Purification and properties of a monoacylglycerol lipase in human erythrocytes

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A membrane-bound monoacylglycerol lipase (MAGL) activity, previously demonstrated in intact human erythrocytes [Boyer, Somma, Vérine, L'Hôte, Finidori, Merger and Arnaud (1981) *J. Clin. Endocrinol. Metab.* **53**, 143–148], has now been purified to apparent homogeneity by a five-step procedure involving solubilization in CHAPS and sequential chromatographies on Sephacryl S-400, DEAE-Trisacryl, Zn²⁺-chelating Sepharose and Superose 12 columns. The purified protein has a molecular mass of 68 ± 2 kDa, as determined by SDS/PAGE and gel filtration, suggesting that the enzyme behaves as a monomer. The concentration-dependence of MAGL activity with mono-

oleoylglycerol, the preferred substrate, showed kinetics typical of an interfacial lipolytic enzyme displaying optimal activity on emulsified substrate particles; apparent K_m values were 0.27 mM and 0.49 mM for the *sn*-1(3)- and *sn*-2-isomers respectively. MAGL had no, or negligible, activity towards tri-oleoylglycerol, di-oleoylglycerol, oleoylcholesterol, oleoyl-CoA and phosphatidylcholine; it was inhibited by di-isopropylfluorophosphate, PMSF and diethyl *p*-nitrophenyl phosphate, suggesting that MAGL is a serine hydrolase. MAGL activity was not modified by bile salt or apolipoprotein C-II, whereas a dose-dependent inhibition was observed with apolipoprotein A-I.

INTRODUCTION

Whereas the conditions of enzymic hydrolysis of triacylglycerol in mammalian tissues have been the subject of numerous studies, those under which partial acylglycerols are metabolized have been much less investigated. In particular, the number and nature of lipases responsible for the hydrolysis of monoacylglycerols *in vivo* are uncertain. Monoacylglycerol lipase (MAGL) activities have been assumed to be distinct enzymes (i.e. monoacylglycerol-hydrolysing activities not attributable to triacylglycerol lipases) in heart [1], small intestine [2], platelets [3], aorta [4] and plasma [5]. In a few instances, MAGL activities have been subjected to various degrees of purification, e.g. from rat [6], human [7] or chicken [8] adipose tissues. In all cases, the precise subcellular localization, as well as the molecular structure of the enzymes, is unknown. Numerous differences in the experimental conditions under which the postulated properties of these lipases are reported, make it difficult to determine if these activities refer to the same (or closely related) proteins and whether they might represent tissue-specific isoenzymes of the erythrocyte MAGL. An answer to these questions will have to await the complete structural elucidation of these catalytic entities.

We have previously identified in intact erythrocytes from rats [9] and human subjects [10] a monoacylglycerol ester lipase (MAGL) acting as a membrane-bound enzyme with its active site externally orientated, as inferred from the ability of the intact cell to hydrolyse an exogenously added lipid substrate. Cell-bound MAGL activity measured in intact erythrocytes from 161 healthy adult subjects had a mean ($\pm$ S.D.) level of 1022 ± 134 m-units/ 10^{12} cells (one m-unit releases 1.0 nmol of acid/min), without significant difference between men and women [10]. MAGL activity was shown to vary with physiological [10,11] or pathophysiological conditions [12–14]. Given the significance of the monoacylglycerol pathway for triacylglycerol metabolism,

we have attempted to purify and characterize MAGL from the human erythrocyte.

The purification of MAGL has been hampered by difficulties due to its low abundance and relative instability. In this report we present the analytical-scale purification of MAGL, which yielded an apparently homogeneous enzyme preparation suitable for further biochemical characterization. MAGL represents the first lipase purified from human erythrocytes.

MATERIALS AND METHODS

[9,10-³H]Oleic acid (10 Ci/mmol), tri-[9,10-³H]oleoylglycerol (³H]TOG; 20 Ci/mmol), 2-[1-¹⁴C]oleoyl-1-palmitoyl-phosphatidylcholine (¹⁴C]PC; 58 mCi/mmol), 1-[1-¹⁴C]-palmitoyl-lysophosphatidylcholine (¹⁴C]lysoPC; 56 mCi/mmol) and [1-¹⁴C]oleoyl-CoA (60 mCi/mmol) were purchased from New England Nuclear Research Products (Les Ulis, France). Apolipoprotein (apo) A-I, apoC-II, oleylethanol (OE), CHAPS, PMSF and diethyl *p*-nitrophenyl phosphate (E 600) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Di-isopropyl-fluorophosphate (DFP) was from Aldrich (Milwaukee, WI, U.S.A.). Defatted BSA was from Calbiochem (La Jolla, CA, U.S.A.). Organic solvents, Florisil and Si-60 TLC plates were from Merck (Darmstadt, Germany). Materials and products for PAGE were from Bio-Rad (Richmond, CA, U.S.A.). All other reagents were of analytical grade.

Substrates

[³H]OE was synthesized by condensation of [³H]oleic acid and ethanol in the presence of boron fluoride at 60 °C for 2 h [15] and was purified on a Florisil column [16] as published previously [10]. The purity of the [³H]OE substrate preparation is critical and should be checked every month; in practice, an additional

Abbreviations used: apo, apolipoprotein; DFP, di-isopropylfluorophosphate; DOG, di-oleoylglycerol; DTT, dithiothreitol; E 600, diethyl *p*-nitrophenyl phosphate; HDLs, high-density lipoproteins; IMAC, ion-metal-affinity chromatography; lyso-PC, 1-palmitoyl-lysophosphatidylcholine; MAGL, monoacylglycerol lipase; MOG, mono-oleoylglycerol; OE, oleylethanol; PC, 2-oleoyl-1-palmitoyl-phosphatidylcholine; TOG, tri-oleoylglycerol.

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Florisil chromatography is needed when the blank value of the MAGL assay increases. Di- ^3H oleoylglycerol (^3H DOG) was obtained by condensation at 180 °C under nitrogen of ^3H oleic acid and glycerol in the presence of crystallized NaOH; the product was first purified on a Florisil column, and then by TLC using ethyl ether/hexane (50:50, v/v) as developing solvent; the final product (^3H DOG) was > 96 % radiochemically pure, as determined by scanning with a Berthold TLC-tracemaster LB 282 (EGG Instruments, Evry, France); it contained approx. 80 % of the *sn*-1,3-isomer and 20 % of the *sn*-1,2(2,3)-isomer. The lipids, *sn*-1(3)-mono- ^3H oleoylglycerol {1(3)- ^3H MOG} and *sn*-2-mono- ^3H oleoylglycerol {2- ^3H MOG} were prepared by partial hydrolysis of ^3H TOG with pancreatic lipase [17]. The mixed isomers were isolated first on a Florisil column then chromatographed by TLC twice using chloroform/methanol (98:2, v/v) as developing solvent on Si-60 plates impregnated with 10 % boric acid in methanol; 1(3)- ^3H MOG and 2- ^3H MOG were about 80 % and 90 % radiochemically pure respectively. All lipid substrates were kept in solution in heptane under nitrogen at -70 °C before being used.

Erythrocyte isolation and ghost preparation

Erythrocytes were obtained from freshly drawn blood (stabilized with EDTA) from healthy volunteers; samples were processed within 48 h. Unless otherwise stated, all procedures were carried out at 4 °C. Each individual sample was first centrifuged (10 min; 800 g) and the cells were resuspended twice in 172 mM Tris/HCl buffer (pH 7.4; TH buffer) with the resulting suspensions being re-centrifuged as above. Each time, the upper layer of the packed cells (containing mainly leucocytes) and the supernatant were removed. Then, the cells obtained from 2–5 individual samples were pooled and further isolated by filtration on a cellulose column as described previously [10]. Isolated erythrocytes were washed twice by alternate centrifugation/resuspension (450 g for 5 min) in TH buffer (pH 7.4). After the last wash, erythrocytes were lysed for 1 h in 11 mM Tris/HCl (pH 7.4) and the ghosts were pelleted by centrifugation for 1 h at 20000 g. The supernatant was discarded and the pellets were washed as above with the same buffer until a colourless (essentially haemoglobin-free) ghost suspension was obtained. The final pellet was resuspended in TH buffer and could be stored at -20 °C for up to 20 days without significant loss of activity.

Enzyme assays

MAGL activity was routinely assayed using an emulsion of ^3H OE as the substrate [10]. The use of OE, which is totally insoluble in water, ascertains that MAGL displays a true lipolytic activity [18]; it has the additional advantage (compared with MOG) that it is more easily separated from oleic acid, a reaction product, in the final extract. A substrate emulsion stock was prepared daily as follows: 50 μmol of ^3H OE ($\sim 4 \times 10^6$ d.p.m./ μmol) in heptane was taken to dryness and 20 ml of a solution of 0.215 M Tris/HCl (pH 7.8) containing 1.25 % defatted BSA was added. The mixture was sonicated for 1 min at room temperature using a Branson sonifier (model B12; Heat System-Ultrasonics, Inc., Plainview, NY, U.S.A.). The reaction was initiated by adding 0.1 ml of enzyme to 0.4 ml of substrate emulsion. The assay mixture (total volume 0.5 ml) contained 0.172 M Tris/HCl (pH 7.8), 1 % BSA ($\approx 150 \mu\text{M}$) and 2 mM emulsified ^3H OE; unless otherwise stated, 1.6 mM CHAPS was introduced in the medium with the enzyme. When MAGL was assayed on MOG or OE emulsions in the absence of BSA, no activity was detected. BSA served as fatty acid acceptor [19] and

was mandatory for the obtention of zero-order kinetics over the 30–60 min assay periods. Unless otherwise stated, assays were carried out in duplicate for 30 min at 37 °C with gentle shaking; blank tubes (buffer replacing the enzyme) were run in parallel. Tri- and di-acylglycerol lipase activities were determined with ^3H TOG and ^3H DOG as substrates respectively, in an assay otherwise identical to that described above. When ^3H MOG was used as substrate, an additional TLC step, carried out with ethyl ether/hexane/acetic acid (50:50:1, by vol.) as developing solvent, was required for the isolation of released ^3H oleic acid. Reactions were initiated by the addition of enzyme. The ^3H oleic acid released during incubation was extracted by using a liquid-liquid partition system [10] and served to estimate the reaction rate. For each activity value, duplicate assays performed either with intact erythrocytes, erythrocyte ghosts, or soluble MAGL as enzyme sources agreed within 10 % of the amounts of ^3H oleic acid released.

Sterol ester activity was assayed with ^3H oleoylcholesterol as substrate, as published previously [20]. Lysophospholipase activity was measured using ^{14}C lysoPC as substrate as described by Tornqvist and Belfrage [6]. Oleoyl-CoA hydrolysis was measured according to Lehner and Kuksis [21]. Phospholipase A activity was measured using ^{14}C PC as substrate according to Poensgen [22].

Purification

All steps were carried out at 4 °C. To solubilize MAGL, ghost suspensions were stirred for 60 min with the zwitterionic detergent CHAPS (8 mM in TH buffer), followed by centrifugation for 30 min at 20000 g. The supernatant (1.2 ± 0.5 mg of protein/ml), which contained 50–70 % of the activity assayed in the ghost suspension, was removed and concentrated in a Centriprep 10 concentrator (Amicon Inc., Beverly MA, U.S.A.; step 1). Re-extraction of the particulate fraction did not improve recovery. Attempts to liberate MAGL from ghosts by the action of a glycosylphosphatidylinositol phospholipase D (Boehringer, Meylan, France; 5 units/ml) were unsuccessful.

The concentrated extract was applied to a size-exclusion Sephacryl S-400 HR column (2.5 cm $\times$ 100 cm; Pharmacia, Orsay, France) equilibrated with 10 mM Tris/HCl buffer/8 mM CHAPS/1 mM dithiothreitol (DTT)/1 mM EDTA, pH 8.0 (solution A). Elution (56 ml/h) was carried out with solution A (450 ml). Fractions (5 ml) containing significant activity were pooled (step 2), supplemented with glycerol (10 %, v/v), adjusted to pH 8.5 and applied to a DEAE-Trisacryl column (1 cm $\times$ 10 cm; Sepracor-IBF, Villeneuve-La-Garenne, France) equilibrated with solution A containing 10 % glycerol, pH 8.5 (solution B). Unbound protein was removed by washing the column with solution B until the absorbance returned to its initial level. Then, the column was developed with an 80-ml linear gradient of NaCl (0–250 mM) in solution B. The peak fractions (3 ml) were eluted between 40 and 60 mM NaCl (step 3) and were pooled, concentrated ('diafiltrated' by Centriprep concentrator; Amicon) to ≈ 5 ml, allowing for solution B being replaced by 20 mM Na_2HPO_4 /0.1 M NaCl/8 mM CHAPS/10 % glycerol, pH 7.4 (solution C). The sample was then loaded on to a Zn^{2+} -chelating Sepharose column [1 cm $\times$ 8 cm; ion metal affinity chromatography (IMAC); Pharmacia] equilibrated with solution C; this column allows for the separation of proteins according to their histidine content. All MAGL activity was recovered with the unbound protein (step 4); further elution with a 100-ml linear gradient of histidine (0–50 mM) in solution C only supplied inactive proteins. The active fractions were concentrated (by Centricon 10) to ≈ 0.5 ml with solution C containing

Figure 1 Reversible inhibition of MAGL activity by CHAPS

CHAPS concentration in the assay medium was varied as shown by diluting one initial enzymic extract containing 8 mM CHAPS. Activity values (mean $\pm$ S.D.) are expressed relative to the activity of MAGL (m-units/ml) incubated without CHAPS (control). Data represent the means of two experiments with assays performed in duplicate.

1 % instead of 10 % glycerol (solution D). The concentrated sample was subjected to FPLC on a Superose 12 HR column (1 cm $\times$ 30 cm; Pharmacia) equilibrated and eluted (0.15 ml/min) with solution D (step 5). Fractions (0.45 ml) were collected and immediately assayed for MAGL activity. Peak fractions were analysed by SDS/PAGE.

Other methods

Protein concentrations were determined either by the Bradford dye-binding assay [23] using BSA as standard or by monitoring the absorbance at 280 nm. SDS/PAGE was performed on vertical 10 % polyacrylamide slab gels according to the method of Laemmli [24]. Proteins were visualized either with Coomassie Brilliant Blue R250 or with Amido Black.

SDS/PAGE of the partially purified enzyme ($\approx$ 120 m-units/mg of protein; $\approx$ 5 μ g) was performed using a vertical Bio-Rad apparatus (Mini Protean II). The enzymic sample was applied as a solution (100 μ l) containing 20 % (v/v) glycerol, 5 mM DTT, 0.1 % SDS, 0.005 % (w/v) Bromophenol Blue in 100 mM Tris/HCl (pH 8.8); the heat treatment (90 $^{\circ}$ C for 3 min) was omitted. PAGE was run at 4 $^{\circ}$ C in 25 mM Tris/HCl/200 mM glycine/0.1 % SDS, pH 8.5, at a constant current of 40 mA. The gel lanes containing the size markers, which were applied and run under the usual Laemmli conditions, were cut and coloured with

Coomassie Blue. The non-coloured gel lane containing the enzyme was sliced horizontally into 30 2-mm-thick fragments, each one being crushed in 0.45 ml of 10 mM Tris/HCl/1 mM EDTA/1 mM DTT/4 mM CHAPS, pH 8.0. Diffusion for 18 h at 4 $^{\circ}$ C was allowed before adding 0.5 ml of substrate emulsion to each tube for assaying MAGL; samples were incubated for 1 h at 37 $^{\circ}$ C. In this way, the SDS concentration in the assay medium was reduced from 0.1 % to approx. 0.004 %, allowing the recovery of detectable amounts of MAGL activity.

RESULTS

Solubilization of human erythrocyte MAGL

The initial purification step involved the solubilization of MAGL in 8 mM CHAPS. Pilot studies had shown that detergent concentrations higher than 0.01 % for Triton X-100, 0.05 % for SDS and 5 mM for CHAPS inhibited MAGL activity in a manner partially reversible upon dilution of the detergent. Figure 1 shows that assaying MAGL in the presence of 8 mM CHAPS results in 63 % inhibition; diluting the CHAPS solution from 8 mM to 0.2 mM produces an 80 % reversal of the inhibitory effect. On the other hand, lowering the CHAPS concentration below 0.1 mM (for instance by dialysis) caused important loss of activity. One of the difficulties of the present work was to avoid either one of these drawbacks.

Purification of MAGL

For a typical five-step purification procedure, 300–500 ml of fresh blood was used. MAGL activity averaged 16 m-units (range 14–19 m-units for six experiments) per ml of packed cells ($\approx$ 1.6×10^{10} cells). Over 80 % of this activity was recovered in the cell ghosts, whereas < 10 % was found in the membrane-free hypotonic lysates, confirming that essentially all MAGL was associated with membranes. Results obtained from a typical purification experiment are presented in Table 1. MAGL was purified at least 808-fold starting from the CHAPS-solubilized extract, with a recovery of 7.6 %. Addition of protease inhibitors to the initial cell suspension did not improve the overall recovery. The elution patterns of MAGL activity obtained in the four steps of chromatography are depicted in Figure 2. Results were consistently reproducible over six similar experiments. A single peak (approx. 80 % of the initial activity) was eluted from the Sephacryl S-400 column (Figure 2a); this material was applied to a DEAE-Trisacryl column from which two active peaks were eluted with NaCl, one in the range 0.06–0.08 M, another at 0.25 M (Figure 2b). The first peak, representing 90 % of the total activity eluted, was applied to the IMAC column (Figure 2c).

Table 1 Purification of MAGL from human erythrocyte

Assays were performed with 2 mM [3 H]OE as substrate, in the presence of 1.6 mM CHAPS.

Enzyme fraction	Protein (mg)	Specific activity (m-units/mg of protein)	Recovery (%)	Purification (-fold)
1. CHAPS solution	348	0.6	100	—
2. Sephacryl S-400	50	3.4	78	5.6
3. DEAE-Trisacryl	6	19.3	51	32
4. IMAC	0.36	244	43	406
5. Superose 12	0.08	485	7.6	808

Figure 2 Chromatographic patterns for the purification of MAGL from human erythrocytes

(a) Sephacryl S-400; (b) DEAE-Trisacryl; (c) IMAC (Zn^{2+} -chelating Sepharose); (d) Superose 12. Experimental details are described in the Materials and methods section. Solid circles indicate absorbance at 280 nm and open circles MAGL activity as determined with [^3H]OE as substrate. The broken lines represent the concentration of NaCl (b, 0–0.25 M) and histidine (c, 0–50 mM). Arrows in (a) and (d) indicate the locations of the BSA marker (67 kDa).

Undesirable yellow material, tentatively identified as deriving from haemoglobin (of molecular mass, 64.5 kDa; about 7% of histidine residues), was tightly bound by the column, whereas approx. 80% of the loaded MAGL activity was not retained; no subsequent peak of activity could be detected. The active fraction was then loaded on to the Superose 12 column. A single peak,

Figure 3 Separation of human erythrocyte MAGL on SDS/PAGE

Peak fractions from the IMAC (Zn^{2+} -chelating) Sepharose column (step 4; 244 m-units/mg of protein) were subjected to SDS/PAGE, and MAGL activity in the gel slices was assayed as indicated in the Materials and methods section. Activity is expressed as d.p.m. of [^3H]oleic acid released enzymically from [^3H]OE. Molecular-mass markers (phosphorylase b, 94 kDa; BSA, 67 kDa) are indicated by vertical arrows. Migration was from left to right. The inset shows denaturing SDS/PAGE of active MAGL fractions at different steps of purification. Lane A, molecular-mass markers (in kDa): phosphorylase b (94), BSA (66.2), ovalbumin (45.0), carbonic anhydrase (31.0) and trypsin inhibitor (21.5); lane B, CHAPS-solubilized extract (0.6 m-units/mg of protein); lane C, peak activity fractions from the Sephacryl S-400 column (step 2; 78 m-units/mg of protein); lane D, peak activity fractions from the Superose 12 column (step 5; 485 m-units/mg of protein).

representing approx. 20% of the loaded activity, was recovered at an exclusion volume corresponding approx. to a 70 kDa protein (Figure 2d).

Purified MAGL (485 m-units/mg of protein; approx. 5 μg of protein) gave a single band on SDS/PAGE gel, with an apparent molecular mass of approx. 68 kDa (Figure 3, inset). As shown by using SDS/PAGE conditions which allow the assay of MAGL in the gel slices (Figure 3), direct correlation of MAGL activity with a protein component of ≈ 70 kDa suggests that MAGL is catalytically active in a monomeric form. The apparent isoelectric point, as determined by chromatofocusing (Mono P column; Pharmacia), was $\text{pI} \approx 7.8$.

Enzyme stability

Whereas cell- or ghost-bound MAGL activity is essentially stable for 8 days at 4 °C or for 24 h at 20 °C [2], the enzyme solubilized in 8 mM CHAPS and stored at 4 °C for 24 h loses 25–30% of its activity. During a 15-min incubation, purified MAGL loses > 60% of its activity at 45 °C and is totally inactivated at 55 °C. At 4 °C, purified MAGL loses 5–7% of its activity per day in 8 mM CHAPS; the decrease is reduced by about 50% in the presence of 10% (v/v) glycerol, as is the case for other lipases [25].

Kinetic properties and inhibitor effects

Assay of the purified enzyme performed at the optimal pH (7.5 ± 0.3) with various concentrations of 1(3)- and 2-MOG indicated apparent K_m values of 0.27 and 0.49 mM respectively,

Figure 4 Effects of substrate concentration on erythrocyte MAGL activity

Purified MAGL activity was measured at 1(3)-[³H]MOG (○) and 2-[³H]MOG (●) concentrations in the range 0.1–8.0 mM, in the presence of 1.6 mM CHAPS. Results (mean ± S.D. of triplicate determinations) are presented as a double-reciprocal Lineweaver–Burk plot. Similar results were obtained in a second experiment with a different lipase preparation.

Table 2 Hydrolysis of various substrates by purified MAGL

Activity was assayed with 3–5 μg of enzyme protein after the Superose 12 step, in the presence of 1.6 mM CHAPS. Values are averages of duplicate or triplicate determinations. Abbreviation: n.d., not detectable.

Substrate	Concn.	Hydrolysis rate (m-units/ml)
Oleoylethanol	2 mM	2.8
Mono-oleoyl-1(3)-glycerol	2 mM	6.7
Mono-oleoyl-2-glycerol	2 mM	7.3
1,2-Di-oleoylglycerol	2 mM	n.d.
Tri-oleoylglycerol	2 mM	n.d.
1-Palmitoyl-lysophosphatidylcholine	1 mM	4.0
Dipalmitoylphosphatidylcholine	2 mM	n.d.
Oleoylcholesterol	0.5 mM	n.d.
Oleoyl-CoA	50 μM	n.d.

with comparable V_{max} values for both isomers (Figure 4). The K_m with OE was 0.47 mM with a V_{max} of about half that measured with MOG. As shown in Table 2, MAGL had practically no activity (< 1% relative to OE) against emulsified DOG, TOG, oleoyl-CoA or oleoylcholesterol. MAGL hydrolysed lysoPC at a V_{max} which was approx. half that with MOG.

Figure 5 shows that MAGL assayed with 1(3)-[³H]MOG displays sigmoidal kinetics: the enzyme has no, or little, activity at low substrate concentrations; however, once substrate saturation is exceeded and a turbid phase appears (above 0.5 mM MOG as determined by optical measurement) there is a sharp increase in activity. Although absorbance values cannot be considered to accurately reflect the physical state (soluble, micellar or emulsified) of the substrate, they reasonably differentiate between substrate over-saturation and under-satu-

Figure 5 Interfacial activation of MAGL

The influence of 1(3)-MOG concentration on MAGL activity (○) was measured in the standard assay system (see the Materials and methods section) containing 1% BSA plus 1.6 mM CHAPS introduced in the assay with the enzyme. Substrate particle formation (●) was monitored by measuring the optical clarity of the medium (containing 1.6 mM CHAPS but no enzyme) at the indicated substrate concentrations, after stirring the mixture for 15 min. The inset shows that no MAGL activity was detected with 0.25 mM MOG as substrate in BSA concentrations varying from 0 to 0.6 mM. All operations were performed at 37 °C. Data are the mean ± S.D. of two experiments.

Table 3 Effects of inhibitors and cofactors

Purified MAGL (4–7 μg of protein) was preincubated with selected inhibitors and cofactors for 15 min at 37 °C. The assay performed with [³H]OE as substrate emulsion contained in each case 1.6 mM CHAPS and the same concentration of inhibitor or cofactor as that used in the incubation step.

Inhibitor	Concn.	MAGL activity (% of control)
None	—	100
PMSF	0.5 mM	57
DFP	5 mM	12
E 600	1 mM	5
SDS	0.05%	15
NaCl	1 M	37
NaF	25 mM	10
ApoA-1	5 μM	30

ration. This type of stimulation, produced by increasing the concentration of substrate above saturation, is typical of most lipolytic enzymes which are activated by a water/lipid interface [26]. To rule out a specific effect of BSA on catalysis, i.e. that the actual substrate particle might require the binding of MOG to BSA, Figure 5 (inset) shows that the addition of BSA at concentrations resulting in a wide range of MOG to BSA molar ratios does not induce the hydrolysis of MOG in 'soluble' form (i.e. at 0.25 mM, a concentration compatible, in the presence of 1.6 mM CHAPS, with an optically clear solution). The reported solubility of long-chain monoacylglycerols in water at 20 °C is in the order of 10^{-6} M, [27]. The higher apparent value found here in the range of 10^{-3} M is likely to be due, on the one hand, to the higher temperature (37 °C) and, on the other, to some degree of 'solubilization' of MOG by BSA [28]; albumin has indeed been shown to bind monoacylglycerol [29].

Selective inhibitors such as DFP, PMSF and E 600 decreased MAGL activity (Table 3), suggesting that MAGL is a serine hydrolase, a feature common to most lipases [30]; NaCl and NaF also inactivated the enzyme. Bile salts (sodium taurocholate 0.4–4.0 mM) and apoC-II (5 μM) had no measurable effect on

Figure 6 Inactivation of MAGL activity by (a) diethyl *p*-nitrophenyl phosphate (E 600) and (b) apoA-I

(a) Purified MAGL was incubated for 15 min at 37 °C with the indicated concentrations of E 600. Then, MAGL activity was assayed using [³H]OE as substrate as indicated in the Materials and methods section, except that the assay medium contained, in each case, the same E 600 concentrations as that used in the incubation step. Control (C) value: no E 600. Inset: same experiment but using intact erythrocytes as the enzyme source. Bars are mean $\pm$ S.D. of triplicate assays. (b) Purified MAGL was incubated for 15 min at 37 °C with the indicated concentrations of apoA-I. Then, MAGL activity was assayed towards 2 mM [³H]OE as indicated in the Materials and methods section, except that the assay medium contained, in each case, the same apoA-I concentration as that used in the incubation step. Inset: reversal of the inhibition produced by 2 μ M apoA-I upon increasing the substrate concentration from 2 mM to 6 mM. Control MAGL activity values (100%; no apoA-I) were 7.0 (C₁) and 7.4 (C₂) m-units/ml with 2 mM and 6 mM [³H]OE respectively. Values are mean $\pm$ S.D. of triplicate assays. In all cases, the assay medium contained 1.6 mM CHAPS.

MAGL activity assayed towards MOG. Two observations were of particular interest: (a) E 600, a non-permeant inhibitor, inhibited MAGL equally whether the enzyme was in the cell-bound or soluble form (Figure 6a), confirming that its active site is exposed at the surface of the erythrocyte membrane [10]; and (b) apoA-I, the major apolipoprotein of high-density lipoproteins (HDLs) was a potent dose-related inhibitor of MAGL (Figure 6b), i.e. inhibition exceeded 60% with 0.5 μ M apoA-I in a medium containing 150 μ M BSA, a 300-fold higher protein concentration.

DISCUSSION

We have obtained, through a five-step purification protocol, a MAGL preparation purified at least 808-fold from human

erythrocytes, with a measured final specific activity of 485 m-units/mg of protein. Actually, a more exact value of the specific activity of the purified enzyme is likely to be much higher, for the following reasons: (a) MAGL stored at 4 °C loses as much as 60% of its activity in 2 weeks (during the purification procedure, which requires 2 to 3 weeks, there is thus a marked fall in specific activity); (b) residual concentrations (up to 2 mM) of CHAPS in the assays performed at each purification step may lower, by up to 50%, the measured values of MAGL activity; and (c) the assay with OE gives values which are about half those measured with MOG, the likely physiological substrate (Table 2).

The results indicate that purified MAGL prefers, with respect to its catalytic efficiency, a substrate in heterogeneous form (Figure 5) and therefore behaves as a true lipase according to Sarda and Desnuelle [18]. Its catalytic properties suggest that it is not simply a 'non-specific' lipase, with regard to the chemical nature of the substrate. Pancreatic cholesterol ester hydrolase, also identified as bile salt-dependent lipase and currently the best known monoester lipase, is able to hydrolyse long- and short-chain triacylglycerols, phospholipids, lysophospholipids, retinyl and cholesteryl esters as well as a non-physiological ester such as *p*-nitrophenyl acetate [31]. In contrast, MAGL has no activity towards either cholesteryl esters or tri- and di-acylglycerols. Noticeably, the enzyme hydrolyses lysoPC but is inactive towards PC, suggesting that two adjacent long-chain acyl groups in the substrate constitute a strong hindrance to catalysis.

MAGL has been detected in erythrocytes of several species [9], suggesting an ubiquitous need for such activity. Whereas whole-enzyme-dispersed fat [32], liver [33,34] or heart [35] cells display membrane-bound hydrolysing activities against tri-, di- and mono-acylglycerols, MAGL is the unique lipolytic activity in the non-nucleated erythrocyte. This suggests that MAGL is a distinct catalytic entity and a phylogenically and ontogenically conserved enzyme. It cannot be totally ruled out, however, that MAGL might be structurally related to triacylglycerol lipase(s). By analogy with rat hepatic lipase, it appears that limited proteolytic digestion of a triacylglycerol lipase can abolish its ability to act on a triacylglycerol substrate, whereas the activity towards monoacylglycerol remains essentially unaltered [36,37].

Our data indicate that MAGL is firmly anchored to the cell membrane, from which it is not releasable by heparin [10]. In the case of MAGL from rat adipose tissue, the activity was found before solubilization in lipid-protein particles of large size resulting from the aggregation with lipid of membrane fragments [38]. It might therefore be that this lipase is, like erythrocyte MAGL, a membrane-bound enzyme.

ApoA-I inhibits the erythrocyte enzyme, as was the case with MAGL from rat heart [39]. ApoA-I contains a high percentage of helical repeats which serve as lipid-associating domains [40] and may compete with the substrate with respect to the active site of the enzyme. Further study is needed to establish the mechanism of this competition with certainty. In any case, several lines of evidence suggest that the inhibitory effect of apoA-I on MAGL might have physiological relevance. These are: (a) inhibition is reversible upon addition of substrate; (b) the inhibitory concentration range is well below the mean value ($\approx 50 \mu$ M) at which total (essentially HDL-bound) apoA-I circulates in human plasma [41] (it may correspond to the minor apoA-I fraction which dissociates, essentially in a lipid-free form, from HDL particles and modulates, as such, lipoprotein metabolism [42]); (c) apoA-I is among the surface components of newly secreted HDLs that vary greatly in concentration when these particles are metabolized in blood [43].

The role of MAGL in erythrocytes has not yet been elucidated. The available data suggest that this enzyme could serve as a

scavenger to completely remove the amount of monoacylglycerol produced by the enzymic hydrolysis of triacylglycerol in blood. This action could be viewed as a protective one against monoacylglycerol, which has detergent properties potentially responsible for membrane damage and cytolysis [44].

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