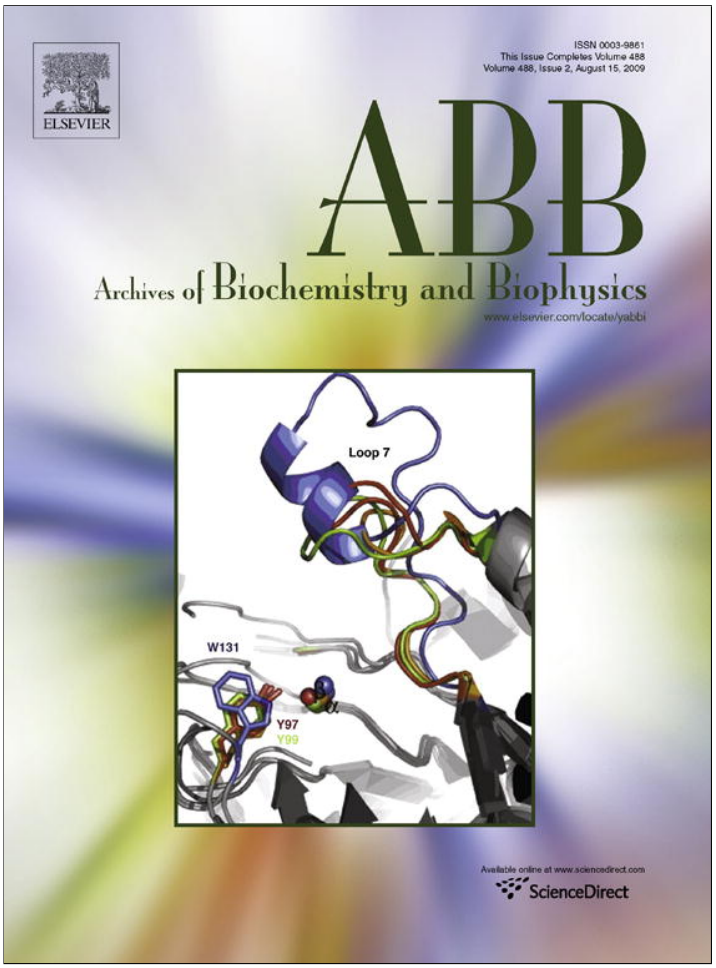

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Age-related changes in the metabolization of phosphatidic acid in rat cerebral cortex synaptosomes

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ABSTRACT

In this study, phosphatidic acid (PA) metabolization is found to generate diacylglycerol (DAG), monoacylglycerol (MAG) and glycerol by the sequential action of lipid phosphate phosphatase (LPP), diacylglycerol lipase (DAGL), and monoacylglycerol lipase (MAGL) in cerebral cortex (CC) synaptosomes. It is also demonstrated that PA is metabolized by phospholipases A (PLA)/lysophosphatidic acid phosphohydrolase (LPAPase) in synaptic endings. Age-related changes in the metabolization of PA have been observed in rat cerebral cortex synaptosomes in the presence of the alternative substrates for LPP, namely LPA, sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P). In addition, LPA and C1P up to concentrations of about 50 μM favor the metabolism in the direction of MAG and glycerol in aged and adult synaptosomes, respectively. At equimolecular concentrations with PA, LPA decreases DAG formation in adult and aged synaptosomes, whereas S1P decreases it and C1P increases it only in aged synaptosomes. Sphingosine (50 μM) or ceramide (100 μM) increase PA metabolism by the pathway that involves LPP/DAGL/MAGL action in aged membranes. Using RHC-80267, a DAGL inhibitor, we could observe that 50% and 33% of MAG are produced as a result of DAGL action in adult and aged synaptosomes, respectively. Taken together, our findings indicate that the ageing modifies the different enzymatic pathways involved in PA metabolization.

Introduction

Aging is accompanied by the impaired functioning of many systems, thus producing a gradual decline in the capacity of various cell types including neurons [1]. Lipids have broad information-carrying functions in the CNS. They form an integral part of membranes and provide messenger molecules that mediate communication among cells. Any modification in their metabolism and/or in the enzymatic activities that metabolize them may therefore affect cell function in physiological aging. Age-related changes in lipid content and in the enzymatic activities involved in lipid metabolism in different brain regions have been documented [2–10]. PA,[1] DAG and MAG are involved in signal transduction [11–13]. In eukaryote cells, these molecules have been associated with neurological disorders such as Alzheimer disease [14]. Previous research from our laboratory demonstrated that lipid phosphate phosphatase (LPP) hydrolyzes PA in synaptosomal cerebral cortex and that the generated DAG is metabolized to MAG by DAGL [6]. LPP also hydrolyzes LPA, S1P and C1P either on the cell surface or inside the cell so the degradation of lipid phosphates by LPP regulates cell signaling under physiological or pathological conditions. This cell signaling occurs via the attenuation of lipid phosphate signaling and the production of bioactive diacylglycerol, monoacylglycerol, sphingosine and ceramide [15]. The precise control of PA, DAG and MAG and the enzymes that metabolize them, LPP, DAGL and MAGL, are necessary for the correct functioning of these molecules in the signaling mechanism. The present study analyzes the formation of lipid mediators generated from [2-^{3}H]PA in synaptosomes prepared from the cerebral cortex (CC) of adult and aged rats. In all instances, [2-^{3}H]PA metabolization was analyzed in the presence of either LPA, S1P or C1P, which are alternative substrates for LPPs [16]. Our results demonstrate that aging modulates PA metabolization and indicate a different utilization of PA in the presence of LPA, S1P and C1P. Advances in our knowledge of lipid metabolism during aging will greatly contribute to a better understanding of the role of lipids in senescence. Imbalances in PA metabolism may be a key event in CNS injuries occurring during the aging process.

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[1] *Abbreviations used:* CC, cerebral cortex; C1P, ceramide 1-phosphate; DAG, diacylglycerol; DAGL, diacylglycerol lipase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; LPA, lysophosphatidic acid, LPP, lipid phosphate phosphatase, MAG, monoacylglycerol; MAGL, monoacylglycerol lipase, NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PLA, phospholipase A; RHC-80267, 1,6-bis(Cyclohexyloximinocarbonyl-amino) hexane; S1P, sphingosine 1-phosphate;TLC, thin-layer chromatography.

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Materials and methods

Male Wistar-strain rats were kept under constant environmental conditions and fed on a standard pellet diet. Animal handling was carried out in agreement with the standards stated in the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care. [2-^{3}H]Glycerol (200 mCi/mmol) and Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA, USA). The DAGL inhibitor, RHC-80267 (1,6-bis(Cyclohexyloximinocarbonyl-amino)) hexane was from Biomol, Horsholm, Denmark. Sphingosine 1-phosphate, ceramide 1-phosphate from bovine brain, oleoyl-L-α-lysophosphatidic acid, D-sphingosine, and non-hydroxy fatty acid ceramide from bovine brain were obtained from Sigma–Aldrich, St. Louis, MO, USA. All other chemicals used were of the highest purity available.

Preparation of synaptosomes

Four-month-old (adult) and 28-month-old (aged) rats were killed by decapitation and their CC was immediately dissected (2–4 min after decapitation).

CC homogenates were prepared in the following way: 20% (w/v) in 0.32 M sucrose, 1 mM EDTA, 5 mM buffer HEPES-Na (pH 7.4). The CC homogenate was centrifuged at 1300*g* for 7 min and the supernatant was carefully poured into another tube. The nuclear pellet was re-suspended with the isolation medium and subsequently spun at 1300*g* for 7 min. The combined supernatant was then centrifuged at 17000*g* for 10 min to obtain the crude mitochondrial pellet (CM). The CM was re-suspended with the isolation medium and layered onto a two-step gradient of 7.5–13% Ficoll solution prepared in the isolation medium. The sample layered onto Ficoll discontinuous gradient was centrifuged at 99000*g* for 30 min using an ultracentrifuge Beckman, model LS-50 with a swinging bucket rotor (SW28). The myelin fraction band is at the interphase between the isolation medium and 7.5% Ficoll medium, the synaptosomal fraction bands are at the interphase between 7.5% and 13% Ficoll medium, and the free mitochondrial fraction is the pellet below 13% Ficoll medium [17].

Preparation of radioactive 1,2-diacyl-sn-glycerol-3-phosphate

Radioactive PA was obtained from [2-^{3}H]glycerol-PC, synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described [18]. Lipids were extracted from the tissue as described elsewhere [19]. [2-^{3}H]glycerol-PC was isolated by mono-dimensional thin-layer chromatography (TLC), eluted [20] and hydrolyzed with phospholipase D [21]. The hydrolysis product [2-^{3}H]glycerol PA was then purified by one-dimensional TLC on silica gel H developed with chloroform/methanol/acetic acid/acetone/water (9:3:3:12:1.5, by vol). The substrate was eluted from silica gel with neutral solvents to avoid the formation of lysophosphatidic acid and was subsequently converted into free acid by washing firstly with an upper phase containing 0.1 M sulfuric acid and then with an upper phase containing water. Radioactivity and phosphorus content [22] were measured to determine specific radioactivity. [2-^{3}H]PA with a specific radioactivity of 0.1–0.2 μCi/μmol was obtained.

Enzymatic assays

For the determination of LPP activity, each assay contained 50 mM Tris–maleate buffer, pH 6.5, 1 mM EDTA plus EGTA, 4.2 mM NEM, and 100 μg synaptosomal protein in a volume of 200 μl. The reaction was started by the addition of 100 μM of [2-^{3}H]-phosphatidate/Triton X-100 mixed micelles in a constant 1:50 M ratio of lipid to Triton X-100. The effect of the alternative substrates on PA metabolization by LPP activity was evaluated using 100 μM [2-^{3}H]PA/Triton X-100 mixed micelles in the presence of unlabeled LPA, S1P or C1P (previously re-suspended in the buffer assay containing Triton X-100) [23]. Radiolabel PA was mixed with unlabeled substrates before drying and re-suspension. The aqueous microdispersions were sonicated by a sonication tip until clarity. Sphingosine and ceramide were solubilized in 0.1% dimethyl sulfoxide (DMSO) as vehicle; the respective controls were made with 0.1% DMSO alone. DAGL activity was determined by monitoring the formation rate of monoacyl [2-^{3}H]glycerol, using diacyl[2-^{3}H]glycerol generated from [2-^{3}H]glycerol-PA by LPP action as substrate [24]. [2-^{3}H]glycerol was determined from [2-^{3}H]glycerol-PA. Standard assays, pH conditions, protein concentration, time and the final volume of incubation were the same as those described for LPP. All the assays were conducted at 37 °C during 30 min. The enzymatic reactions were stopped by adding chloroform/methanol (2:1, by vol). Blanks were prepared identically except that the membrane fraction was boiled for 5 min before use.

Lipids were extracted with chloroform/methanol (2:1, by vol) and washed with a 0.2 volume of $CaCl_2$ (0.05%) [19]. Neutral lipids were separated by TLC on silica gel G [25] and developed with hexane/diethyl ether/acetic acid (35:65:1, by vol). To separate MAG from phospholipids, the chromatogram was rechromatographed up to the middle of the plate by using hexane/diethyl ether/acetic acid (20:80:2.3, by vol) as developing solvent. Once the chromatogram was developed, [2-^{3}H]glycerol-PA and phospholipids were retained at the spotting site. To separate [2-^{3}H]glycerol, the aqueous phase from a Folch extraction was chromatographed by TLC on silica gel G using chloroform/acetone/5 NH_3 (10:80:10) as developing solvent [26] and the chromatograms visualized as described [27]. The chromatograms were visualized by exposure to iodine vapors and scraped off for counting by liquid scintillation after the addition of 0.4 ml water and 10 ml 5% Omnifluor in toluene/Triton X-100 (4:1, by vol) using a WALLAC 1214 RACKBETA liquid scintillation counter.

Other methods

Protein and lipid phosphorus were determined according to Bradford [28] and Rouser [22], respectively.

Statistical analysis

All data are given as means ± SD and were evaluated using the Studenťs *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The statistical analysis was performed using GraphPad software, San Diego, CA, USA, www.graphpad.com. Statistical significance was set at $p < 0.05$.

Results

Metabolization of [^{3}H]-phosphatidic acid in the presence of LPA in CC synaptosomes from adult and aged rats

LPP hydrolyzes [^{3}H]-phosphatidic acid generating DAG which is, in turn, partially hydrolyzed by DAGL, thus yielding MAG. DAG generation and its partial degradation by DAGL occur immediately and have been extensively studied in our laboratory [18,24]. In our research, it was observed that MAG is hydrolyzed by MAGL to glycerol. DAGL and MAGL are coupled to LPP and these enzymes appear to work as an enzymatic complex. The assay conditions for the determination of LPPs (time, substrate and protein concentration) were usually used for this enzymatic assay [8,9,15,29,30]. The LPPs

activity in synaptosomal membranes shows a linear relation up to 1 mM PA. The activity was linear for 60 min with a protein concentration of 0.3 mg. A pH optimum was observed around 7 (data not shown). Fig. 1 shows the rate of DAG, MAG and glycerol formation in the presence of LPA in adult (circular symbols) and aged (square symbols) CC synaptosomes. DAG formation in the former decreases as of an LPA concentration of 30 μM, inhibition being 25% and 40% at 50 and 100 μM LPA, respectively. In aged CC synaptosomes DAG generation is diminished by 27% at 100 μM LPA (Fig. 1a). LPA has opposite effects on MAG formation in aged and adult synaptosomes, as it stimulates production at 25 μM LPA by 45% in the former while it inhibits it by 30% in the latter. No differences in MAG formation between adult and aged CC synaptosomes were observed at 100 μM LPA (Fig. 1b). At 50 μM LPA, glycerol formation was inhibited by 52% in adult synaptosomes but stimulated by 132% in aged synaptosomes. These opposite effects are not observed at 100 μM PA (Fig. 1c).

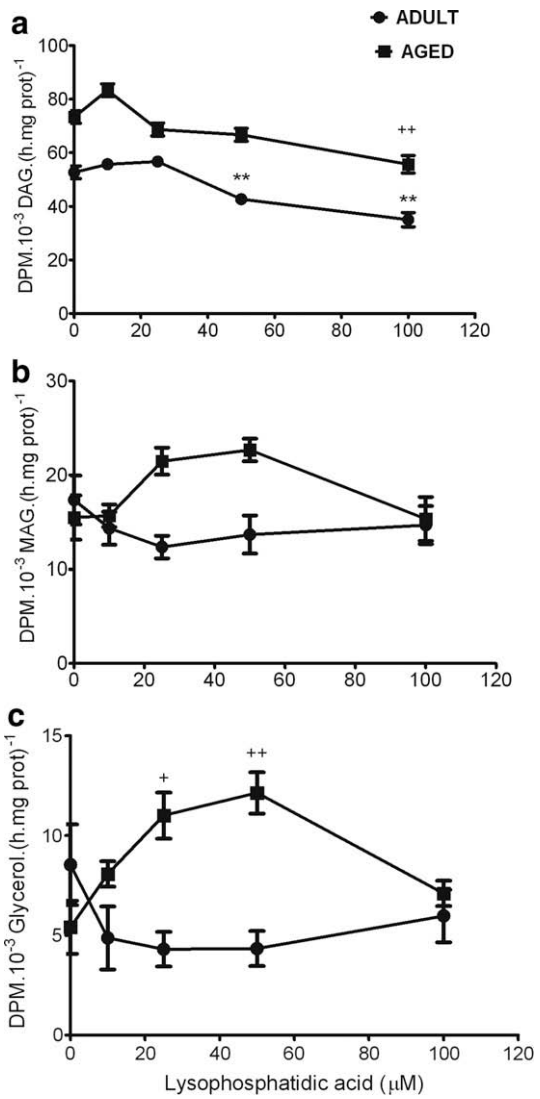

Fig. 1. Metabolization of [^{3}H]-phosphatidic acid in the presence of LPA in CC synaptosomes from adult and aged rats. DAG, MAG and Glycerol were determined using synaptosomes from 4-month-old (adult) and 28-month-old (aged) rats as enzyme source as specified in Materials and methods. The effects of LPA on PA hsydrolysis were evaluated using 100 μM [2-^{3}H]PA/Triton X-100 (1:50 M ratio) mixed micelles in the presence of LPA at the indicated concentrations. All assays were conducted at 37 °C for 30 min. The enzymatic reactions were stopped by adding chloroform/methanol (2:1, by vol). Incubation products were subsequently extracted and separated by gradient-thickness thin-layer chromatography and by TLC on silicagel G to separate [2-^{3}H]PA. The bands corresponding to PA, DAG, MAG and glycerol were scraped and quantitated by liquid scintillation spectroscopy. Results represent the mean ± SD of nine individual samples. Each sample comes from a different animal. $^{**}p < 0.005$ with respect to "0 adult"; $^{+}p < 0.05$, $^{++}p < 0.005$ with respect to "0 aged".

Metabolization of [^{3}H]-phosphatidic acid in the presence of C1P in CC synaptosomes from adult and aged rats

In adult CC synaptosomes DAG formation is stimulated in the presence of C1P, reaching a 38% stimulus at 50 μM. Equimolecular concentrations of C1P and PA generate a DAG level similar to that observed with PA alone (Fig. 2a). MAG and glycerol generation in the presence of C1P at concentrations reaching up to 50 μM are lower in aged than in adult CC synaptosomes. Equimolecular concentrations of C1P and PA generate a higher production of MAG and glycerol in aged with respect to adult CC synaptosomes

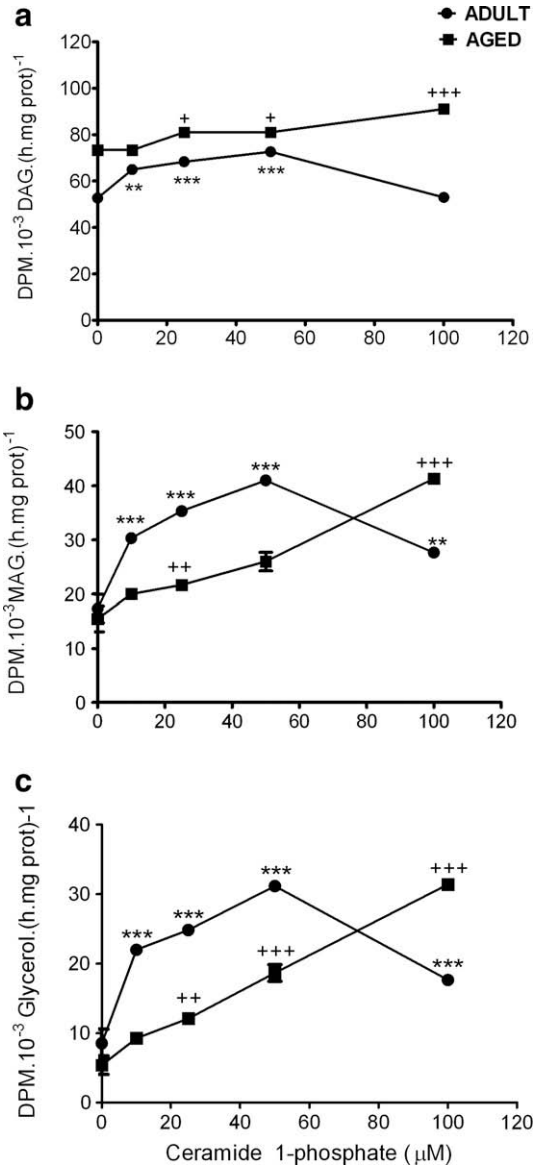

Fig. 2. Metabolization of [^{3}H]-phosphatidic acid in the presence of C1P in CC synaptosomes from adult and aged rats. The effects of C1P on PA hydrolysis were evaluated using 100 μM [2-^{3}H]PA/Triton X-100 (1:50 M ratio) mixed micelles in the presence of C1P at the indicated concentrations. Other considerations are specified in Fig. 1. Results represent the mean ± SD of nine individual samples. Each sample comes from a different animal. $^{**}p < 0.05$, $^{***}p < 0.005$ with respect to "0 adult"; $^{+}p < 0.05$, $^{++}p < 0.005$;$^{+++}p < 0.0005$ with respect to "0 aged".

(Fig. 2b and c). In adult synaptosomes, MAG and glycerol formation are stimulated by 141% and 244%, respectively, at 50 μM C1P (Fig. 2b and c, circular symbols) and diminish at higher C1P concentrations, reaching values similar to those observed in the absence of C1P (Fig. 2b and c, circular symbols). In aged synaptosomes MAG and glycerol increase as a function of C1P concentration. The formation of these products are 1.6- and 5.2- fold higher, respectively, at 100 μM PA plus 100 μM C1P than at 100 μM PA alone (Fig. 2b and c, square symbols).

Metabolization of [^{3}H]-phosphatidic acid in the presence of S1P in CC synaptosomes from adult and aged rats

DAG production is inhibited as a function of S1P concentration in aged CC synaptosomes, the maximal inhibition (21%) being produced at 100 μM S1P. No changes in DAG production by S1P are observed in adult CC synaptosomes (Fig. 3a). MAG formation in adult and aged CC synaptosomes underwent no changes in the presence of S1P (Fig. 3b). Glycerol production is higher in adult than in aged membranes in the presence of increased S1P concentrations. At 50 μM S1P, the stimulus for glycerol production is 76% and 120% in adult and aged CC synaptosomes, respectively. At S1P concentrations higher than 50 μM, glycerol generation decreases in adult membranes and continued to increase in aged synaptosomes (Fig. 3c).

Metabolization of [^{3}H]-phosphatidic acid in the presence of sphingosine and ceramide in CC synaptosomes from adult and aged rats

In order to assess whether or not the effect of S1P and C1P on DAG, MAG, and glycerol production is due to sphingosine and ceramide generated by LPP on S1P or C1P, we evaluated the effect of sphingosine and ceramide on these products in CC synaptosomes from adult and aged rats (Fig. 4a–f). To this end, sphingosine and ceramide are added using dimethylsulfoxide as a vehicle in a concentration that produced no modifications in enzymatic activity. Sphingosine and ceramide produce no changes in DAG production in adult membranes. However, in aged membranes, DAG production is stimulated by 22% and by 19% at 50 μM sphingosine and at 100 μM ceramide, respectively (Fig. 4a and b). Sphingosine produces no changes in MAG production in adult synaptosomes but stimulates it by 69% in aged CC synaptosomes at 50 μM (Fig. 4c). Ceramide, on the other hand, inhibits MAG generation by 29% at all concentrations assayed in adult membranes and stimulates its formation in aged membranes (Fig. 4d). The highest stimulus (81%) are observed at a 100 μM ceramide concentration (Fig. 4d). In the presence of ceramide, glycerol formation is inhibited by 44% in adult membranes (Fig. 4f) while it is stimulated in aged membranes, the stimulus being maximal (2.6 times) at 100 μM ceramide (Fig. 4f). A similar level of stimulus in the same membrane preparations is observed in the presence of 50 μM sphingosine (Fig. 4e).

[^{3}H]-monoacylglycerol and [^{3}H]-glycerol formation in the presence of DAGL inhibitor, RHC-80267, in CC synaptosomes from adult and aged rats

In order to assess the contribution of the DAGL/MAGL pathway to MAG and glycerol, we determined the formation of these products in the presence of RHC-80267, a DAGL inhibitor. Synaptosomes are firstly pre-incubated at a final concentration of 30 μM of this inhibitor in DMSO (0.1%) during 5 min. and they are subsequently incubated in the presence of [^{3}H]PA plus 100 μM LPA, C1P or S1P. RHC-80267 inhibits MAG formation by 47% in adult synaptosomes and by 20% in aged synaptosomes (Fig. 5a). The presence

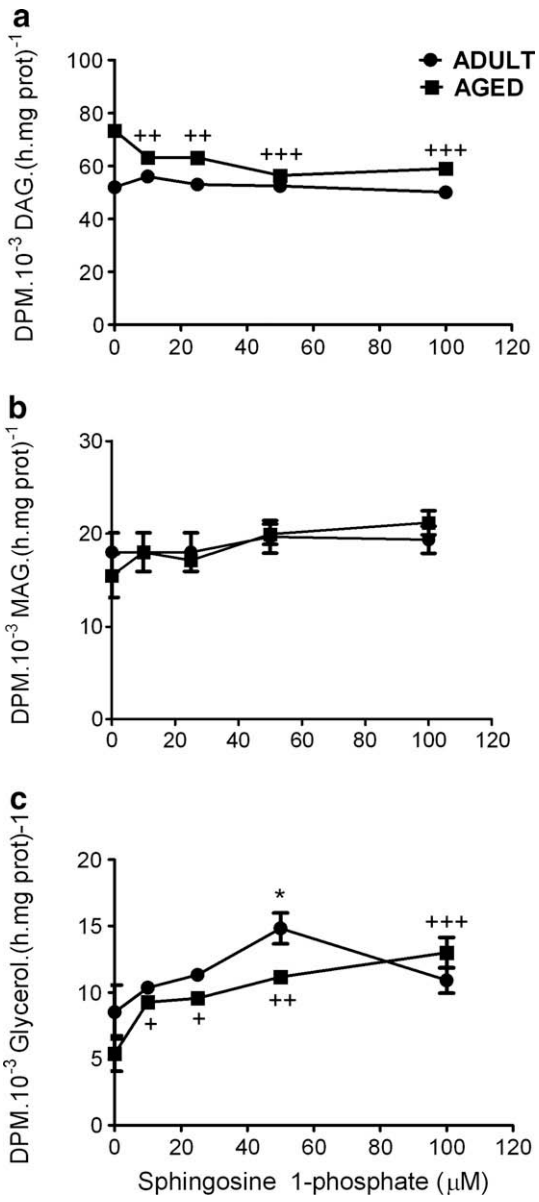

Fig. 3. Metabolization of [^{3}H]-phosphatidic acid in the presence of S1P in CC synaptosomes from adult and aged rats. The effects of S1P on PA hydrolysis were evaluated using 100 μM [2-^{3}H]PA/Triton X-100 (1:50 M ratio) mixed micelles in the presence of S1P at the indicated concentrations. Other considerations are specified in Fig. 1. Results represent the mean ± SD of nine individual samples. Each sample comes from a different animal. $^{*}p < 0.05$ with respect to "0 adult"; $^{+}p < 0.05$, $^{++}p < 0.005$, $^{+++}p < 0.0005$ with respect to "0 aged".

of LPA, C1P or S1P do not modify the effect of RHC on adult synaptosomes whereas in aged synaptosomes, S1P produces an additional inhibitory effect of 39% over and above the RHC inhibitory effect (Fig. 5a). RHC do not modify glycerol production in adult synaptosomes. However, in the presence of both RHC and LPA, C1P or S1P markedly diminishes (70%) glycerol production in adult membranes. In aged synaptosomes RHC inhibits glycerol production by 50% (Fig. 5b), its inhibitory effect being restored by LPA and C1P (Fig. 5b).

Metabolization of [^{3}H]-diacylglycerol in the presence of LPA, C1P and S1P in CC synaptosomes from adult and aged rats

Fig. 6 shows the transformation of [^{3}H]-DAG into [^{3}H]-MAG plus [^{3}H]-glycerol in the presence of LPA, C1P and S1P in synaptosomas

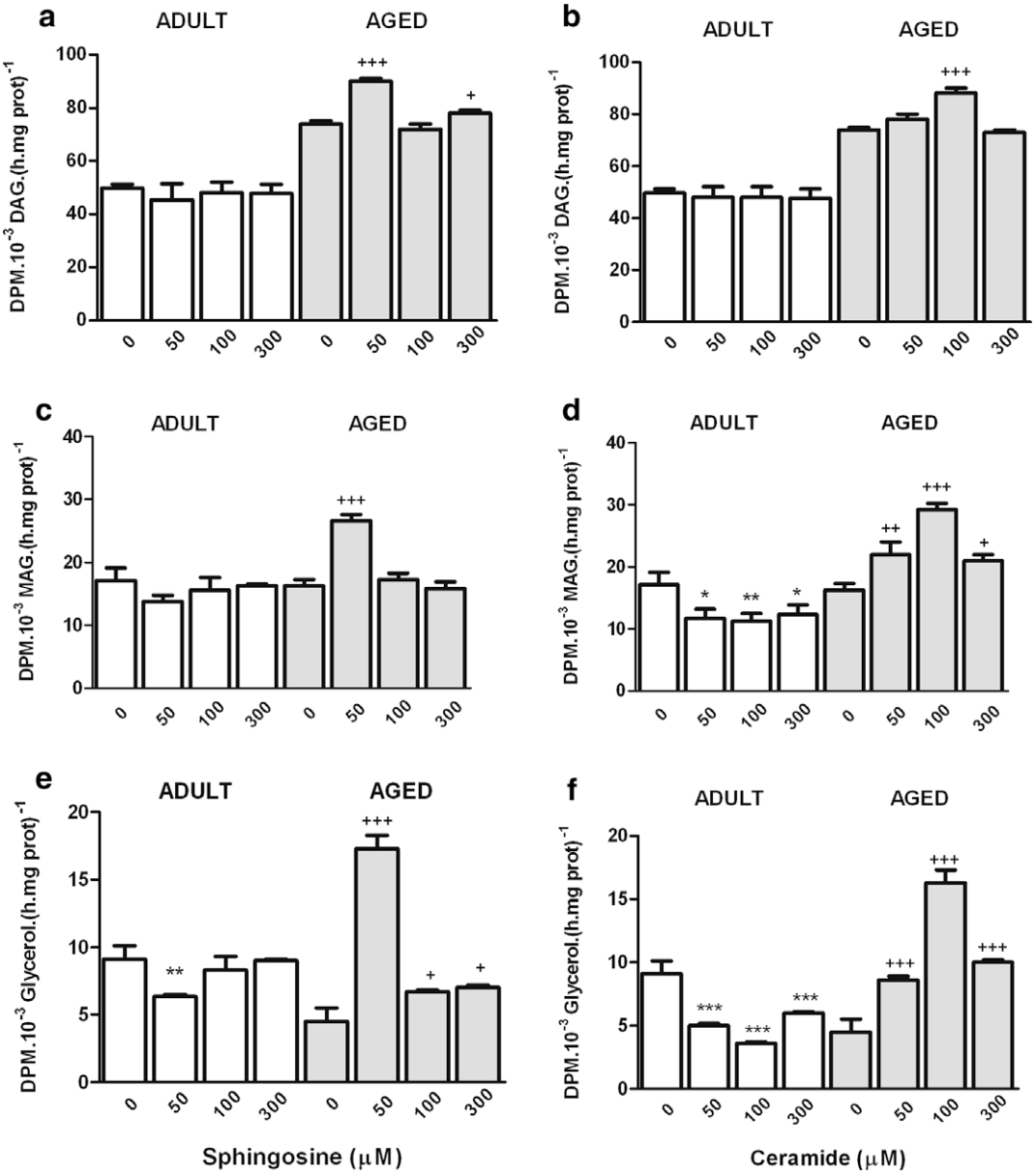

Fig. 4. Metabolization of [^{3}H]-phosphatidic acid in the presence of sphingosine and ceramide in CC synaptosomes from adult and aged rats. Adult and aged synaptosomes were pre-incubated with sphingosine (50, 100 and 300 μM) or ceramide (50, 100 and 300 μM) for 10 min. [2-^{3}H]PA (100 μM)/Triton X-100 mixed micelles were subsequently added and the products from PA hydrolysis were determined as specified in Fig. 1. Sphingosine and ceramide were added in DMSO 0.1%. Results represent the mean ± SD of nine individual samples. Each sample comes from a different animal. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ with respect to "0 adult"; $^{+}p < 0.05$, $^{++}p < 0.005$, $^{+++}p < 0.0005$ with respect to "0 aged".

pre-incubated with RHC-80267, allowing us to better visualize the differences in DAG metabolization between adult and aged CC synaptosomes. In the former, 21% of DAG is transformed into MAG and glycerol while in the latter only 8% (Fig. 6a and b, "0" LPA) is transformed into MAG and Glycerol. In the presence of LPA the greatest change occurred at 100 μM concentration where DAG transformation into MAG and glycerol is 35% in adult membranes as against 12% in aged membranes (Fig. 6a and b). In the presence of C1P, DAG transformation into MAG and glycerol ranges between 23% and 32% in adult membranes. In aged membranes the transformation is markedly lower, ranging between 3% and 10% (Fig. 6c and d). No significant differences were observed in DAG metabolization between adult and aged membranes in the presence of S1P (Fig. 6e and f). The metabolization of [^{3}H]-diacylglycerol in the presence of different concentrations of ceramide and sphingosine was also evaluated. In the presence of ceramide the greatest change occurres at 300 μM concentration, at which 18% of DAG is transformed into MAG and glycerol in adult membranes and 9.5% in aged membranes (data not shown). At 300 μM sphingosine concentration a lower amount of DAG is transformed into MAG and glycerol in aged (6%) than in adult (16%) synaptosomes (data not shown).

Discussion

PA [31], LPA [32], DAG [33], MAG [34], sphingosine, ceramide and their phosphorylated products [35,36] have been defined as key inter- and intracellular lipid signaling molecules. All of them and their related enzymes participate in the regulation of many functions of the central nervous system (CNS) [37,38].

Phosphatidic acid metabolization in CC synaptosomes involves two possible pathways: (i) the sequential action of LPPs, DAGL

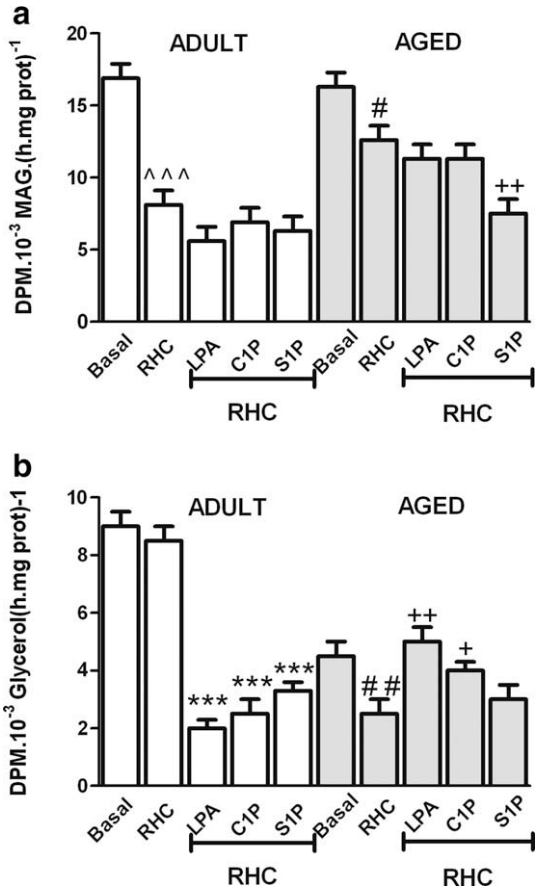

Fig. 5. [^{3}H]-monoacylglycerol and [^{3}H]-glycerol formation in the presence of DAGL inhibitor, RHC-80267, in CC synaptosomes from adult and aged rats. Adult and aged synaptosomes were pre-incubated at a final concentration of 30 μM of RHC-80267 in DMSO (0.1%) during 5 min. They were subsequently incubated in the presence of [^{3}H]PA plus 100 μM of LPA, C1P or S1P as specified in Fig. 1. Results represent the mean ± SD of nine individual samples. Each sample comes from a different animal. $^{\wedge\wedge\wedge}p < 0.0005$ with respect to basal; $^{*}p < 0.05$, $^{***}p < 0.0005$ with respect to RHC; $^{\#}p < 0.05$, $^{\#\#}p < 0.005$ with respect to basal; $^{+}p < 0.05$, $^{++}p < 0.005$ with respect to RHC.

and MAGL [8,9] generating DAG, MAG and glycerol, respectively; and (ii) the action of phospholipase A, LPA phosphohydrolase, and MAGL, generating LPA, MAG and glycerol, respectively. The fact that the product of an enzymatic reaction could be used as a substrate for the subsequent enzyme is indicative that these enzymes are acting as an enzymatic complex. A sequential action of LPP and DAG lipase producing DAG and MAG, respectively, was observed in rat CC synaptosomes [9]. It has been reported that endogenously produced DAG by LPP action is further hydrolyzed to MAG and glycerol [24,39]. Furthermore it has been demonstrated that [^{3}H]-diacylglycerol and [^{3}H]-diacylglycerol co-emulsified with PC are poor substrates for DAG lipase [40]. The extremely hydrophobic nature of DAG molecules induces their localization inside vesicles, from where they are inaccessible to the enzyme [41]. The significant hydrophobicity of bioactive lipids also restricts their action to their site of production [42]. Knowledge of these properties and of the hydrophobic nature of lipid metabolism enzymes is necessary in order to understand how to determine enzymatic activity when lipids are used as substrate. Taking the foregoing into account, the endogenous generation of DAG/MAG or LPA/MAG by LPP/DAGL/MAGL or PLA/LPAPase action provides substrates with a suitable physical state for the expression of enzymatic activity, confirming the adequacy of our assay conditions for the determination of LPPs [8,9,15,29,30].

LPP are the enzymes responsible for the dephosphorylation of PA and the other phosphorylated substrates such as LPA, S1P and C1P. Four mammalian LPP isoforms, namely LPP1 (PAP2a), LPP1a, LPP2 (PAP2c) and LPP3 (PAP2b) have been cloned and each of them has been detected at the plasma membrane [43]. Intracellular pools of LPPs have also been identified [44]. Phosphatidic acid represents 1.7% of total phospholipid in adult synaptosomal membranes and this percentage was unchanged in aged membranes. Lysophosphatidic acid (LPA), ceramide 1-phosphate (C1P), sphingosine 1-phosphate (S1P), ceramide and sphyngosine are in negligible concentrations in synaptosomes (Zulian, Ilincheta de Boschero and Giusto, unpublished observations). In general, tracer lipids such as S1P are present in low nanomolar concentrations in cells but in high nanomolar concentrations in serum [45]. Ceramide often constitutes 0.1–1% of total membrane lipids and sphingosine is often detected in concentrations that are less than an order of magnitude lower than those of ceramide [42]. LPA on the other hand can be detected in various fluids including cerebrospinal fluid [46] and is abundant in whole rat brain [47].

A marked competitive effect between PA and LPA by LPPs is observed in adult and aged synaptosomes and between PA and S1P only in aged synaptosomes. LPP1, LPP2 and LPP3 show the maximal catalytic efficiency for LPA, PA and S1P, respectively [44,48]. The degree of competitiveness observed between PA/LPA and PA/S1P suggests that LPP1 is the most active isoform in adult synaptosomes while LPP1 and LPP3 isoforms are most active in aged synaptosomes. As a result, LPP1 in adult synaptosomes and LPP1/LPP3 in aged synaptosomes could either restrict the effects of LPA or S1P to their respective receptors and/or participate in their uptake, thus exerting their influence on synaptosomal functions [1]. DAG generation is quantitatively different from that observed in MAG and glycerol formation in the presence of LPA, C1P and S1P (Figs. 1–3), thus suggesting that these effects on MAG and glycerol are related to DAGL/MAGL or PLA/LPA phospholipase/MAGL themselves and that they are not a consequence of different degrees of DAG availability.

Under our assay conditions, it is observed that MAG and glycerol from PA involve two possible routes: (i) the sequential action of LPP/DAGL/MAGL, and (ii) PLA/LPA phosphohydrolase/MAGL [8]. The use of RHC-80267, a specific DAGL inhibitor [49], enabled us to determine the different pathways involved in MAG and glycerol production. In adult CC synaptosomes, the two pathways contribute equally to MAG generation whereas in aged CC synaptosomes the second pathway is predominant.

Though MAG availability is similar in adult and aged synaptosomes, its metabolization to glycerol is lower in aged membranes, which would appear to indicate that ageing diminishes the catalytic efficiency of MAGL for its substrate. When the DAGL pathway is inhibited, glycerol production decreases only in aged synaptosomes, thus corroborating the above-mentioned hypothesis. It was also observed that under lower availability of the substrate, MAGL becomes more sensitive to the presence of LPA, C1P and S1P, reducing the activity in adult synaptosomes and increasing it in aged synaptosomes. The analysis of the metabolization of DAG to MAG plus glycerol by DAGL/MAGL (Fig. 6) indicates that ageing diminishes this metabolization, whereas in the presence of LPA, C1P or S1P, metabolization underwent no changes. Based on these results and on those shown in Figs. 1–3 it can be hypothesized that LPA, C1P and S1P modulate MAG and glycerol formation by the pathway involving PLA/LPAPase/MAGL. Two DAGL isoforms, α and β, have been cloned and their expression in adult animals becomes restricted to synaptic fields [40]. The product of the DAGL-catalyzed reaction 2-AG functions as an endogenous CB-1 receptor agonist [50]. Our findings therefore seem to be indicative of a possible role of the endocannabinoid system in the aging process [51].

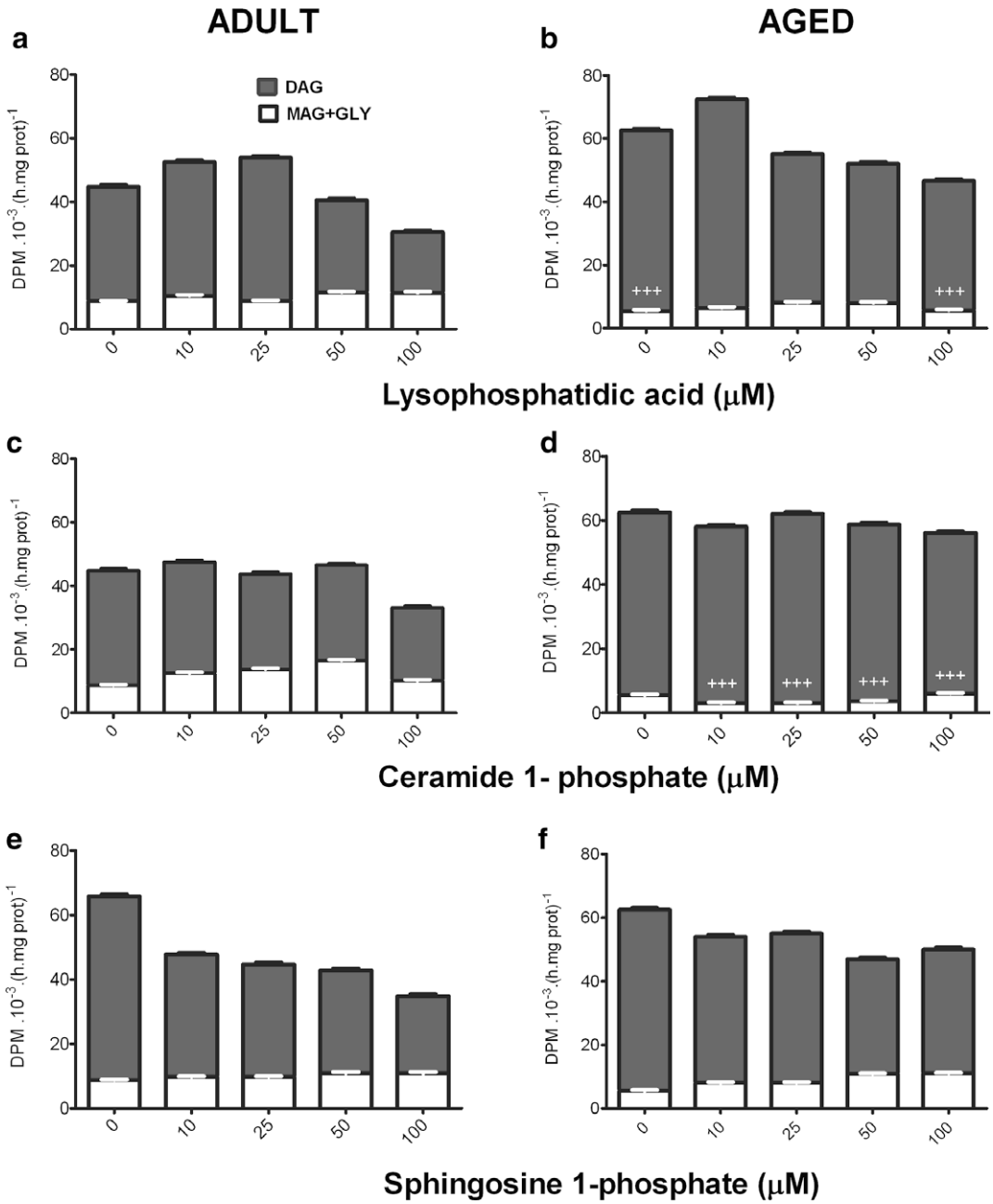

Fig. 6. Metabolization of [^{3}H]-diacylglycerol in the presence of LPA, C1P and S1P in CC synaptosomes from adult and aged rats. The degree of DAG metabolization is expressed as the sum of MAG plus glycerol, as a function of LPA, C1P and S1P concentrations. Membranes were pre-incubated with RHC-80267 and incubated with 100 μM of [2-^{3}H]PA/Triton X-100 mixed micelle or with [2-^{3}H]PA in the presence of different concentrations of LPA, C1P or S1P as specified in Figs. 5 and 1, respectively. Results represent the mean ± SD of nine individual samples. Each sample comes from a different animal. $^{+++}p < 0.0001$ with respect to adult.

In view of the above, and in order to assess whether or not the effect of S1P and C1P on DAG, MAG, and glycerol production is due to the sphingosine and ceramide generated by LPP on S1P or C1P, we evaluated the effect of the two lipids on these products. Our results demonstrate differential effects of S1P and sphingosine on glycerol generation from adult synaptosomes and on DAG and MAG generated from aged membranes. In addition, C1P and ceramide exert differential effects on DAG/MAG/glycerol in adult membranes. The different effects of S1P and C1P on PA metabolism from those of sphingosine and ceramide suggest that these lipids modulate the enzymatic activities that metabolize PA by independent mechanisms. LPA and S1P exert their effects mainly through related membrane receptors [52,53], whereas PA, DAG, C1P and ceramide do so by the recruitment of cytosolic proteins [54].

Summarising, our study evaluates the ageing effect on PA metabolization of the sequential action of LPPs/DAGL/MAGL and of the pathway involving PLA/LPAPase. These pathways are analyzed in the absence and presence of LPA, S1P and C1P, all of which are substrates for LPPs. Analyzing DAG production using PA and LPA, S1P or C1P at equimolecular concentrations brings out the competitive effect between PA and the alternative substrates. However, the effects of the alternative substrates at concentrations other than equimolecular with PA on MAG and glycerol production may be due not only to the different DAG availability but also to the effect of the alternative substrate itself on the enzymes that

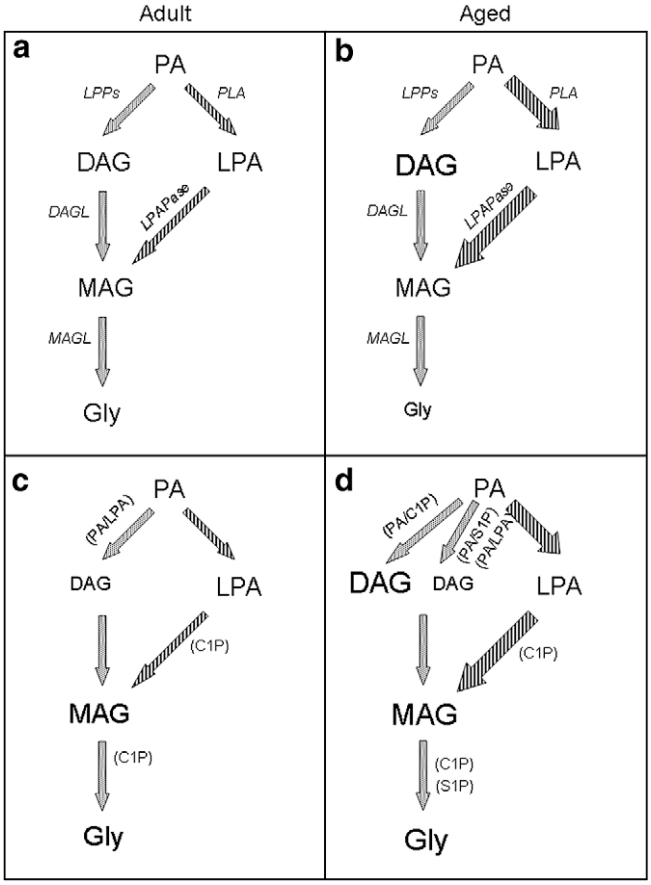

Fig. 7. Age-related changes in the Phosphatidic acid metabolization. Basal PA metabolism in adult (a) and aged (b) cerebral cortex synaptosomes. PA metabolism in the presence of LPA, S1P and C1P in adult (c) and aged (d) cerebral cortex synaptosomes. The enzyme name is in italic script. The relative size of the letters used to write the products indicates their level of production. The relative size of arrows indicates the predominance of PA metabolism pathway. LPPs, lipid phosphate phosphatases; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase; PLA, Phospholipase A; LPAPase, lysophosphatidic acid phosphohydrolase.

subsequently metabolize DAG or generate MAG and glycerol. Fig. 7 summarizes these findings.

Recent advances in neuroscience have demonstrated that lipids have extensive information-carrying functions in the central nervous system both as ligands and as substrates for proteins [55]. PA and PA-lipid derivates mediate a diverse range of biological processes in CNS. PA and DAG alter the properties of the membrane, control traffic, and serve as messenger molecules mediating communication between cells [56]. MAG functions as an endogenous CB-1 receptor agonist [50]. An imbalance of PA, DAG or MAG may induce alterations both in neurotransmission and in the neuronal dysfunction observed in senescense and in neurological disorders such as Parkinson and Alzheimer's diseases, demonstrating the crucial role of lipids in tissue pathophysiology and cell signaling [57,58]. Signalling lipid-generating enzymes from PA may thus provide pharmacologically potential targets for the treatment of aging and neurological dysfunctions. Further research in this novel area will therefore lead to a better understanding of the mechanisms controlling PA metabolism in CNS and will provide potential targets for diagnostic or therapeutic strategies for the treatment of aging and neurological diseases.

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