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Differential modulation of phospholipase D and phosphatidate phosphohydrolase during aging in rat cerebral cortex synaptosomes

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Abstract

Phosphatidylcholine (PC) hydrolysis generates two important second messengers: phosphatidic acid (PA) and diacylglycerol (DAG). Phospholipase D (PLD) and phosphatidate phosphohydrolase (PAPase) are involved in their generation and therefore are key enzymes in signal transduction. Specific isoforms of these enzymes are activated by receptor occupancy in brain. Phosphatidylinositol 4,5-bisphosphate-dependent PLD (PIP_2-PLD) and *N*-ethylmaleimide-insensitive PAPase (PAP2) have been suggested to act in series to generate the biologically active lipids PA and DAG. In the present study we examine age-induced changes mainly in PIP_2-PLD and PAP2 activities in cerebrocortical synaptosomes from adult (4 months) and aged (28 months) Wistar rats. Aging increases the activity of both enzymes. Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and cytosol (from cerebral cortex) stimulate PLD activity in adult and senescent synaptosomal membranes, the effect being greater in the latter. Under the same experimental conditions PAP2 activity was stimulated in aged membranes whereas in adult membranes GTPγS had no effect and cytosol showed a slight inhibitory effect. Diacylglycerol lipase (DGL) activity differs from that of PAP2 in aged rats and it was 21% inhibited with respect to synaptosomal membranes from adult rats. Increased sinaptosomal PLD activity in aged membranes appears to be independent of G protein regulation, whereas PAP2 activity is differentially regulated by GTPγS in aged membranes with respect to adult membranes. Our results suggest that under G-protein activation conditions, DAG production by the serial activation of PLD and PAP2 activities is increased in synaptosomal membranes from aged brain. The present paper demonstrates that PA generation (PLD activity) and degradation (PAPase activity) are differentially modulated during the aging process.

Keywords: Aging; Phospholipase D; Phosphatidate phosphohydrolase; Phosphatidylcholine; Phosphatidic acid; Diacylglycerol

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Abbreviations: CC, cerebral cortex; CM, crude mitochondrial pellet; CNS, central nervous system; Cyt., cytosolic fraction from cerebral cortex; DAG, diacylglycerol; DGL, diacylglyceride lipase; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; MAG, monoacylglycerol; NEM, *N*-ethylmaleimide; PAPase, phosphatidate phosphohydrolase; PAP2, *N*-ethylmaleimide-insensitive PAPase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP_2, phosphatidylinositol(4,5)bisphosphate; PLC-PIP_2, phospholipase C phosphoinositide specific; PIP_2-PLD, phosphatidylinositol (4,5) bisphosphate dependent phospholipase D; TLC, thin layer chromatography; TRIS, tris[hydroxymethyl]aminomethane

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1. Introduction

Phospholipids not only regulate plasma membrane properties but may also be hydrolyzed by certain phospholipases, as effectors of agonist-receptor occupancy, generating second messenger molecules that participate in different signal transduction mechanisms (Divecha and Irvine, 1995).

The generation of diacylglycerol (DAG) and inositoltriphosphate (IP_3) by phosphoinositide-specific phospholipase C is the best characterized signaling pathway. DAG can also be produced by the sequential action of phospholipase D (PLD) and phosphatidate phosphohydrolase (PAPase) (Singer et al., 1997). PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline, and PA can be hydrolyzed by PAPase to generate DAG. Molecular characterization indicates that there are at least three distinct isoforms of PLD in central nervous system (CNS), an oleic acid-dependent isoform and two phosphatidylinositolbisphosphate (PIP_2)-modulated isoforms (PLD1 and PLD2, respectively) (Kobayashi and Kanfer, 1987; Massemburg et al., 1994; Liscovitch et al., 1999). The regulation of oleate-dependent PLD remains unclear, but the modulation mechanism of PLD1 and PLD2 by small G proteins and other cytosolic factors is well known (Liscovitch et al., 1999).

Two isoforms of PAPase have been described in mammalian cells, one Mg^{2+}-dependent and *N*-ethylmaleimide-sensitive (PAP1) and the other Mg^{2+}-independent and *N*-ethylmaleimide-insensitive (PAP2) (Jamal et al., 1991; Kanoh et al., 1992; Brindley and Waggoner, 1996). Three isoforms of PAP2 (PAP2a, PAP2b, PAP2c) have recently been cloned that hydrolyze other lipid phosphates besides PA (Waggoner et al., 1996; Kai et al., 1996; Roberts et al., 1998; Hooks et al., 1998). A functional role for PAP2b was demonstrated in the metabolism of PA derived from PLD activation, and DAG generation from this pathway was shown to participate in different processes such as vesicular trafficking and signal transduction (Sciorra and Morris, 1999).

We demonstrated that enzyme activities involved in alternative pathways for phospholipid synthesis, such as PLD, are modified by the effect of aging in different brain areas (Ilincheta de Boschero et al., 2000). PLC and PLA_2 enzymes bound to aged synaptosomal membranes show higher activity than in adult membranes (Strosznajder et al., 1994). The formation and accumulation of very potent lipid messengers such as DAG, lysophospholipids and arachidonic acid in neurotransmission processes may play a role in the deterioration of neuronal function during aging.

It has been suggested that the formation of PA by PLD, its conversion to DAG by PAPase activity, and the metabolization of DAG by diacylglyceride lipase (DGL) comprise an important signaling cascade (Freeman, 2000). We have found that aging differentially modulates the PAPase and DGL activities in microsomal and cytosolic fractions from brain cerebral cortex (CC) (Pasquaré et al., 2001). For this reason we decided to study the effect of aging on PAPase and DGL activities under the same regulation conditions assayed for PIP_2-dependent PLD in synaptosomal membranes. It is well known that PIP_2-dependent PLD is activated in the presence of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (a known G-protein activator) and cytosolic factors (comprising small G proteins) (Liscovitch et al., 1999; Exton, 1997). We determined the effect of GTPγS and/or cytosolic proteins on PLD and PAP2 activities assayed independently using [^{3}H]PC and [^{3}H]PA as substrates, in sinaptosomal membranes from adult and aged rat CC.

2. Materials and methods

Wistar-strain male rats were kept under constant environmental conditions and fed on a standard pellet diet.

[2-^{3}H]Glycerol (200 mCi/mmol), PC, L-α-dipalmitoyl-[choline-methyl-^{3}H], (43 Ci/mmol) and omnifluor were obtained from New England Nuclear–Dupont, Boston, MA. Dithiothreitol (DTT), guanosine 5′-*O*-(3-thiotriphosphate (GTPγS), were obtained from Sigma–Aldrich, St Louis, MO, USA. All other chemicals were of the highest purity available.

2.1. Preparation of subcellular fractions

Homogenates were prepared from the CC of 4- (adult) and 28-month-old (aged) rats. Rats were killed by decapitation and all brain areas were 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 (pH 7.4). The CC homogenate was centrifuged at 1300*g* for 3 min, and the supernatant was carefully poured into another tube. The nuclear pellet was resuspended with the isolation medium and was then spun at 1300*g* for 3 min. The combined supernatant was subsequently centrifuged at 17,000*g* for 10 min to obtain the crude mitochondrial pellet (CM). The CM was resuspended with the isolation medium and layered onto a two-step gradient of 7.5–13% Ficoll solution prepared in the isolation medium (Lai and Clark, 1979). The sample layered onto Ficoll discontinuous gradient was centrifuged at 99,000*g* for 30 min in a swinging bucket rotor. The myelin fraction band is at the interface between the isolation medium and the 7.5% Ficoll medium, the synaptosomal fraction (syn) bands at the interface between the 7.5% and the 13% Ficoll media, and the free mitochondrial fraction is the pellet below the 13% Ficoll medium (Lai and Clark, 1979).

2.2. Determination of phospholipase D activity

For determination of PIP_2-dependent PLD activity, PC hydrolysis was determined using an assay described by Brown et al. (1993) with slight modifications. Briefly, 50 μl of mixed lipid vesicles (PE/PIP_2/dipalmitoylphosphatidylcholine (DPPC), molar ratio 16:1.4:1) with [choline-methyl-^{3}H]DPPC to yield 200,000 cpm per assay was added to 100 μl of synaptosomal membranes (200 μg of protein) in a total volume of 200 μl containing 50 mM Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl, 2.5 mM $MgCl_2$. Lipid vesicles were prepared by mixing the lipids in chloroform, drying under a stream of nitrogen, and resuspending by sonication for 10 min at 25 °C in buffer solution without divalent cations. The final concentration of DPPC was 8.5 μM (Brown et al., 1993). The reaction was incubated at 37 °C for 30 min and stopped by the addition of 1 ml of chloroform/methanol/concentrated HCl, 50:50:0.3 (by vol.) and 0.35 ml of 1 M HCl/5 mM EGTA. The aqueous phase was obtained after centrifugation for 5 min at 3000 rpm and the choline was separated from the other water soluble products using thin layer chromatography (TLC) on silicagel G plates and a mobile phase made up of methanol/NaCl 0.5%/NH_3 50:50:1 (by vol.) (Yavin, 1976). Choline was scraped off the plate and quantified by liquid scintillation spectroscopy.

For the determination of oleate-dependent PLD activity the assay was performed using a lipid microdispertion of DPPC 200,000 cpm containing 10 nmol of DPPC in a buffer containing 50 mM Hepes pH 7.5, 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl and 4 mM sodium oleate. The reaction was carried out as described for PIP_2-dependent PLD activity. Choline quantification was performed as described earlier.

2.3. Determination of phosphatidate phosphohydrolase activities

PAPase activities were differentiated on the basis of NEM-sensitivity (Jamal et al., 1991; Hooks et al., 1998). For the determination of NEM-insensitive PAPase activity, each assay contained 50 mM Tris–maleate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA plus 1 mM EGTA, 4.2 mM NEM and 100 μg of synaptosomal membrane protein in a volume of 0.2 ml. The reaction was started by adding 0.6 mM of [2-^{3}H]phosphatidate.

NEM-sensitive PAPase activity was determined in an assay containing 50 mM Tris–maleate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA and 1 mM EGTA, 0.2 mM $MgCl_2$ and 100 μg of synaptosomal membrane protein in a volume of 0.2 ml. The reaction was started by adding 0.6 mM of [2-^{3}H]phosphatidate plus 0.4 mM PC. Parallel incubations were carried out after pre-incubating the enzyme for 10 min with 4.2 mM NEM. NEM-sensitive PAPase was calculated as the difference between the two types of assay.

All the assays for the determination of PAPase activities were conducted at 37 °C for 30 min. The enzyme assay was stopped by adding chloroform/methanol (2:1, by vol.). Blanks were prepared identically, except that membranes were boiled for 5 min before being used.

The PAPase activity products 1,2-diacyl-2-[^{3}H]glycerol and [^{3}H]monoacylglycerol were isolated and measured as described later. PAPase activity was expressed as the sum of nmol of ([^{3}H]diacylglycerol and [^{3}H]monoacylglycerol) $\times\ h^{-1} \times$ (mg of protein)$^{-1}$.

2.4. Determination of diacylglyceride lipase activity

DGL activity was determined by monitoring the formation rate of monoacylglycerol (MAG), using

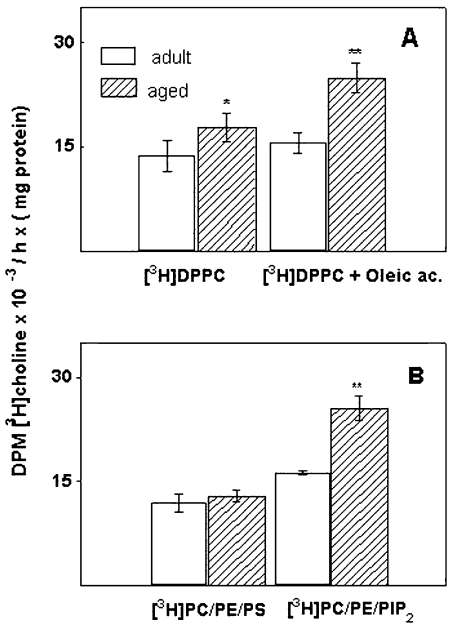

Fig. 1. Effect of aging on PLD isoforms in CC synaptosomal membranes. (A) Oleate-dependent PLD activity was determined by measuring choline liberation from [^{3}H]DPPC with or without 4 mM sodium oleate as described in Section 2. (B) PIP_2-dependent PLD activity was assayed using a mixed lipid vesicle formed by PE/PIP_2/DPPC in a molar ratio 16:1.4:1 with [^{3}H]DPPC to yield 200,000 cpm per assay, as described in Section 2. Enzyme activity is expressed as $(\text{DPM} \times 10^{-3}) \times \text{h}^{-1} \times (\text{mg protein})^{-1}$. Data are the mean ± SD of six individual samples per condition. Each sample was obtained from a different adult or aged animal. (*) Adult vs. aged synaptosomal membranes $*p < 0,05$, $**p < 0,001$.

PAPase-generated DAG as substrate. Standard assay pH condition, protein concentration, time and final volume of incubation were the same as those described for PAPase assay (Pasquaré and Giusto, 1993).

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

Radioactive PA was obtained from [2-^{3}H]phosphatidylcholine, synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described (Pasquaré de García and Giusto, 1986). Lipids were extracted from the tissue as described in Folch et al., 1957. [2-^{3}H]phosphatidylcholine was isolated by mono-dimensional TLC and eluted therefrom (Arvidson, 1968). Then, [2-^{3}H]PC was hydrolyzed with PLD (Kates and Sastry, 1969) and the hydrolysis product PA, 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 silicagel with neutral solvents to avoid the formation of lysophosphatidic acid and then converted into free acid by washing it twice using an upper phase containing 0.1 M sulfuric acid and then an upper phase containing water. Radioactivity and phosphorous content (Rouser et al., 1970) were measured to determine specific radioactivity. [2-^{3}H]PA had a specific radioactivity of 0.1–0.2 μCi/μmol. To determine NEM-sensitive PAPase activity, the substrate was prepared by sonicating 3.33 mM [2-^{3}H]phosphatidate (0.1–0.2 μCi/μmol) and 2.22 mM dipalmitoyl phosphatidylcholine in 5.56 mM EGTA and 5.56 mM EDTA. For the determination of NEM-insensitive PAPase activity, an identical emulsion to the above was prepared except that PC was omitted (Jamal et al., 1991; Hooks et al., 1998).

2.6. Extraction and isolation of lipids

Lipids were extracted with chloroform/methanol (2:1, by vol.) and washed with 0.2 volumes of $CaCl_2$ (0.05%) (Folch et al., 1957). Neutral lipids were separated by gradient–thickness thin-layer chromatography on silicagel G (Giusto and Bazán, 1979) 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]PA and phospholipids were retained at the spotting site. Lipids were visualized by exposure of the chromatograms 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.).

2.7. Other methods

Protein and lipid phosphorus were determined according to Bradford (1976) and Rouser et al. (1970), respectively.

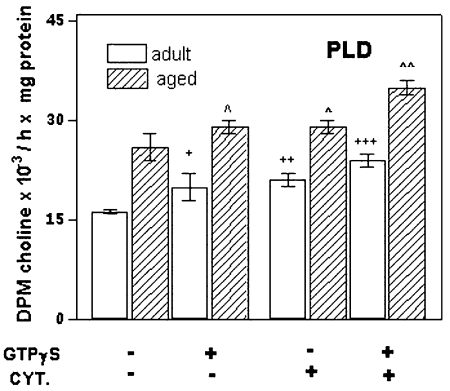

Fig. 2. Effect of aging on PIP_2-PLD activity in the presence of GTPγS and/or cytosolic fraction. PIP_2-PLD-dependent activity was assayed as described in Fig. 1B. The membranes were incubated in the presence of 100 μM GTPγS, or cytosolic fraction (80 μg protein) or GTPγS plus cytosolic fraction. All conditions were assayed in adult and aged membranes. Adult membranes were incubated with cytosol from adult CC and senescent membranes with cytosol from senescent CC. Enzyme activity is expressed as $(\text{DPM} \times 10^{-3}) \times \text{h}^{-1} \times (\text{mg protein})^{-1}$. Data are the mean ± SD of six individual samples per condition. Each sample was obtained from a different adult or aged animal. (+) Basal vs. stimulation conditions in adult membranes, $^{+}p < 0,05$, $^{++}p < 0.025$, $^{+++}p < 0.001$; (^) basal vs. stimulation conditions in aged membranes $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0,001$.

2.8. Statistical analysis

Statistical analysis was performed using student's *t*-test, with the values representing the mean ± SD of six individual samples per condition. Each sample was obtained from a different adult or aged animal.

3. Results

3.1. Phospholipase D activity in cerebral cortex synaptosomes from adult and aged rats

PLD activity was measured by quantification of choline generation from [^{3}H]DPPC under different experimental conditions. Choline liberation from [^{3}H]DPPC without the addition of exogenous activators was evaluated both in adult and senescent rats. A slight increase in PLD activity was observed in samples from senescent animals (Fig. 1A).

The first isoform of PLD activity described in synaptosomal membranes was an oleate-dependent form that has been extensively characterized by Kobayashi and Kanfer (1987). However, the regulation mechanism of oleate-dependent PLD remains unknown. In the presence of exogenous oleic acid (4 mM) choline generation increased strongly in synaptosomes from aged rats. PLD activity in the presence of this fatty acid was 60% higher in synaptosomes from senescent rats than that found in adult membranes (Fig. 1A).

Another PLD isoform characterized in the CNS is a PIP_2-dependent PLD (Liscovitch and Chalifa-Caspi, 1996). When PLD assays were carried out in the presence of PIP_2, we used liposomes constructed in the presence of an acidic phospholipid such as PS as controls. Under control conditions (liposome with PE/DPPC/PS) we observed no variation in PLD activity as a result of aging. However, in the presence of PIP_2, a known activator of PLD1/2 isoforms, choline generation in synaptosomes from aged rats was 57% higher than the values reported in synaptosomes from adult rats (Fig. 1B).

It is well known that PLD activated by PIP_2 is regulated by the presence of non-hydrolyzable analogs of GTP and of cytosolic factors such as ADP-ribosylation factor (ARF) and other small G proteins (Liscovitch et al., 1999). In order to evaluate the effect of aging on the regulation of PIP_2-dependent PLD, the assays were carried out in the presence of the above-mentioned activator in synaptosomal membranes from both adult and aged rats. When enzyme assays were performed in the presence of GTPγS (100 μM), there was a 23% increase in PIP_2-dependent PLD activity in adult rat synaptosomal membranes ($p < 0.05$) and a 12% increase in those from senescent rats with respect to basal conditions ($p < 0.05$) (without the addition of exogenous analogs). The presence of cytosol stimulated PIP_2-dependent PLD activity by 28% in adult rat membranes ($p < 0.025$) and by 12% in those from senescent rats ($p < 0.05$). In the presence of GTPγS plus cytosol, PIP_2-dependent PLD activity was, respectively, 46% ($p < 0.001$) and 34% ($p < 0.001$) higher in adult rat and aged rat membranes as compared to the basal conditions (without GTPγS and cytosol). The presence of GTPγS plus cytosol had an additive effect on PIP_2-dependent PLD. The increased activity under these latter conditions was 21% higher in synaptosomes from both adult and aged rats than in the membranes incubated in the presence of GTPγS or cytosol (Fig. 2).

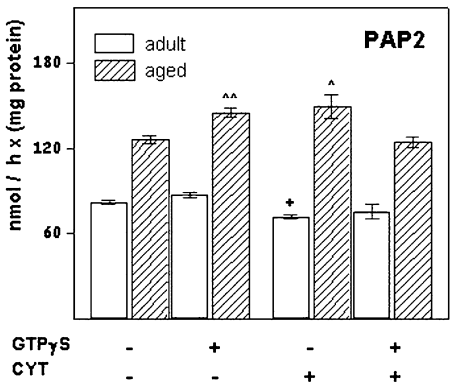

Fig. 3. Effect of aging on PAP2 activity in the presence of GTPγS and/or cytosolic fraction. For the determination of NEM-insensitive PAPase (PAP2) activity the assay was carried out with 4.2 mM NEM and the reaction started by adding 0.6 mM of [^{3}H]PA. The membranes were incubated in the presence of 100 μM GTPγS, or cytosolic fraction (80 μg protein) or GTPγS plus cytosolic fraction. All conditions were assayed in adult and aged membranes. Adult membranes were incubated with adult cytosolic CC fraction and senescent membranes with senescent cytosolic CC fraction. Other assay conditions are described in Section 2. Activity is expressed as the sum of nmol of [^{3}H]DAG plus [^{3}H]MAG $\times h^{-1} \times$ (mg protein)$^{-1}$. Data are the mean ± SD of six individual samples per condition. Each sample was obtained from a different adult or aged animal. (+) Basal vs. stimulation conditions in adult membranes, $^{+}p < 0.05$; (^) basal vs. stimulation conditions in aged membranes $^{\wedge}p < 0.005$, $^{\wedge\wedge}p < 0,001$.

3.2. Phosphatidate phosphohydrolase activity in cerebral cortex synaptosomes from adult and aged rats

PAPase activity is mainly coupled to PLD activity, since the main product of hydrolytic PLD activity, PA, is the substrate for PAPase to yield DAG. The PAP2 isoform, implicated in signal transduction and coupled to PLD, was directly assayed using [^{3}H]PA as substrate. In order to establish the effect of aging on the degradation of PA by PAPase we analyzed enzymatic activity under the same experimental conditions as PIP_2-dependent PLD activity (i.e. GTPγS and GTPγS plus cytosol).

PAP 1 (NEM-sensitive) activity was absent from synaptosomes; the only isoform present in these membranes was the PAP2 form (NEM-insensitive) (data not shown). When synaptosomes from adult membranes were incubated in the presence of GTPγS (100 μM), no significant change was found in PAP2 activity (Fig. 3). The presence of cytosol had the effect of inhibiting PAP2 activity by 12% in adult membranes ($p < 0.05$). This effect was sustained when cytosol plus GTPγS were added.

Interestingly, aging stimulated PAP2 activity by 56% (Fig. 3). In addition, synaptosomal PAP2 from aged rats was stimulated by 15% in the presence of 100 μM GTPγS ($p < 0.001$). The presence of cytosol exerted the same effect on PAP2 activity as 100 μM GTPγS ($p < 0,005$). When cytosol plus 100 μM GTPγS were added to the incubation medium, PAP2 activity returned to the basal values for synaptosomal membranes from aged rats (Fig. 3).

3.3. Diacylglyceride lipase activity in cerebral cortex synaptosomes from adult and aged rats

The DAG formed by PAP2 activity is partially hydrolyzed by DGL activity, yielding MAG. MAG

Table 1
Effect of aging on DAG and MAG production in synaptosomal membranes. MAG was measured by monitoring the release of [2-^{3}H]monoacylglycerol from of 1,2-diacyl-[2-^{3}H]glycerol (produced by PAP2). Enzymatic activities were expressed as DPM of MAG or DAG released $\times h^{-1} \times$ (mg protein)$^{-1}$. The third column shows the percentage of MAG transformation with respect to DAG generated by PAP2 in each condition

	Condition	PAP2 activity	DGL activity	% MAG generated
Adult	Basal	18467 ± 307	12031 ± 16	65.1
Aged	Basal	28669 ± 705	12576 ± 205	43.9
Adult	GTPγS	19847 ± 409	9370 ± 182	47.2
Aged	GTPγS	33078 ± 660	20695 ± 227	62.6
Adult	Cytosol	16261 ± 387	8187 ± 682	50.4
Aged	Cytosol	33906 ± 1819	22469 ± 672	66.3
Adult	Cytosol + GTPγS	17091 ± 1205	8642 ± 296	50.6
Aged	Cytosol + GTPγS	28117 ± 819	11940 ± 500	42.5

generation was not modified by aging; however, the percentage of MAG generated from DAG was inhibited under basal conditions (Table 1). DGL activity in adult membranes was inhibited by 22% in the presence of 100 μM GTPγS. The presence of cytosol or cytosol plus GTPγS produced a similar decrease in DGL activity (30% inhibition with respect to adult basal conditions). In synaptosomal membranes from aged rats, 100 μM GTPγS and cytosol stimulated DGL activity by 65 and 79%, respectively, compared to basal conditions (without GTPγS or cytosol). GTPγS plus cytosol returned DGL activity to its basal values in synaptosomes from senescent rats (Table 1).

4. Discussion

PC is a major source of lipid-derived second messenger molecules that function in both intracellular and extracellular signaling. PC-specific PLD and PAPase are two pivotal enzymes in this signaling system and they act in series to generate the biologically active lipids PA and DAG (Exton, 1997; Brindley and Waggoner, 1996).

Neuronal PLD activity is hypothesized to be involved in vesicle trafficking, endocytosis and possibly transmitter release. It has been demonstrated that depolarization inhibits PLD activity. This inhibition mechanism could be due to the generation of inositol phosphates and a reduction in the synaptosomal pool of PIP_2, suggesting that neuronal PLD activity is regulated by synaptic activity (Waring et al., 1999). In this work, two isoforms of PLD (oleate-dependent and PIP_2-dependent) showed increased activity in aged synaptosomal membranes with respect to adult membranes. Although both isoforms were significantly stimulated by the effect of aging, PIP_2-dependent PLD showed the highest activity. Sequestration of PIP_2 within membrane domains may control PLD activity and it has been demonstrated that PIP_2 is selectively enriched in microdomains from distinct cellular types (Hope and Pike, 1996).

Narang et al. (1996) have demonstrated decreased carbachol-induced PIP_2-PLC activity in the CC of aged rats, suggesting that during aging there is an uncoupling of muscarinic stimulated PIP_2 hydrolysis, which is accompanied by compensatory changes in PLC expression. Muscarinic-stimulated PI responses are lower in senescent brain primarily due to an uncoupling of the receptor–G-protein and/or G-protein–PLC link (Ayyagari et al., 1998). These results are in agreement with our findings showing an increase in PIP_2-dependent PLD activity in synaptosomal membranes during aging. This may be a consequence of the greater availability of PIP_2 microdomains owing to a decrease in PIP_2-PLC activity as a result of aging.

Our results are in agreement with previous reports showing an additive effect of GTPγS plus cytosol on PIP_2-dependent PLD activity (Liscovitch et al., 1999). This effect was present not only in synaptosomes from adult animals but also in synaptosomes from aged rats. However, enzyme activity in aged membranes was higher than in adult membranes under all assay conditions as compared to the corresponding control. This may suggest that aging exerts a stimulatory effect on this isoform that is independent of regulation by GTP binding proteins or cytosolic factors. These results support the hypothesis that the higher PLD activity observed in synaptosomes from aged rats could be due to the greater availability of PIP_2.

Most tissues are capable of dephosphorylating PA, the specific activity being highest in rat brain (Brindley and Waggoner, 1998; Waggoner et al., 1995). With respect to the PAPase isoform present in synaptosomal membranes, our results are in agreement with previous studies on brain cortex demonstrating that NEM-insensitive PAPase (PAP2) is the only isoform located in synaptosomes (Fleming and Yeaman, 1995).

Our results show that PAP2 activity in synaptosomal membranes from aged animals is significantly higher than that found in membranes from adult rats. This, together with the results on PIP_2-PLD, would suggest that the PC hydrolysis pathway yielding PA and subsequently DAG is stimulated by aging. In the presence of GTPγS, PAP2 activity was differently modulated in synaptosomal membranes from aged rats with respect to adult rats. These results suggest that the increased level of PA found in synaptosomes from adult rats is due to a GTPγS-mediated stimulation of PLD which is not paralleled by changes in PAP2 activity. However, aging could provoke a remotion of PA levels produced by PLD, increasing DAG generation by the stimulation of PAP2 activity.

The diminished level of PA under stimulation conditions owing to higher PAP2 activity in synaptosomes from aged rats could indicate that lower PA levels or higher DAG generation are involved in some mechanism leading to the synaptic dysfunction reported in aging (Toescu and Verkhratsky, 2000).

The effect of cytosolic fraction plus GTPγS on PAP2 activity indicates that a GTP-dependent cytosolic factor is necessary in adult membranes for the maintenance of PA levels, inhibiting PAP2 and stimulating PLD activities. In aged membranes, the level of PA was diminished by the cytosolic fraction and by GTPγS, stimulating PAP2 activity. The effect of cytosol plus GTPγS suggests the presence of a PAP2-inhibitory factor, regulated by GTPγS, that restores the level of PAP2 activity to that of the aged membrane control. Our findings indicate that cytosolic factors and/or G protein activators differently regulate PAP2 activity in synaptosomal membranes from aged rats during aging.

In most cases, DGL activity is coupled to PAPase activity (Farooqui et al., 1993). The DAG generated by PAPase is hydrolyzed to MAG. DAG generated by PAP2 activity in synaptosomal membranes is partially hydrolyzed by DGL activity to MAG. Under basal conditions (without GTPγS and/or cytosol) DGL activity was the same in synaptosomes from adult and aged rats. However, when the results are expressed in terms of the percentage of MAG generated with respect to DAG produced by PAP2 activity, we can see that degradation of DAG by DGL is strongly inhibited by aging (21% of inhibition with respect to adult values) (Table 1). These results indicate that despite the greater availability of DAG during aging, the enzyme under basal conditions does not additionally metabolize the DGL substrate.

In the presence of GTPγS, cytosol or cytosol plus GTPγS, DGL activity was affected differently in synaptosomes from aged rats with respect to synaptosomes from adult rats. Under the same experimental conditions, DGL activity in aged membranes was stimulated to a higher degree than PAP2 indicating that such stimulation is not a consequence of the changes in PAP2 activity. These findings lead us to conclude that under stimulation conditions the removal of DAG by DGL increases during aging, whereas in adult membranes the maintenance of PA levels seems to be the principal physiological event in this pathway.

The PLD/PAPase pathway is operational in microdomains, a type of specialized membrane compartment implicated in vesicular trafficking and signal transduction (Brown and London, 1998). PLD (most likely PLD 2) and PAP2 (most likely PAP2b) activities localize to and act sequentially to generate DAG in these specialized domains (Sciorra and Morris, 1999). Other DAG-binding proteins besides protein kinase C have been identified, and they constitute the target for the DAG generated by the PLD/PAPase pathway (Topham and Prescott, 1999).

Rab3A, a Ras-related GTP-binding protein, has been reported in rat neuronal cytosol or located on the synaptic vesicles, cycling between the cytosol and the membranes. PA was found to be required for the translocation of Rab3A to the membranes in a GTPγS-dependent form and it has been suggested that PA produced by PLD can in turn recruit small GTPases and stimulate PLD, resulting in the acceleration of PA production (Jung et al., 1999). In accordance with our results the increase in PLD activity during aging could be related with an increased association of Rab3A or another small G protein with the synaptosomal membranes.

It is well known that in rat cerebrocortical synaptosomes, several membrane receptors such as glutamate and muscarinic receptors are coupled to the PLD pathway (Shinomura et al., 2000; Qian and Drewes, 1989). As previously mentioned, PLD is coupled to PAP2 and DGL. A marked increase has been described in DGL activity in plasma membrane and synaptosomal membrane preparations from nucleus basalis and hippocampal regions of brain from patients with Alzheimer disease compared to control samples (Farooqui et al., 1990). Moreover, it has been reported that the treatment of neuron cultures with glutamate or *N*-methyl-D-aspartate also results in a marked increase in DGL activity, suggesting that DAG may be involved in the cross-talk between cannabinoid and glutamate receptors (Farooqui et al., 1993).

The fact that the PLD/PAP2/DGL pathway is differentially regulated by G protein activators or cytosolic factors during aging could be the cause of the alteration in neurotransmission and in the neuronal dysfunction observed in senescence.

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