J. Biochem. 125, 1077-1085 (1999)

Purification and Characterization of Diacylglycerol Lipase from Human Platelets[1]

Tatsuya Moriyama,[2] Reiko Urade, and Makoto Kito

Research Institute for Food Science, Kyoto University, Uji, Kyoto 611-0011

Received February 15, 1999; accepted March 12, 1999

Diacylglycerol lipase (DGL) was solubilized from human platelet microsomes with heptyl-β-D-thioglucoside, and purified to homogeneity on SDS-PAGE using a combination of chromatographic and electrophoretic methods. The molecular mass of the purified DGL was estimated to be 33 kDa. Its apparent pI was pH 6.0, as determined by Immobiline isoelectrofocusing. The enzymatic activity of the partially purified DGL was investigated in the presence of a variety of inhibitors and reagents, as well as its pH and calcium dependence. Thiol reagents such as *p*-chloromercurubenzoic acid (*p*CMB), *N*-ethylmaleimide (*N*EM), and $HgCl_2$ inhibited the activity, while dithiothreitol (DTT) and reduced glutathione (GSH) enhanced it. In addition, the enzymatic activity was inhibited by two serine blockers, phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP), and by a histidine modifying reagent, *p*-bromophenacyl bromide (*p*BPB). These results suggest that cysteine, serine and histidine residues are required for the enzymatic activity of DGL. DGL was optimally active in the pH range of 7-8 and its activity did not change significantly in the presence of various calcium concentrations, even in the presence of 2 mM EGTA. This indicates that DGL can hydrolyze substrates with a basal cytosolic free Ca^{2+} level in the physiological pH range. A DGL inhibitor, RHC-80267, inhibited DGL activity in a dose-dependent manner with an IC_{50} (the concentration required for 50% inhibition) of about 5 μM. Unexpectedly, several phospholipase A_2 (PLA_2) inhibitors were potent inhibitors of DGL activity ($IC_{50} < 5$ μM), suggesting that the catalytic mechanisms of DGL and PLA_2 may be similar. Finally, we show that DGL activity was inhibited by 2-monoacylglycerols (2-MGs), the reaction products of this enzyme. Among the three 2-MGs tested (2-arachidonoyl glycerol, 2-stearoyl glycerol, and 2-oleoyl glycerol), 2-arachidonoyl glycerol was the most potent inhibitor.

Key words: diacylglycerol lipase, platelet, signal transduction, purification.

When cells are stimulated with hormones, neurotransmitters, or growth factors, phosphoinositide (PI) metabolism is accelerated (*1*). The initial cellular reaction in response to such stimulation is the hydrolysis of PI by PI-specific phospholipase C (PI-PLC) (*2*). One product of this reaction, inositol 1,4,5-trisphosphate (IP_3), mobilizes ionized calcium from intracellular stores (*3*). The other product of this reaction, diacylglycerol (DG), activates protein kinase C (*4*).

DG is also produced from phosphatidylcholine *via* phospholipase D and the phosphatidic acid hydrolase pathway (*5*). DG lipase (DGL) cleaves the *sn*-1 acyl chain of DG and the resultant 2-acyl monoacylglycerol (MG) is hydrolyzed at the *sn*-2 position by MG lipase (MGL). The presence of this sequential DG breakdown pathway has been reported in various tissues and cells, including platelets (*6-12*), neutrophils (*13*), brain (*14-16*), pancreatic islets (*17*), smooth muscle cells (*18-20*), fetal membranes and decidua vera (*21*), and amniotic tissue (*22*). The rate-limiting step in this pathway is thought to be the first step, *i.e.*, the DGL-mediated step (*12, 13*). Since PI contains a high level of arachidonic acid at the *sn*-2 position (*23*), the fatty acids of DG derived from PI are mainly of this type. Consequently, through the combined actions of DGL and MGL, 2-arachidonyl DG derived from PI is hydrolyzed to produce arachidonic acid, which is the precursor of eicosanoids which amplify the initial signal transduction step. Physiologically significant arachidonic acid release through the

[1] This work was supported in part by a Grant-in-Aid for Scientific Research, 09760133, from the Ministry of Education, Science, Sports, and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-774-38-3753, Fax: +81-774-38-3752, E-mail: moriyama@soya.food.kyoto-u.ac.jp

Abbreviations: A_{280}, absorbance at 280 nm; $AACOCF_3$, arachidonoyl trifluoromethyl ketone; BEL, bromoenol lactone; DFP, diisopropyl fluorophosphate; DG, diacylglycerol; DGL, diacylglycerol lipase; IC_{50}, concentration required for 50% inhibition; IEF, isoelectrofocusing; MAFP, methyl arachidonoyl fluorophosphonate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MG, monoacylglycerol; MGL, monoacylglycerol lipase; *N*EM, *N*-ethylmaleimide; ONO-RS-082, 2-(*p*-amylcinnamoyl)amino-4-chloro benzoic acid; PBA, phenyl boronic acid; *p*BPB, *p*-bromophenacyl bromide; *p*CMB, *p*-chloromercuru-benzoic acid; PI, phosphoinositide; PLA_2, phospholipase A_2; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; RHC-80267, (1,6-di(*O*-carbamoyl)cyclohexanone oxime)hexane; U-73122, 1H-pyr-role-2,5-dione,1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-.

© 1999 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

DGL/MGL pathway has been reported in platelets (*6, 8, 24*), pancreatic islets (*17*), mast cells (*25, 26*), and macrophages (*27*). Furthermore, DG metabolism by DGL and/or DG kinase results in the attenuation of protein kinase C-mediated signal transduction.

In various studies focused on the metabolic fate of DG (*18-20, 22*), it has been demonstrated that DGL, as well as DG kinase, contributes significantly to DG catabolism. Recently, DGL was also shown to play a possible role in the formation of 2-arachidonoyl glycerol (2-AG), which is a putative endogenous cannabinoid receptor ligand (*28, 29*). The significance of DGL, therefore, appears to extend beyond cellular lipid metabolism to intracellular and/or extracellular signal transduction.

In the last two decades, DGL activity has been studied using subcellular fractions derived from various tissues and cells. Although several groups have tried to purify this enzyme from human platelets (*8*), bovine brain (*14*), and bovine aorta (*30, 31*), complete purification of the human enzyme has never been achieved. DGL activity in platelets has been extensively characterized (*6-12, 24*), and we previously showed that, in human platelets activated with collagen, DGL plays a significant role in the resulting release of arachidonic acid (*24*). In this study, therefore, we attempted to purify DGL to homogeneity from human platelet microsomes and to characterize its enzymatic properties.

MATERIALS AND METHODS

Materials—Phosphatidylethanolamine plasmalogen (bovine brain) and 2-oleoyl glycerol were purchased from Serdary Research Laboratory (London, Ontario, Canada). 2-Arachidonoyl glycerol was from RBI (Natick, MA, USA). 2-Stearoyl glycerol was from Larodan Fine Chemicals (Malmo, Sweden). Aquasol-2 and [1-^{14}C]stearic acid (58.0 mCi/mmol) were from Du Pont-New England Nuclear (Boston, MA, USA). Leupeptin was from the Peptide Institute (Osaka). Heptyl-β-D-thioglucoside was from Dojindo Laboratories (Kumamoto). ONO-RS-082, aristolochic acid, RHC-80267, and AACOCF$_3$ were from BIOMOL (Plymouth Meeting, PA, USA). U-73122, MAFP, and BEL were from Cayman Chemical (Ann Arbor, MI, USA). Phospholipase C (grade I, from *Bacillus cereus*) was purchased from Boehringer Mannheim. The TSK gel DEAE-Toyopearl 650M, TSK gel heparin-Toyopearl, and TSK gel G3000SW HPLC columns were from Tosoh (Tokyo). The Hi-Load 26/60 Superdex 200 gel filtration column and Immobiline IEF gel were from Pharmacia (Uppsala, Sweden). The Rotofor preparative IEF cell, protein assay kit and silver stain kit (Silver Stain Plus) were from Bio-Rad (Richmond, CA, USA). All other chemicals were of reagent grade and obtained from commercial sources.

Preparation of Labeled Substrates—1-[^{14}C]Stearoyl-diacylglycerol was obtained as described previously (*24*). Briefly, 1-[^{14}C]stearoylphosphatidylethanolamine was first prepared biosynthetically from rat liver microsomes using [1-^{14}C]stearic acid (2.1 GBq/mmol) and 2-acyllysophosphatidylethanolamine as described by Lands and Merkl (*32*). 2-Acyllysophosphatidylethanolamine was generated from phosphatidylethanolamine plasmalogen by acid treatment (0.05 N HCl, 0.1 M H_3BO_4 in ethanol for 30 min). 1-[^{14}C]Stearoyl-diacylglycerol was obtained by treating 1-[^{14}C]stearoyl-phosphatidylethanolamine with phospholipase C (*Bacillus cereus*) for 3 h at 30°C. The obtained labeled diacylglycerol was dissolved in ethanol and stored at -20°C to keep the isomerization of 2- to 1-acyl migration to a minimum.

Diacylglycerol Lipase Assay—The standard assay for DGL activity was performed as follows. The assay mixture [40 mM MES-NaOH (pH 7.0), 0.2% heptyl-β-D-thioglucoside, 1-[^{14}C]stearoyl-diacylglycerol (approx. 40,000 cpm; 10 nmol per assay)] was prepared by vortexing and sonication twice with a Branson sonicator model 250 at setting 8 for 1 min. The assay mixture (100 μl) containing DGL was incubated for 60 min at 37°C. In the case of DGL assays involving gel pieces after native PAGE or Immobiline IEF, the assay was carried out for longer (3 h or 20 h, respectively). The reaction was terminated by the addition of 1.5 ml of chloroform/methanol/heptane (125:140:100, by volume). Then unlabeled stearic acid (20 μl of a 25 mM solution in chloroform) as a carrier and 0.5 ml of 50 mM K_2CO_3-$KHCO_3$ (pH 10.0) were added, followed by mixing by vortexing for 10 s. Phase separation was performed by centrifugation to obtain the released fatty acids in the upper aqueous phase (*6*). Aliquots of the upper phase (0.9 ml) were used for scintillation counting in 4 ml of Aquasol-2. Various free Ca^{2+} concentrations were obtained using Ca^{2+}-EGTA buffers (pH 7.0) containing 2 mM EGTA (final concentration) and an appropriate amount of $CaCl_2$ (*33*). Certain variations of this standard assay are indicated in the figure legends. For the pharmacological studies, H_2O, ethanol, or DMSO was used as the solvent, and their concentrations ($<$2%) in the assay mixture were reflected in the control samples as well. Assay mixtures containing reagents or inhibitors were made up on ice and then incubated for 60 min at 37°C.

Purification Procedure—All purification steps were carried out at 4°C except for Immobiline IEF, which was performed at 6°C. Outdated human platelets (200 units) drawn within four days were suspended in lysis buffer [20 mM Tris/HCl (pH 7.4), 20 mM EGTA, 0.5 mM leupeptin, 1 mM dithiothreitol], followed by two rounds of sonication (Branson sonicator model 250) in an ice bath at setting 3 for 30 s. The lysate was centrifuged at 100,000 × g for 1 h. The resultant pellet (about 3.8 g protein) was solubilized with buffer A [20 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1.5% heptyl-β-D-thioglucoside, 10% glycerol, 0.5 mM leupeptin, 1 mM dithiothreitol]. Five milliliter of buffer A was added to 100 mg of pellet membrane protein and then solubilization was achieved by stirring the mixture (about 200 ml) at 4°C for 1 h, followed by centrifugation at 100,000 × g for 1 h and removal of the supernatant from the insoluble pellet.

The solubilized supernatant (about 200 ml) was concentrated to 25 ml with a Centriprep 10 (Amicon), and then loaded on a Hi-Load 26/60 Superdex 200 gel filtration column (26 mm × 60 cm) which had been equilibrated with buffer A. The flow rate for this column was 0.5 ml/min. The fractions (about 30 ml) containing DGL activity were pooled and treated as a batch, being mixed with about 5 ml each of TSK gel DEAE-Toyopearl 650M and TSK gel heparin-Toyopearl equilibrated with buffer A. The batch mixture was stirred gently at 4°C for 1 h and then the unbound fraction was separated by centrifugation (1,500 × g, 10 min at 4°C). The mixed gel was washed once with

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

buffer A and the washing were combined with the unbound pool. Under these conditions, DGL activity was recovered in the unbound fraction. This fraction was concentrated to a volume of 10 ml with a Centriprep 10 and then loaded on a Rotofor IEF cell using 2% Bio-Lyte 3/10 (pH 3-10). Rotofor IEF was performed on a 50 ml scale [4 mM Tris/HCl (pH 7.4), 1.5% heptyl-β-D-thioglucoside, 2% Bio-Lyte 3/10, 15% glycerol, 0.1 mM leupeptin, 1 mM dithiothreitol, 1 mM EDTA]. Focusing was carried out at 12 W constant power for 4 h with circulation of ice-cold water. Twenty fractions of approximately 2.5 ml each were collected, and their pH and DGL activity were measured. The pooled active fractions obtained on Rotofor IEF were concentrated with a Centriprep 10 and then injected onto a G3000SW gel filtration HPLC column (7.5 mm × 60 cm) that had been equilibrated with buffer A. The flow rate was 0.3 ml/min. The DGL-active fractions obtained from this column were mixed and rechromatographed on the same column under the same conditions. The eluted active fractions were pooled and stored in multiple aliquots at −80°C. This fraction is referred to as "partially purified DGL" and was used in the experiments of Figs. 4-7 and Table II. The above purification process, involving approximately 200 units of human platelets, was repeated five times.

Aliquots (70 μg protein each) of the mixed active fractions from the second G3000SW column were concentrated to 0.5 ml with a Centricon 10 (Amicon) and then applied to native PAGE gels (*34*). Electrophoresis was carried out in 1.0 mm thick, 7% (w/v) polyacrylamide gels containing 1.5% heptyl-β-D-thioglucoside at a constant voltage of 100 V for 4-6 h at 4°C. Following electrophoresis, a vertical strip (0.7 cm × 5 cm) of the gel (5 cm × 5 cm) was cut out for protein staining, and the residual gel (4.3 cm × 5 cm) was cut horizontally from top to bottom into 14 strips and corresponding gel pieces (0.3 cm × 0.35 cm). The gel pieces were used in the DGL assay to identify the strips containing DGL activity. The gel strips, equilibrated with Immobiline buffer (2 mM acetate, 1.5 mg/ml dithiothreitol, 1.5% heptyl-β-D-thioglucoside, 15% glycerol, 0.4% Bio-Lyte 3/10, 10 μg/ml orange G), were directly applied onto an Immobiline gel plate (pH 5.6-6.6). This gel was also swollen with the same Immobiline gel buffer. Isoelectrofocusing was carried out as described in the instruction manual using a Multiphor II (Pharmacia) electrophoresis system at 6°C. After electrophoresis, the Immobiline gel was cut into two series of gel pieces, one of which was used for the DGL assay, and the other of which was treated with 1 × SDS-sample buffer (*34*) and applied directly to an SDS-PAGE gel (10%). Since the enzyme proteins were trapped in the gel matrix, the DGL assay was performed for 20 h at 37°C.

Other Methods—SDS-PAGE was carried out by the method of Laemmli (*34*) in 10% polyacrylamide slab gels under reducing conditions. A silver-staining kit (Bio-Rad) was used to visualize protein bands. Proteins were assayed with the Bio-Rad protein assay kit with γ-globulin as the standard.

RESULTS

Detergent Solubilization of DGL from Human Platelets—In our preliminary studies, about 90% of the DGL activity in human platelets was found in the microsomal fraction. DGL activity was only minimally extracted from microsomes on extensive washing with salt (2 M KCl) (data not shown). Therefore, detergents were then tested as to the solubilization of DGL. Among the detergents tested, heptyl-β-D-thioglucoside (1.5%) was able to solubilize about 70% of the membrane-bound DGL activity. Under these conditions, about 30% of total microsome proteins were extracted (Table I).

Partial Purification of Solubilized DGL—Solubilized microsome fractions were concentrated and applied onto a

TABLE I. **Partial purification of DGL from human platelet microsomes.**

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (-fold)
Crude microsomes	18,705	7,999	0.428	100	1
Solubilized supernatant	5,415	6,172	1.140	77.2	2.7
Hi-Load Gel filtration	983	1,026	1.044	12.8	2.4
Batch treatment	174	656	3.770	8.2	8.8
Rotofor IEF	31.6	325	10.28	4.1	24.0
1st G3000SW	1.91	213	111.5	2.7	260.5
2nd G3000SW	0.73	112	154.5	1.4	361.0

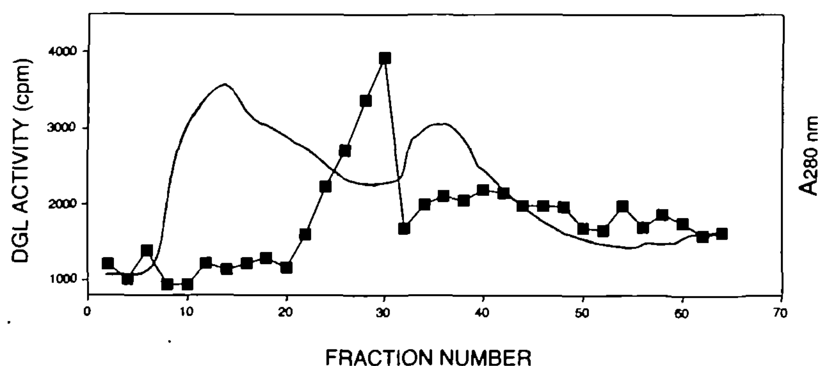

Fig. 1. **Hi-Load gel filtration column chromatography.** Human platelet microsomes (approx. 3.8 g of protein) were solubilized with 1.5% heptyl-β-D-thioglucoside, concentrated to 25 ml, and then applied to a Hi-Load 26/60 Superdex 200 gel filtration column (26 mm × 60 cm) equilibrated with buffer A. The elution speed was 0.5 ml/min. Eluted fractions (3 ml/fraction) were assayed for DGL activity using 1-[^{14}C]stearoyl-DG (100 μM) as the substrate. The DGL assay was carried out as described under "MATERIALS AND METHODS." -■-, DGL activity; —, absorbance at 280 nm.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

Hi-Load gel filtration column. DGL activity was eluted as a single peak separate from the void volume (Fig. 1). The sharpness profile of the DGL peak suggests that the solubilized enzyme had not aggregated. Although it is unclear why, the recovery of DGL activity was poor (Table I). Fractions eluted from this column were then treated by stirring with a mixture of ion-exchange gel (DEAE Toyopearl 650M gel) and heparin affinity gel (heparin Toyopearl gel) media. About 80% of the total protein was absorbed to the mixed gel media, while the DGL activity was not. The recovery of DGL activity was about 70%, allowing for its purification from the major contaminating proteins. After this batch treatment, the unbound material was applied to a Rotofor IEF system. As shown in Fig. 2A, the major DGL activity was focused at a position corresponding to around pH 6. An aliquot of each fraction obtained on Rotofor IEF was separated by SDS-PAGE and the gel was stained with silver (Fig. 2B), which indicated that the applied proteins were efficiently separated by the Rotofor IEF system. The DGL-active fractions were collected, concentrated, and then applied to a G3000SW gel filtration HPLC column, which gave two peaks of DGL activity (Fig. 3A). The fractions comprising the major peak were pooled and rechromatographed, and an almost single peak of activity was eluted (Fig. 3B). The apparent molecular size of the protein in this peak was estimated to be 30-60 kDa from a calibration curve obtained using molecular standards. A summary of the partial purification is presented in Table I. The specific activity increased 361-fold and the yield was 1.4%, as compared with in the case of the starting crude microsomes. DGL activity was unstable throughout the purification.

Determination of the Molecular Mass and pI of DGL by Sequential Electrophoresis—Since the partially purified DGL preparation from the second G3000SW column still gave many protein bands, as detected on silver staining, we attempted further purification using the sequential electrophoresis protocol. An aliquot of the partially purified DGL

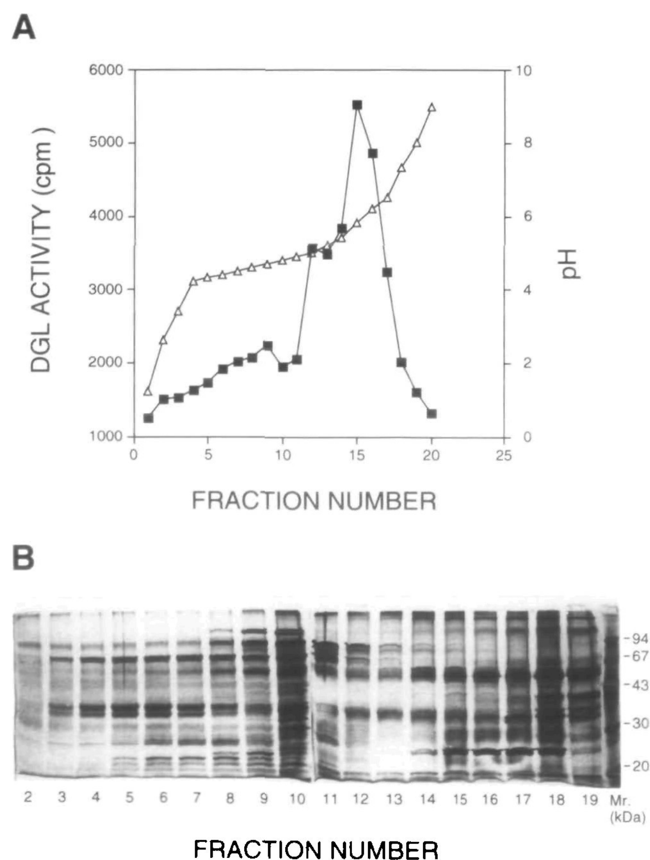

Fig. 2. **Rotofor IEF of DGL.** (A) DGL activity and pH value of each fraction. (B) SDS-PAGE of each fraction. (A) DGL obtained on batch treatment was applied to a Rotofor IEF system. After electrophoresis, 20 separate fractions were assayed for DGL activity using 1-[^{14}C]-stearoyl-DG (100 μM) as the substrate. The DGL assay was carried out as described under "MATERIALS AND METHODS." The pH of each fraction was measured with a pH meter (HORIBA). ■, DGL activity; , pH value. (B) Separate fractions from the Rotofor IEF system were subjected to SDS-PAGE. Protein bands were visualized by silver staining.

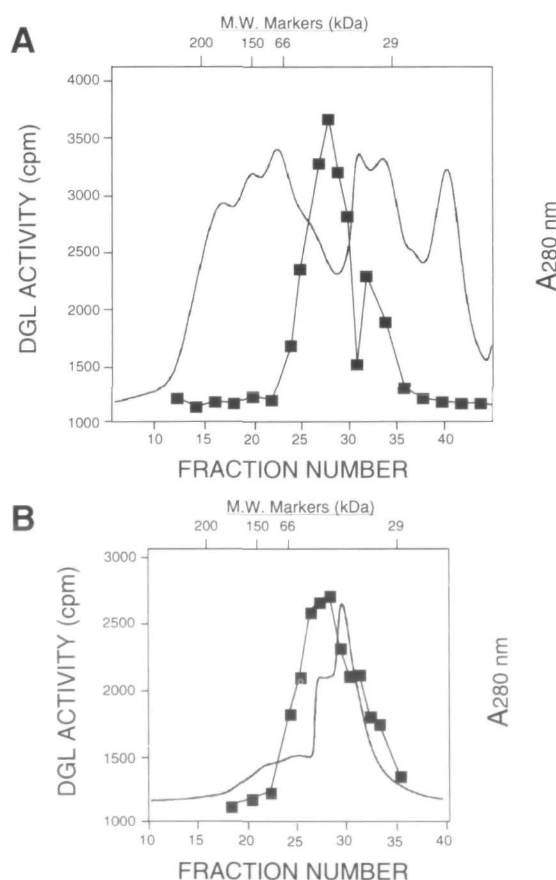

Fig. 3. **First (A) and second (B) G3000SW HPLC gel filtration column chromatography of DGL.** (A) DGL obtained from the Rotofor IEF system was applied to a G3000SW HPLC column. the elution speed was 0.3 ml/min. The eluted fractions were assayed for DGL activity using 1-[^{14}C]stearoyl-DG (100 μM) as the substrate. The DGL assay was carried out as described under "MATERIALS AND METHODS." ■, DGL activity; , absorbance at 280 nm. (B) The DGL-active fractions from the first G3000SW HPLC column were again applied to the same column. The eluted fractions were assayed for DGL activity using 1-[^{14}C]stearoyl-DG (100 μM) as the substrate. The DGL assay was carried out as described under "MATERIALS AND METHODS." ■, DGL activity; , absorbance at 280 nm. M.W. markers: β-amylase (200 kDa), alcohol dehydrolase (150 kDa), BSA (66 kDa), carbonic anhydrolase (29 kDa).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

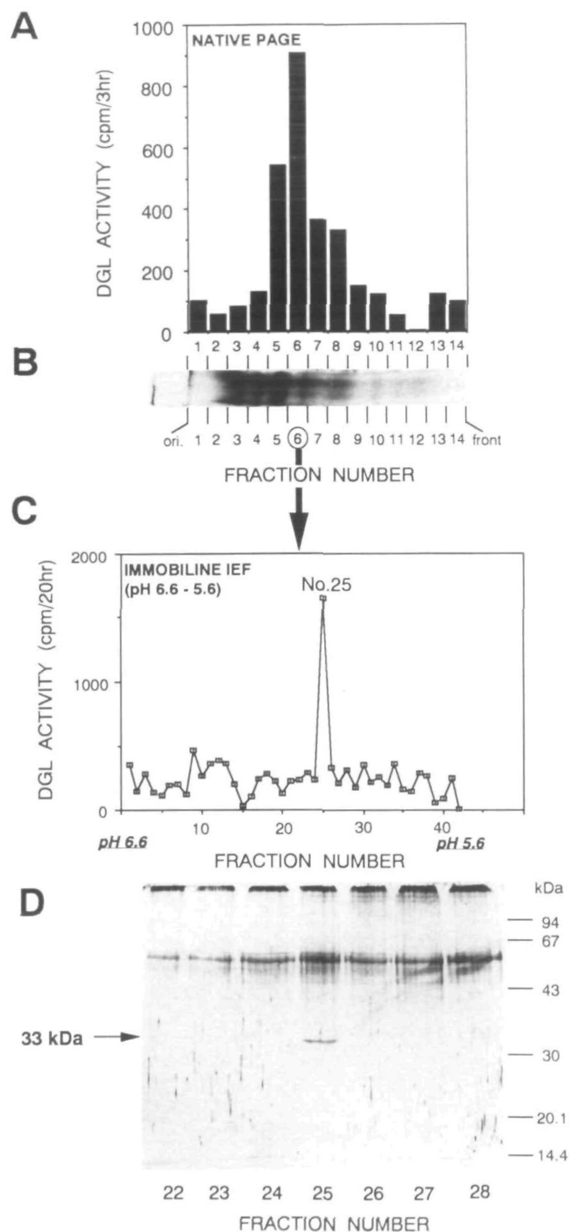

Fig. 4. **Identification of the purified DGL protein by sequential electrophoresis.** (A) DGL activity in sliced native PAGE gel pieces. (B) Silver staining of native PAGE gel strips. (C) DGL activity in sliced Immobiline IEF gel pieces (pH 6.6 5.6). (D) SDS-PAGE of sliced Immobiline IEF gel pieces. An aliquot of the partially purified DGL preparation obtained from the second G3000SW HPLC column was concentrated and then applied onto a native polyacrylamide gel in the presence of 1.5% heptyl-β-D-thioglucoside. After electrophoresis, the gel was cut into gel pieces and gel slices. The DGL assay was performed on the gel pieces (A). A vertically cut gel strip was stained with silver (B). The gel strip containing DGL activity (No. 6) was applied directly onto an Immobiline IEF gel plate (pH 6.6 5.6). After electrophoresis, the Immobiline gel was cut into two series of 42 gel pieces. One series of gel pieces was assayed for DGL activity (C), and the other series was subjected to SDS-PAGE. Protein bands were detected by silver staining (D). The DGL assay was carried out as described under "MATERIALS AND METHODS" using 1-[^{14}C]-stearoyl-DG (100 μM).

was subjected to native PAGE in the presence of heptyl-β-D-thioglucoside. To ensure that the applied proteins were separated properly under these conditions, a gel strip was cut from the slab gel and stained with silver. Figure 4B shows that several protein bands appeared on native PAGE. Following electrophoresis, the gels were cut into 14 strips and pieces as described previously. The gel strips were stored at 4°C while the gel pieces were used for the DGL assay. The most active gel piece was found to be No. 6 (Fig. 4A). Then, the stored No. 6 gel strip was applied directly to an Immobiline IEF gel plate (pH range, 5.6-6.6). Since the pI of DGL was estimated to be around pH 6, as determined by Rotofor IEF (Fig. 2A), we used this pH range. After focusing for 31 h, the Immobiline gel plate was cut vertically into two gel strips which were then each cut horizontally into 42 gel pieces from top (pH 6.6) to bottom (pH 5.6). One series of gel pieces was used for the DGL assay and the other series was analyzed by SDS-PAGE. As shown in Fig. 4C and D, DGL activity was detected in gel piece No. 25, and a single band of 33 kDa was visualized on silver staining in the corresponding gel piece. The position of gel piece No. 25 corresponded to a pI of 6.0, assuming a linear pH gradient in the Immobiline gel. Similar results were

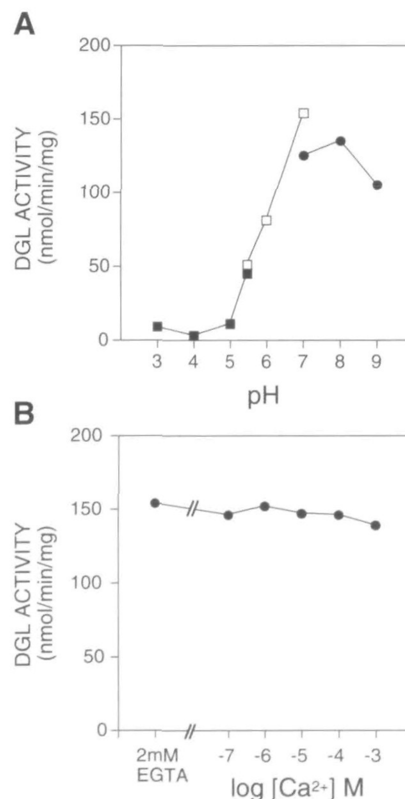

Fig. 5. **pH (A) and calcium (B) dependence of the partially purified DGL.** (A) DGL activity was measured at various pHs using 40 mM sodium acetate buffer (pH 3.0 5.5; ■), MES-NaOH buffer (pH 5.5 7.0; □), and Tris-HCl buffer (pH 7.0 9.0; ●). Except for the buffer used, the assay conditions were the same as for the standard assay method described under "MATERIALS AND METHODS" using 1-[^{14}C]stearoyl-DG (100 μM). (B) DGL activity was measured at various free Ca^{2+} concentrations or in the presence of 2 mM EGTA. The various free Ca^{2+} concentrations were obtained using Ca^{2+}-EGTA buffer (pH 7.0) (*33*).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

obtained when an Immobiline gel ranging from pH 3-10 was used. Moreover, when the partially purified DGL preparation from the second G3000SW column was applied directly to the Immobiline gel (pH 3-10), the 33 kDa band was detected parallel to the DGL activity at approximately the pH 6 position (data not shown). These observations strongly indicate that the 33 kDa protein band is DGL.

Activity of Partially Purified DGL: Dependence on pH and the Ca^{2+} Concentration—DGL activity was measured at various pHs and Ca^{2+} concentrations using partially purified DGL. As shown in Fig. 5A, the optimum pH for DGL activity was in the neutral range (pH 7-8). The effects of different Ca^{2+} concentrations on the partially purified enzyme were measured using Ca^{2+}-EGTA buffer (*33*). The enzymatic activity did not change significantly in the presence of various Ca^{2+} concentrations, even in the presence of 2 mM EGTA (Fig. 5B).

Effects of Inhibitors and Reagents on the Activity of Partially Purified DGL—DGL activity was determined in the presence of various reagents (Table II). All of the SH inhibitors tested (*p*CMB, *N*EM, and $HgCl_2$) had an inhibitory effect on the activity, while DTT and GSH had a stimulatory effect (125 and 208% at 10 mM, respectively). This suggests that DGL activity is dependent on free thiol group(s). Serine blockers such as DFP and PMSF also inhibited DGL activity. The sensitivity of DGL to SH reagents and serine blockers has been reported previously (*10*). Boronic acid derivatives are known to inhibit lipases (*35*). In the current study, phenylboronic acid (PBA) reduced DGL activity to 38% of the control level at the concentration of 4 mM. Similar results were reported by Lee *et al.* (*31*) for partially purified DGL from bovine aorta. It has been reported that *p*-bromophenacyl bromide (*p*BPB), an alkylating agent which modifies a specific histidine residue of phospholipase A_2 (*36*), inhibits the DGL activity of human platelet microsomes (*10*). According to our results with highly purified DGL, 1 mM *p*BPB reduced DGL activity to 22% of the control level. Farooqui *et al.* have reported the effects of nucleotides and NaF on the activity of DGL derived from bovine brain (*37*). In the present study, therefore, we examined the effects of high concentrations of ATP (25 mM), AMP (25 mM), and NaF (75 mM) on the activity of platelet-purified DGL for comparison. ATP had no significant effect (107% relative to control activity), whereas AMP had a slightly inhibitory effect (72%). Slight stimulation (127%) was also observed in the presence of NaF. These results differed significantly from the results of Farooqui *et al.* (Table III).

Effects of Phospholipase Inhibitors on Partially Purified DGL—DGL activity was measured in the presence of various concentrations of inhibitors which are known to inhibit phospholipases. As shown in Fig. 6, inhibitors of phospholipase A_2, MAFP (*38*), $AACOCF_3$ (*39*), BEL (*40*), and ONO-RS-082 (*41*), inhibited DGL activity. Among these inhibitors, MAFP was the most potent inhibitor of DGL activity ($IC_{50} < 0.1\ \mu M$). These phospholipase A_2 inhibitors were more potent than RHC-80267, a DGL inhibitor (*42*), with an IC_{50} of approximately 5 μM in this study. Aristolichic acid [an inhibitor of viperous venom

TABLE II. **Effects of various reagents on partially purified DGL activity.**

Reagent	Concentration (mM)	Relative activity (% of control)
None		100
(SH reagents)		
*p*CMB	1	4
$HgCl_2$	5	5
*N*EM	1	36
DTT	10	125
GSH	10	208
(Serine inhibitors)		
DFP	1	2
PMSF	1	27
(Histidine inhibitors)		
*p*BPB	1	22
(Others)		
PBA	4	38
ATP	25	107
AMP	25	72
NaF	75	127

Data are the averages of four measurements.

TABLE III. **Comparison of DGLs from various sources.**

	DG lipase activity of				
	Human platelets (present data)	Human platelets (*8, 10*)	Bovine aorta (*30, 31*)	Bovine brain (*14, 15, 37*)	
				Microsomal	Plasma membrane
M_r size ($\times 10^{-3}$)	33	NA	NA	27	52
pI value	6.0	NA	NA	NA	NA
Optimum pH	7-8	7.0	7-8	7.4	7.4
Ca^{2+} dependence	no effect	no effect	changed under conditions	no effect	no effect
SH inhibitors	inhibited (*p*CMB, Hg^{2+}, *N*EM)	inhibited (*p*CMB, *N*EM)	NA	NA	NA
Serine inhibitors	inhibited (DFP, PMSF)	inhibited (DFP, PMSF)	inhibited (THL)	NA	NA
RHC-80267	inhibited ($IC_{50} = 5\ \mu M$)	inhibited ($IC_{50} = 15\ \mu M$)	NA	inhibited	inhibited
ATP	no effect (25 mM)	NA	no effect (2 mM)	inhibited (25 mM)	inhibited (25 mM)
AMP	inhibited (25 mM)	NA	NA	no effect (25 mM)	no effect (25 mM)
NaF (75 mM)	slightly stimulated	NA	NA	inhibited	inhibited
2-MGs	inhibited	NA	inhibited	NA	NA
*p*BPB	inhibited	inhibited	NA	NA	NA
Note	inhibited by PLA_2 inhibitors			suggested to be a glycoprotein	suggested to be a glycoprotein

NA: data not available, THL: tetrahydrolipstatin.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

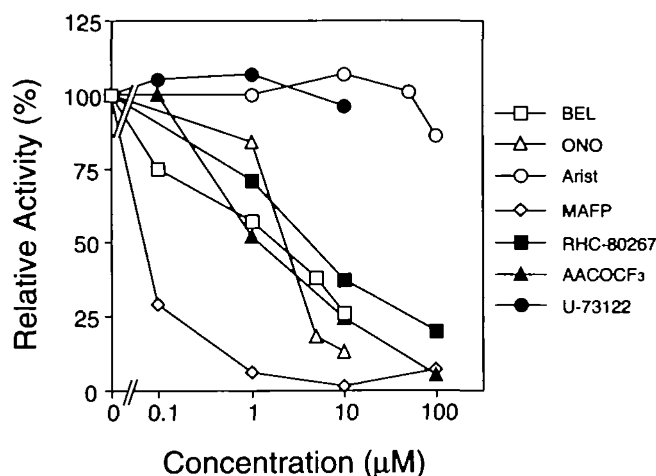

Fig. 6. **Effects of phospholipase inhibitors on the partially purified DGL.** DGL activity was measured in the presence of various phospholipase inhibitors. The DGL assay was carried out as described under "MATERIALS AND METHODS" using 1-[^{14}C]stearoyl-DG (100 μM). The inhibitors were dissolved in ethanol or DMSO, and then added to the assay mixtures. The inhibitors used were as follows. Phospholipase A_2 inhibitors: BEL (□), ONO-RS-082 (△), aristolochic acid (○), MAFP (◇), $AACOCF_3$ (▲); phospholipase C inhibitor: U-73122 (●); DGL inhibitor: RHC-80267 (■). Data are expressed as the averages of four measurements.

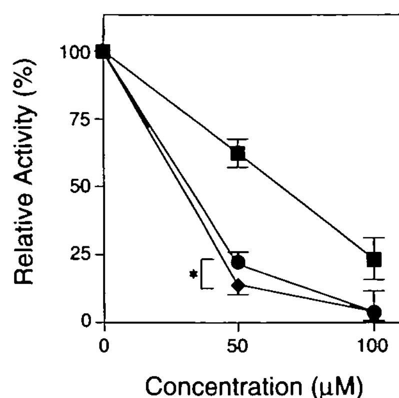

Fig. 7. **Effects of various 2-monoacylglycerol species on the partially purified DGL.** DGL activity was measured in the presence of 2-stearoyl glycerol (■), 2-oleoyl glycerol (●), or 2-arachidonoyl glycerol (◆). These 2-monoacylglycerols were added as ethanol or hexane solutions to the tubes for substrate preparation, followed by drying under N_2 and co-sonication with 1-[^{14}C]stearoyl-DG. The final concentration of 1-[^{14}C]stearoyl-DG was 100 μM. Other conditions were the same as for the standard assay method described under "MATERIALS AND METHODS." Data are expressed as the means± SD for three independent determinations. *$p<0.05$ (Student's t-test.)

phospholipase A_2 (*43*)] and U-73122 [a phospholipase C inhibitor (*44*)] had little effect on DGL activity up to concentrations of 100 and 10 μM, respectively.

Effects of 2-Monoacylglycerols on Partially Purified DGL—The effects on DGL activity of 2-monoacylglycerols (2-MGs), products of the DGL reaction, were determined using three molecular species of 2-MGs: 2-stearoyl, 2-oleoyl, and 2-arachidonoyl glycerols. As shown in Fig. 7, all 2-MGs tested inhibited DGL activity. However, 2-arachidonoyl glycerol and 2-oleoyl glycerol were more significantly inhibitory than 2-stearoyl glycerol, completely abolishing DGL activity at the concentration of 100 μM. The inhibitory effect of 2-arachidonoyl glycerol was significantly greater than that of 2-oleoyl glycerol ($p<0.05$, at the concentration of 50 μM).

DISCUSSION

In the current paper we describe the solubilization and purification from human platelet microsomes of diacylglycerol lipase (DGL), a key enzyme in diacylglycerol hydrolysis. The purified enzyme was shown to have a molecular mass of 33 kDa and a pI of 6.0. In addition, we compared various enzymatic properties of the partially purified DGL with results published previously for other preparations of DGL.

Since the majority of DGL activity in human platelets was detected in the microsomal fraction, we attempted to solubilize DGL activity from platelet microsomes using heptyl-β-D-thioglucoside as a detergent. About 70% of the total DGL activity was solubilized with this detergent. Heptyl-β-D-thioglucoside is a non-ionic detergent with a high cmc value (30 mM) and a small micelle scale (*45*). These properties are beneficial for subsequent protein purification steps, including electrophoresis, column chromatography, and concentration. However, DGL activity was found to be unstable during these post-solubilization purification steps. A marked loss of activity, for example, occurred after column chromatography (Table I). Among the columns tested, the gel filtration column maintained the greatest amount of DGL activity and consequently was used for each of the three main purification steps. In addition to gel filtration column chromatography, the purification protocol included batch treatment using a gel mixture (ion-exchange and heparin affinity gels) and a preparative isoelectrofocusing system (Rotofor). The overall purification scheme allowed DGL to be partially purified 361-fold. This DGL preparation was then used in a variety of enzymatic assays to characterize the properties of human platelet DGL. Aliquots of the partially purified DGL were also further purified to homogeneity by sequential electrophoresis, a technique that may facilitate the purification of proteins which are difficult to purify by column chromatography alone.

The purified DGL had an estimated molecular size of 33 kDa, which is the first such size reported in human lipase families (*46*). This suggests that platelet DGL may represent a novel lipase family in humans. Interestingly, 33 kDa is close to the size of MG lipase (MGL) isolated from mouse adipose tissue (*47*) or DGL/MGL from fungi (*48*) (33 and 30 kDa, respectively).

The optimal pH value for the enzymatic activity of the partially purified DGL was 7-8. Similar pH dependencies have been found using DGL-containing microsomes from human platelets (*10*), partially purified bovine aorta DGL (*30*), and DGL from bovine brain (*14, 15, 37*) as the assay source material. Under the current assay conditions, the activity of the partially purified DGL was not affected by the Ca^{2+} concentration. This finding is supported by a previous study involving human platelets (*10*). These pH and Ca^{2+} dependencies indicate that DGL can act under physiological conditions with a basal free Ca^{2+} level.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

DGL activity was further characterized by testing various residue-specific reagents. Enzymatic activity was inhibited by *p*CMB, *N*EM, and Hg^{2+}, whereas DTT and GSH enhanced the activity. These results indicate that free thiol group(s) of cysteine are necessary for DGL activity. Similar inhibition was observed with the serine blockers, DFP and PMSF. The histidine inhibitor, *p*BMB, which is known to modify histidine residues through alkylation (*36*), also reduced DGL activity. Taken together, serine and histidine residues might also be important for DGL activity. It is notable that the 33 kDa MGL from adipose tissue was similarly inhibited by serine and cysteine blockers (*47*). It is not clear, however, whether this MGL is the same as platelet DGL or whether platelet DGL has MGL activity; future experiments should answer these questions. Our preliminary experiments showed that purified platelet DGL has significant MGL activity (data not shown). Further studies on these issues are needed.

Unexpectedly, the partially purified DGL was potently inhibited by several PLA_2 inhibitors, including MAFP, ONO-RS-082, $AACOCF_3$, and BEL (Fig. 6). MAFP and $AACOCF_3$ are known to be inhibitors of $cPLA_2$ (Ca^{2+}-dependent cytosolic PLA_2), and BEL is known to be an inhibitor of $iPLA_2$ (Ca^{2+}-independent cytosolic PLA_2). This is the first report describing such inhibition. Although these inhibitors were approximately 10-fold less potent against DGL than PLA_2, they were in fact more potent inhibitors of DGL ($IC_{50} < 5\ \mu M$) than a known DGL inhibitor, RHC-80267 ($IC_{50} = 5\ \mu M$, present data). RHC-80267 has also been reported to inhibit PLA_2 as well as DGL (*49*). These results suggest that platelet DGL and the cytosolic type of PLA_2, both glycerol ester hydrolases, may share certain structural or catalytic features. In support of this hypothesis, it is interesting to note that a multi-functional enzyme exhibiting phospholipase, lysophospholipase, and DGL activity has been isolated from human neutrophils (*50*). Confirmation, however, awaits further DGL structural studies.

As shown in Fig. 7, DGL activity was inhibited by 2-MGs, the enzymatic reaction products of DGL. Bovine aortic DGL activity was also inhibited by 2-MG (2-oleoyl glycerol) (*31*). In our study, we found that 2-arachidonoyl glycerol was a more potent inhibitor of DGL than 2-stearoyl glycerol or 2-oleoyl glycerol. This is the first report showing differential inhibition by the 2-MG molecular species. It is significant that 2-arachidonoyl glycerol, a putative physiological reaction product (*28, 29*), preferentially reduces DGL activity. This may indicate a physiological mechanism for DGL regulation.

Finally, the properties of DGL obtained from various tissues were compared, as shown in Table III. The enzymatic activity of DGL from human platelets was originally investigated by Prescott *et al.* through a series of extensive studies (*8, 10*). Their findings correlate well with our present results (Table III), suggesting that the DGL activity detected by them may be identical to that of DGL purified here. In addition, the DGL purified from human platelets here has properties similar to those of bovine aortic DGL, which was partially purified by Lee and Severson (*30, 31*). On the other hand, DGLs purified from bovine brain by Farooqui *et al.* (*14, 15, 37*) exhibit different molecular masses and different sensitivities toward ATP, AMP, and NaF, compared with our present data. The apparent differences in the enzymatic properties of DGLs purified from various sources may reflect species-specific differences in the enzyme, or perhaps merely variations in the assay conditions used. However, the possibility of several DGL isozymes with different enzymatic properties cannot be excluded. Further studies, including cloning of the DGL genes, will be necessary to examine the DGL diversity at the molecular level.

The authors wish to thank Manabu Oki of Kyoto Red Cross Blood Center for providing the outdated human platelets, and Kazuteru Wada, Juichi Fukushima, and Megumi Sugiura for the help in the purification.

REFERENCES

1. Michell, R.H., Drummond, A.H., and Downes, C.P. (eds.) (1989) *Inositol Lipids in Cell Signaling*, pp. 1-515, Academic Press, London
2. Katan, M. (1998) Families of phosphoinositide-specific phospholipase C: structure and function. *Biochim. Biophys. Acta* **1436**, 5-17
3. Berridge, M.J. and Irvine, R.F. (1989) Inosito phosphates and cell signalling. *Nature* **341**, 197-205
4. Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607-614
5. Exton, J.H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta* **1212**, 26-42
6. Bell, R.L., Kennerly, D.A., Stanford, N., and Majerus, P.W. (1979) Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. USA*. **76**, 3238-3241
7. Chau, L.Y. and Tai, H.H. (1981) Release of arachidonate from diglyceride in human platelets requires the sequential action of a diglyceride lipase and a monoglyceride lipase. *Biochem. Biophys. Res. Commun.* **100**, 1688-1695
8. Prescott, S.M. and Majerus, P.W. (1983) Characterization of 1,2-diacylglycerol hydrolysis in human platelets. Demonstration of an arachidonoyl-monoacylglycerol intermediate. *J. Biol. Chem.* **258**, 764-769
9. Mauco, G., Fauvel, J., Chap, H., and Douste-Blazy, L. (1984) Studies on enzymes related to diacylglycerol production in activated platelets. II. Subcellular distribution, enzymatic properties and positional specificity of diacylglycerol- and monoacylglycerol-lipases. *Biochim. Biophys. Acta* **796**, 169-177
10. Majerus, P.W. and Prescott, S.M. (1982) Characterization and assay of diacylglycerol lipase from human platelets. *Method Enzymol.* **86**, 11-17
11. Authi, K.S., Lagarde, M., and Crawford, N. (1985) Diacylglycerol lipase activity in human platelet intracellular and surface membranes. Some kinetic properties and fatty acid specificity. *FEBS Lett.* **180**, 95-101
12. Chau, L.Y. and Tai, H.H. (1988) Monoglyceride and diglyceride lipases from human platelet microsomes. *Biochim. Biophys. Acta* **963**, 436-444
13. Balsinde, J., Diez, E., and Mollinedo, F. (1991) Arachidonic acid release from diacylglycerol in human neutrophils. Translocation of diacylglycerol-deacylating enzyme activities from an intracellular pool to plasma membrane upon cell activation. *J. Biol. Chem.* **266**, 15638-15643
14. Farooqui, A.A., Rammohan, K.W., and Horrocks, L.A. (1989) Isolation, characterization, and regulation of diacylglycerol lipases from the bovine brain. *Ann. N.Y. Acad. Sci.* **559**, 25-36
15. Farooqui, A.A., Taylor, W.A., and Horrocks, L.A. (1986) Characterization and solubilization of membrane bound diacylglycerol lipases from bovine brain. *Int. J. Biochem.* **18**, 991-997
16. Cabot, M.C. and Gatt, S. (1977) Hydrolysis of endogenous diacylglycerol and monoacylglycerol by lipases in rat brain microsomes. *Biochemistry* **16**, 233-234
17. Konrad, R.J., Major, C.D., and Wolf, B.A. (1994) Diacylglycerol

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

hydrolysis to arachidonic acid is necessary for insulin secretion from isolated pancreatic islets: sequential actions of diacylglycerol and monoacylglycerol lipases. *Biochemistry* **33**, 13284-13294
18. Chuang, M., Dell, K.R., and Severson, D.L. (1990) Protein kinase C does not regulate diacylglycerol metabolism in aortic smooth muscle cells. *Mol. Cell Biochem.* **96**, 69-77
19. Migas, I., Chuang, M., Sasaki, Y., and Severson, D.L. (1997) Diacylglycerol metabolism in SM-3 smooth muscle cells. *Can. J. Physiol. Pharmacol.* **75**, 1249-1256
20. Chuang, M. and Severson, D.L. (1998) Metabolic fate of exogenous diacylglycerols in A10 smooth muscle cells. *Biochim. Biophys. Acta* **1390**, 149-159
21. Okazaki, T., Sagawa, N., Okita, J.R., Bleasdale, J.E., MacDonald, P.C., and Johnston, J.M. (1981) Diacylglycerol metabolism and arachidonic acid release in human fetal membranes and decidua vera. *J. Biol. Chem.* **256**, 7316-7321
22. Sagawa, N., Okazaki, T., MacDonald, P.C., and Johnston, J.M. (1982) Regulation of diacylglycerol metabolism and arachidonic acid release in human amnionic tissue. *J. Biol. Chem.* **257**, 8158-8162
23. Takamura, H., Narita, H., Park, H.J., Tanaka, K., Matsuura, T., and Kito, M. (1987) Differential hydrolysis of phospholipid molecular species during activation of human platelets with thrombin and collagen. *J. Biol. Chem.* **262**, 2262-2269
24. Moriyama, T., Wada, K., Oki, M., Matsuura, T., and Kito, M. (1994) The mechanism of arachidonic acid release in collagen-activated human platelets. *Biosci. Biotechnol. Biochem.* **58**, 93-98
25. Koike, T., Mizutani, T., Hirai, K., Morita, Y., and Nozawa, Y. (1993) SCF/c-kit receptor-mediated arachidonic acid liberation in rat mast cells. Involvement of PLD activation associated tyrosine phosphorylation. *Biochem. Biophys. Res. Commun.* **197**, 1570-1577
26. Ishimoto, T., Akiba, S., and Sato, T. (1996) Importance of the phospholipase D-initiated sequential pathway for arachidonic acid release and prostaglandin D_2 generation by rat peritoneal mast cells. *J. Biochem.* **120**, 616-623
27. Moscat, J., Herrero, C., Garcia-Barreno, P., and Municio, A.M. (1986) Phospholipase C-diglyceride lipase is a major pathway for arachidonic acid release in macrophages. *Biochem. Biophys. Res. Commun.* **141**, 367-373
28. Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., and Waku, K. (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* **215**, 89-97
29. Stella, N., Schweitzer, P., and Piomelli, D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature* **388**, 773-778
30. Lee, M.W. and Severson, D.L. (1994) Partial purification of a diacylglycerol lipase from bovine aorta. *Biochem. J.* **298**, 213-219
31. Lee, M.W., Kraemer, F.B., and Severson, D.L. (1995) Characterization of a partially purified diacylglycerol lipase from bovine aorta. *Biochim. Biophys. Acta* **1254**, 311-318
32. Lands, W.E.M. and Merkl, I. (1963) Metabolism of glycerolipids. *J. Biol. Chem.* **238**, 898-903
33. Schatzmann, H.J. (1973) Dependence on calcium concentration and stoichiometry of the calcium pump in human red cells. *J. Physiol. (Lond.)* **235**, 551-569
34. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
35. Garner, C.W. (1980) Boronic acid inhibitors of porcine pancreatic lipase. *J. Biol. Chem.* **255**, 5064-5068
36. Kudo, I., Murakami, M., Hara, S., and Inoue, K. (1993) Mammalian non-pancreatic phospholipase A_2. *Biochim. Biophys. Acta* **1170**, 217-231
37. Farooqui, A.A., Taylor, W.A., and Horrocks, L.A. (1984) Separation of bovine brain mono- and diacylglycerol lipases by heparin Sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.* **122**, 1241-1246
38. Balsinde, J. and Dennis, E.A. (1997) Function and inhibition of intracellular calcium-independent phospholipase A_2. *J. Biol. Chem.* **272**, 16069-16072
39. Street, I.P., Lin, H.K., Laliberte, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N.M., Huang, Z., Weech, P.K., and Gelb, M.H. (1993) Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. *Biochemistry* **32**, 5935-5940
40. Hazen, S.L., Zupan, L.A., Weiss, R.H., Getman, D.P., and Gross, R.W. (1991) Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase A_2. Mechanism-based discrimination between calcium-dependent and -independent phospholipases A_2. *J. Biol. Chem.* **266**, 7227-7232
41. Banga, H.S., Simons, E.R., Brass, L.F., and Rittenhouse, S.E. (1986) Activation of phospholipases A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. *Proc. Natl. Acad. Sci. USA* **83**, 9197-9201
42. Sutherland, C.A. and Amin, D. (1982) Relative activities of rat and dog platelet phospholipase A_2 and diglyceride lipase. Selective inhibition of diglyceride lipase by RHC 80267. *J. Biol. Chem.* **257**, 14006-14010
43. Vishwanath, B.S. and Gowda, T.V. (1987) Interaction of aristolochic acid with Vipera russelli phospholipase A_2: its effect on enzymatic and pathological activities. *Toxicon* **25**, 929-937
44. Thompson, A.K., Mostafapour, S.P., Denlinger, L.C., Bleasdale, J.E., and Fisher, S.K. (1991) The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for Gp in receptor compartmentation. *J. Biol. Chem.* **266**, 23856-23862
45. Shimamoto, T., Saito, S., and Tsuchiya, T. (1985) Value of heptyl-beta-D-thioglucoside, a new nonionic detergent, in studies on membrane proteins. *J. Biochem.* **97**, 1807-1810
46. Cygler, M., Schrag, J.D., Sussman, J.L., Harel, M., Silman, I., Gentry, M.K., and Doctor, B.P. (1993) Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins. *Protein Sci.* **2**, 366-382
47. Karlsson, M., Contreras, J.A., Hellman, U., Tornqvist, H., and Holm, C. (1997) cDNA cloning, tissue distribution, and identification of the catalytic triad of monoglyceride lipase. Evolutionary relationship to esterases, lysophospholipases, and haloperoxidases. *J. Biol. Chem.* **272**, 27218-27223
48. Yamaguchi, S., Mase, T., and Takeuchi, K. (1991) Cloning and structure of the mono- and diacylglycerol lipase-encoding gene from *Penicillium camembertii* U-150. *Gene* **103**, 61-67
49. Oglesby, T.D. and Gorman, R.R. (1984) The inhibition of arachidonic acid metabolism in human platelets by RHC 80267, a diacylglycerol lipase inhibitor. *Biochim. Biophys. Acta* **793**, 269-277
50. Munford, R.S. and Hunter, J.P. (1992) Acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides, has phospholipase, lysophospholipase, diacylglycerollipase, and acyltransferase activities *in vitro*. *J. Biol. Chem.* **267**, 10116-10121

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012