step (results not shown).

Reaction rates were linear with respect to total protein for both S100 (5–50 μg) and octyl-Sepharose (0.025–0.20 μg) fractions. The presence of 0.1% Triton X-100 in assays with the 1-[^{14}C]POG substrate enhanced the linearity of DG lipase assays with respect to total protein and time of incubation. The partially purified DG lipase exhibited a neutral to slightly alkaline pH optimum (pH 7.0–8.0), with very little acid lipase activity (results not shown).

Although MG lipase activity could be measured in the S100 fraction (Figure 1a), MG lipase activity could not be detected in the octyl-Sepharose fraction. However, the sensitivity of MG lipase assays of partially purified fractions was compromised by the inhibitory effects of Triton X-100 (Figure 1a), which precluded assays with larger sample volumes. Similar difficulties were encountered in assays for *sn*-2 lipase activity (release of ^{14}C-labelled fatty acids) using 2-[^{14}C]POG or 2-[^{14}C]SAG substrates.

Triacylglycerol lipase activity was detected in both the S100 and octyl-Sepharose fractions. S100 triacylglycerol lipase activity (measured with [^{14}C]triolein) was increased from 2.8 ± 0.6 to 8.5 ± 3.2 units/mg (both $n = 3$) by addition of 0.1% Triton X-100. By comparison, octyl-Sepharose triacylglycerol lipase activity was 1390 ± 254 units/mg ($n = 3$), and was not enhanced by Triton X-100 (1400 ± 531 units/mg; $n = 3$). Cholesterol esterase activity was not detectable in the octyl-Sepharose fraction.

DG lipase and kinase activities can be measured simultaneously when assay incubations contain ATP and $MgCl_2$ [10,11,17]. DG kinase activity in the octyl-Sepharose fraction was undetectable when assays utilized either [^{3}H]diC_8 or 1-[^{14}C]POG (results not shown). The presence of ATP and $MgCl_2$ had no effect on lipase assays with the [^{3}H]diC_8 substrate, but the hydrolysis of 1-[^{14}C]POG was increased 9-fold (octyl-Sepharose fraction) under these conditions. Consequently, the effects of $MgCl_2$ and ATP added individually and in combination were determined. Addition of Mg^{2+} produced a concentration-dependent stimulation of DG lipase activity in assays with 1-[^{14}C]POG, resulting in an 11-fold increase in lipase activity (from 383 to 4380 units/mg; means of two expts.) at 5 mM $MgCl_2$. ATP alone (2 mM) did not have any effect, and there was no further effect on lipase activity when ATP and $MgCl_2$ were combined. The partially purified DG lipase was also stimulated to a similar degree by 2 mM $CaCl_2$ (from 396 to 5080 units/mg; means of two expts.). Addition of other bivalent metal ions either completely abolished hydrolysis of 1-[^{14}C]POG (i.e. Cu^{2+}, Zn^{2+}) or produced little effect (i.e. Ni^{2+}).

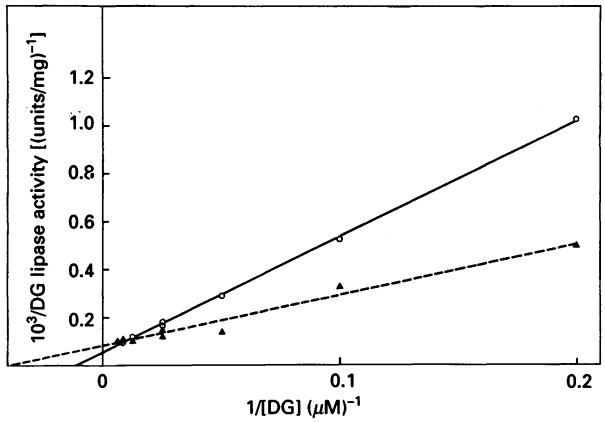

Figure 5 Effect of substrate concentration on aortic DG lipase activity

Octyl-Sepharose-purified DG lipase activity was measured at [^{3}H]diC_8 (▲) and 1-[^{14}C]POG (○) concentrations in the ranges 5–160 μM and 5–120 μM respectively; hydrolysis of 1-[^{14}C]POG was measured in the presence of 0.1% Triton X-100. Results are presented as a double-reciprocal Lineweaver–Burk plot. The specific radioactivities of both substrates ranged from 1000 to 10000 d.p.m./nmol. Similar results were obtained with a second experiment with a different lipase preparation.

Figure 5 shows a representative double-reciprocal plot for the hydrolysis of [^{3}H]diC_8 and 1-[^{14}C]POG by the same octyl-Sepharose preparation. Although the apparent K_m for [^{3}H]diC_8 (27 μM) was less than that for 1-[^{14}C]POG (98 μM), the $V_{max.}$ for the long-chain substrate assayed in the presence of 0.1% Triton X-100 (20000 units/mg) was higher than the $V_{max.}$ for the short-

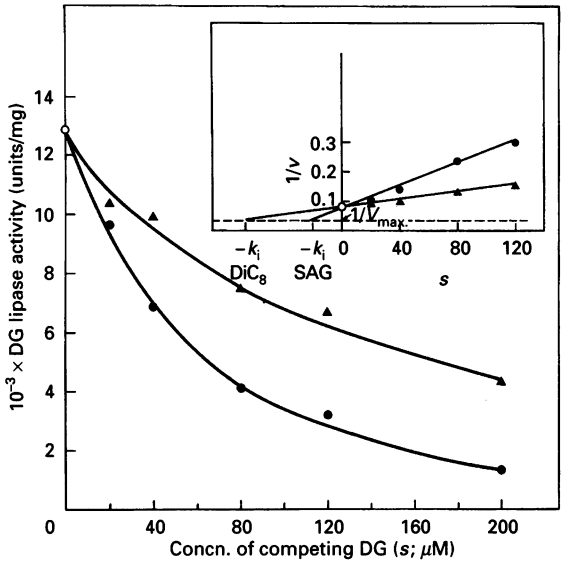

Figure 6 Effect of diC_8 and SAG on hydrolysis of 1-[^{14}C]POG by aortic DG lipase

DG lipase activity was measured at 80 μM 1-[^{14}C]POG in the presence of 0.1% Triton X-100 and in the presence of unlabelled diC_8 (▲) and SAG (○) concentrations ranging from 0 to 200 μM. Insert: results are presented as a Dixon plot ($1/v$ versus competing DG concentration), with the $1/V_{max.}$ of the preparation indicated by the dashed line.

chain substrate (12600 units/mg). Cross-competition experiments utilizing unlabelled DG were also performed. The addition of either SAG or diC_8 produced a concentration-dependent decrease in the hydrolysis of 1-[^{14}C]POG (Figure 6); the inhibition by SAG was much greater than that produced by diC_8. The Dixon plot (Figure 6, insert) indicates that the inhibition was competitive, with apparent K_i values of 20 μM for SAG and 67 μM for diC_8.

DISCUSSION

Previous studies with vascular smooth muscle have shown that elimination of the DG second messenger occurs mainly by the DG lipase pathway, based on determinations *in vitro* of DG-metabolizing enzymes [10,11,17,18], and flux through the DG lipase pathway measured with intact [17,18] and permeabilized [33] aortic smooth-muscle cells. Therefore, the objective of this investigation was to purify DG lipase activity from bovine aorta.

DiC_8 has been used previously as an effective substrate for measuring DG lipase activity *in vitro* [14,15,17,34]. Furthermore, diC_8 has been used to determine the biochemical pathways responsible for the metabolism of DG second messengers by intact cells [14,17–19]. SAG has been termed a 'physiological' DG in signal-transduction pathways involving phosphoinositide breakdown [1–3,5]. However, levels of SAG increase transiently in response to receptor stimulation, so that turnover of phosphoinositides enriched in arachidonic acid may contribute only modest quantities of DG in relation to the total cellular DG pool [35,36]. Molecular-species analysis has shown that other DGs, including POG and 1,2-dioleoyl-*sn*-glycerol, were the predominant species generated as part of a sustained increase in DG mass, likely from the catabolism of phosphatidylcholine [36]. Therefore we considered it important to characterize our partially purified DG lipase preparation with both short-chain (diC_8) and long-chain (POG, SAG) substrates.

The interpretation of changes in the relative degree of purification for aortic DG lipase (Table 1) is complicated by substrate-specific effects of Triton X-100 that also depend on the particular enzyme fraction. The stimulation of 1-[^{14}C]POG hydrolysis by Triton X-100 was much greater in the octyl-Sepharose preparation than in the S100 fraction (Figure 1). In contrast, Triton X-100 inhibited [^{3}H]diC_8 hydrolysis in the S100 fraction, but produced a slight stimulation of the DG lipase activity in the octyl-Sepharose preparation. Assays conducted with 1-[^{14}C]POG in the presence of Triton X-100 gave rates of hydrolysis that were very similar to diC_8 hydrolysis (Figure 1, Table 1); DG lipase activities determined with 1-[^{14}C]POG and 2-[^{14}C]SAG were also nearly identical.

The degree of purification for DG lipase in the final octyl-Sepharose fraction of 155-fold, based on 1-[^{14}C]POG hydrolysis in the presence of 0.1% Triton X-100, was significantly greater than the 60-fold increase in relative specific activity when assays were conducted with [^{3}H]diC_8 in the absence of Triton X-100. This difference depending on the substrate and assay conditions could indicate the existence of distinct enzymes with specific substrate preferences, resulting in selectivity for either short-chain (esterase activity) or long-chain (detergent-activated lipase activity) substrates. However, since diC_8 was a competitive inhibitor of 1-[^{14}C]POG hydrolysis (Figure 6), separate enzyme activities are not very likely. Further purification is needed to establish this with certainty. The selective ability of detergents to stimulate hydrolysis of long-chain substrates would also be consistent with an action on the substrate emulsion rather than on the enzyme protein. The selective stimulatory effect of Mg^{2+} and Ca^{2+} on 1-[^{14}C]POG hydrolysis may also arise from changes in the substrate emulsion's physical state. For example, bivalent cations could promote phase changes and reorganization of phosphatidylserine bilayers [37,38] in the long-chain DG substrate preparation.

Farooqui et al. [26] initially observed that DG lipase activity was enriched in plasma-membrane and microsomal fractions from bovine brain. Membrane-bound lipases solubilized with 0.25% Triton X-100 had specific activities of 330–450 units/mg, when assayed with a thioester analogue of didecanoylglycerol as substrate [39]. By comparison, the specific activity of DG lipase in the aortic S100 fraction was 170 units/mg (Table 1) with a [^{3}H]diC_8 substrate. Solubilized brain preparations were purified by chromatography on heparin–Sepharose [25] or concanavalin A–Sepharose [26], resulting in 5–10-fold increases in specific activity. Our degree of purification obtained for aortic DG lipase (Table 1) was therefore significantly higher than that obtained for the brain enzyme. Furthermore, purified brain DG lipase preparations have not been characterized with respect to activity against long-chain DG substrates.

Further purification will be required to determine unequivocally if DG (*sn*-1 hydrolysis) and MG (*sn*-2 hydrolysis) lipase activities result from separate enzymes. MG lipase activity could not be measured directly with 2-[^{14}C]mono-olein or indirectly with 2-[^{14}C]POG and 2-[^{14}C]SAG in the octyl-Sepharose fraction. This could partly have been the result of a lack of assay sensitivity due to inhibition by Triton X-100. Farooqui et al. [26] reported that DG and MG lipases could be completely resolved by chromatography on heparin-Sepharose. In adipose tissue, both a hormone-sensitive triacylglycerol lipase with positional specificity for *sn*-1(3)-ester bonds and an MG lipase are required for complete degradation of triacylglycerols [40,41]. Preliminary work has shown that partially purified aortic DG lipase is sensitive to active site-directed inhibitors such as phenylboronic

acid and tetrahydrolipstatin (M. W. Lee and D. L. Severson, unpublished work). These agents may serve as useful affinity probes in the further purification and characterization of the aortic DG lipase.

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