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Activation of phosphatidylcholine signalling during oxidative stress in synaptic endings

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ABSTRACT

The purpose of the present study was to investigate the involvement of phosphatidylcholine (PC) signalling in synaptic endings incubated under oxidative stress conditions. Synaptosomes purified from adult rats (4 months old) cerebral cortex were exposed to oxidative insult ($FeSO_4$, 50 μM) or vehicle, and diacylglycerol (DAG) generation and free fatty acid (FFA) release were subsequently evaluated using exogenous [^{14}C]PC as substrate. DAG formation increased after 5, 30, and 60 min of Fe^{2+}-exposure with respect to the control conditions. The contribution of PC-specific phospholipase C (PC-PLC) and phospholipase D (PLD) pathways to DAG generation was evaluated using ethanol in the enzyme assays. Phosphatidylethanol (PEth) production was measured as a marker of PLD activity. In the presence of ethanol (2%) iron significantly stimulated DAG and PEth production at all times assayed. FFA release from PC, however, was inhibited after 5 and 60 min of iron exposure. Similar results were observed in aged animals (28 months old) when compared with adult animals. DAG generation from PC was also evaluated in the presence of the tyrosine kinase inhibitors genistein and herbimycin A. Inhibition of tyrosine kinase activity did not modify the stimulatory effect exerted by iron on PC-PLC and PLD activities. Moreover, the presence of LY294002 (a specific PI3K inhibitor) did not alter DAG production. Our results demonstrate that oxidative stress induced by free iron stimulates the generation of the lipid messenger DAG from PC in synaptic endings in adult and aged rats.

1. Introduction

Oxidative stress and abnormally high levels of iron in the brain have been demonstrated to be present in several neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Berg et al., 2001; Ong and Farooqui, 2005; Berg and Youdim, 2006; Quintana et al., 2006). Oxidative stress, resulting from increased brain iron levels, and possibly also from defects in antioxidant defence mechanisms, is widely believed to be associated with neuronal death in these pathologies (Aracena et al., 2006; Ke and Qian, 2007).

Though a number of reports have described the intracellular events triggered by oxidative stress very little is known about the role of lipid signal transduction during oxidative injury (Petersen et al., 2007). Phospholipases specifically hydrolyze membrane phospholipids and generate bioactive lipid second messengers which participate in numerous cell signalling events (Eyster, 2007). Phosphatidylcholine (PC) is the most abundant class of glycerophospholipids in mammalian cell membranes and it plays a key role in membrane structure, cell death, and cellular signalling. In regard to signal transduction, PC is the main substrate for phospholipase D (PLD), yielding phosphatidic acid (PA) and choline upon cleavage (Exton, 2000; Foster and Xu, 2003). Involvement of the PLD pathway has been proposed in several cellular events such as cytoskeletal rearrangement, vesicle trafficking, exocytosis, phagocytosis, oncogenesis, and neuronal and cardiac stimulation (Hattori and Kanfer, 1984;

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Abbreviations: βA, β-amyloid peptide; AD, Alzheimer's disease; CC, cerebral cortex; DAG, diacylglycerol; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FFA, free fatty acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; LDH, lactate dehydrogenase; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; D609, tricyclodecan-9-yl-xanthate potassium salt; MAG, monoacylglycerol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD^+, nicotinamide dinucleotide; NADH, reduced nicotinamide dinucleotide; TBARS, thiobarbituric acid reactive substances; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PBS, phosphate buffered saline; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine specific-phospholipase C; PEth, phosphatidylethanol; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol bisphosphate; PLA, phospholipase A; PLD, phospholipase D; PMSF, phenylmethylsulfonilfluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; Syn, synaptosomes; TBM, tris buffer medium; TLC, thin-layer chromatography.

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Liscovitch et al., 2000; Klein, 2005). PA generated by PLD can be further hydrolyzed by PA phosphatase type 2 (PAP2) in order to generate another lipid second messenger, diacylglycerol (DAG). DAG derived from PC can be also generated by a specific phospholipase C (PC-PLC). In contrast to DAG generated from phosphatidylinositol (4, 5) bisphosphate (PIP_2) by PIP_2-PLC activity, the wave of DAG elicited from PC hydrolysis is generated more slowly and occurs without elevation of intracellular Ca^{2+}. PC-PLC activity has been associated with cellular events such as glutamate-induced nerve cell death, Fas-induced apoptosis, and cell mitogenic responses triggered by platelet-derived growth factor (Li et al., 1998; Kim et al., 2001; Ramoni et al., 2004). Furthermore, PC-PLC appears to act downstream Ras but upstream Raf-1 during mitogenic signal transduction (Cai et al., 1993). Though the physiological role of PC-PLC in immune cells has been described extensively, very little is known about its function in the central nervous system (Spadaro et al., 2006; Cecchetti et al., 2007).

The coexistence of PLD and PC-PLC pathways has already been reported in cerebral cortex synaptic endings and the existence of PC-PLC in the nervous system has been documented in immature cortical neurons and in a hippocampal nerve cell line, HT22 (Li et al., 1998; Mateos et al., 2006). However, the role of PC-derived lipid signalling in the central nervous system constitutes a newly emerging field whose significance in pathological and physiological processes remains to be elucidated (Salvador et al., 2002, 2005; Klein, 2005; Adibhatla et al., 2006). In this work we present evidence for the first time of the activation of PLD and PC-PLC pathways in synaptic endings subjected to oxidative injury triggered by $FeSO_4$ overload.

2. Materials and methods

Wistar-strain adult (4 months old) and aged (28 months old) rats were kept under constant environmental conditions and fed on a standard pellet diet *ad libitum* until decapitation.

1-[^{14}C]palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]-DPPC) (111 mCi/mmol), was purchased from New England Nuclear-Dupont, Boston, MA, USA. Preblended dry fluor 2a70 (98% PPO and 2% bis-MSB) was obtained from Research Products International Corp., USA. Triton X-100, tricyclodecan-9-yl-xanthate potassium salt (D609), DAG lipase inhibitor (RHC80267), DAG kinase inhibitor (R59022), PIP_2-PLC inhibitor (U73122), PI3K inhibitor (LY294002), genistein, herbimycin A, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and thiobarbituric acid (TBA) were obtained from Sigma–Aldrich St. Louis, MO, USA. The kit (LDH-P UV AA) for measuring lactate dehydrogenase (LDH) activity was kindly supplied by Wiener laboratory, Rosario, Argentina. All other chemicals were of the highest purity available.

2.1. Preparation of synaptosomal fraction

Total homogenates were prepared from the cerebral cortex (CC) of 4 months old (adults) and 28 months old (aged) rats. Rats were killed by decapitation and CC was immediately dissected (2–4 min after decapitation). All proceedings were in accordance with *Principles of Use of Animals* and *Guide for the Care and Use of Laboratory Animals* (NIH regulation).

Synaptosomal fraction (Syn) was obtained as previously described by Cotman with slight modifications (Cotman, 1974; Salvador et al., 2002). Briefly, CC homogenate (20% w:v) was prepared in a medium containing 0.32 M sucrose, 1 mM EDTA, 10 mM HEPES buffer (pH 7.4) in the presence of 1 mM DTT, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.1 mM PMSF. The cerebral cortex was homogenized by 10 strokes with a Thomas tissue homogenizer. The homogenate was centrifuged at 1800 × *g* for 7.5 min at 4 °C using a JA-21 rotor in a Beckman J2-21 centrifuge. The pellet was discarded, and the supernatant was retained and centrifuged at 14,000 × *g* for 20 min at 4 °C. The resulting pellet was washed and resuspended in 3 ml of 0.32 M sucrose isolation buffer, layered over a discontinuous ficoll gradient (8.5% pH 7.4, 13% pH 7.4 ficoll solutions, each prepared in isolation buffer) and spun at 85,500 × *g* for 30 min at 4 °C using a SW 28.1 rotor in a Beckman Optima LK-90 ultracentrifuge. Synaptosomes in the 8.5–13% ficoll interface were removed, resuspended in isolation buffer, and centrifuged at 33,000 × *g* for 20 min at 4 °C using a JA-21 rotor in a Beckman J2-21 centrifuge. Washed synaptosomal fraction was used for the experiments detailed below. Protein content of the synaptosomal fraction was determined by a previously published method (Lowry et al., 1951).

2.2. Experimental treatments

For all the experiments, synaptosomes were diluted in Tris base buffer medium (TBM) containing: 120 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 10 mM glucose, 20 mM Tris (pH 7.2). Synaptosomal suspensions were aliquoted into tubes and incubated at 37 °C under an O_2:CO_2 (95:5, v:v) atmosphere during experimental treatments. Oxidative stress was induced by incubating the Syn with $FeSO_4$ for 5, 30 and 60 min as previously described (Uranga et al., 2007). The final concentration of $FeSO_4$ was 50 μM for all the conditions assayed. $FeSO_4$ was prepared as a 10 mM stock in ultra pure water immediately prior to use.

Synaptosomes exposed to 50 μM $FeSO_4$ or vehicle for 5 and 60 min were co-incubated with 30 μM RHC80267, 20 μM R59022 or 10 μM U73122 to study the involvement of DAG metabolizing pathways. To study the role of tyrosine phosphorylation, Syn were co-incubated with 100 μM genistein for 5 and 60 min or with 10 μM herbimycin A for 60 min. The involvement of PI3K was studied co-incubating with 10 μM LY294002 for 5 min. The concentrations of the different inhibitors had been previously determined in our laboratory (Salvador et al., 2005; Mateos et al., 2006; Zulian et al., 2006; Uranga et al., 2007).

2.3. Determination of total DAG generation from PC and phospholipase A (PLA) activities

PC hydrolysis was determined using lipid vesicles containing [^{14}C]-DPPC and cold DPPC to yield 45,000 dpm and 0.125 mM per assay in a buffer containing 0.2% Triton X-100 and 0.1 M Tris (pH 7.2). 100 μl of these lipid vesicles were added to 100 μl of preincubated Syn (150 μg of protein) in a final volume of 200 μl. The reaction was carried out at 37 °C for 20 min and stopped by the addition of 5 ml of chloroform/methanol (2:1, v:v). Blanks were prepared identically, except that membranes were boiled for 5 min before use. Lipids were extracted and separated as described below (Mateos et al., 2006).

2.4. Determination of PC-PLC and PLD activities

To evaluate the contribution of the PC-PLC pathway to total DAG formation the enzyme reaction was carried out in the presence of 2% ethanol (Salvador and Giusto, 1998; Salvador et al., 2002). For all the experiments carried out in the presence of ethanol, lipid vesicles were prepared containing [^{14}C]-DPPC to yield 60,000 dpm.

To assay PLD activity, the transphosphatidylation reaction was measured in Syn preincubated under the different experimental conditions. Lipid vesicles were prepared as described above but the assay was conducted in the presence of 2% ethanol and 60,000 dpm of [^{14}C]-DPPC per condition (Mateos et al., 2006). The reaction was incubated at 37 °C for 20 min and stopped by the addition of 5 ml of chloroform/methanol (2:1, v:v). Lipids were extracted and separated as described below.

2.5. Extraction and isolation of lipids

Lipids were extracted according to Folch et al. (1957). Briefly, the lipid extract was washed with 0.2 volumes of 0.05% $CaCl_2$ and the lower phase was obtained after centrifugation at 900 × *g* for 5 min. Neutral lipids: monoacylglycerol (MAG), DAG, and free fatty acid (FFA) were then separated by one-dimensional thin-layer chromatography (TLC) using silica gel G plates (Merck) in a mobile phase consisting of hexane/diethyl ether/acetic acid (50:50:2.6, v:v). PC was retained at the spotting site. Lipids were visualized by exposure of the plate to iodine vapours. DAG and FFA spots were scraped off the plate and quantified by liquid scintillation.

Phosphatidylethanol (PEth) was separated by one-dimensional TLC on silica gel H (Merck) and developed with chloroform/methanol/acetone/acetic acid/water (50:15:15:10:5, v:v) up to 70% of the plate. Then the plate was rechromatographed up to the top using hexane/diethyl ether/acetic acid (70:30:2.6, v:v) as previously described (Salvador and Giusto, 1998). Radioactivity of lipid spots was determined by liquid scintillation as previously described (Salvador and Giusto, 1998).

2.6. MTT reduction assay

To determine the synaptosomal reducing capacity, the extent of MTT reduction to insoluble, intracellular formazan crystals was measured. This reduction depends on the activity of intracellular dehydrogenases and is independent of changes in the integrity of the plasma membrane. MTT reduction was measured in Syn exposed to 50 μM $FeSO_4$ or vehicle and in Syn co-incubated for 5 and 60 min with 2% ethanol or 250 μg/ml D609. The methods employed in the present study are similar to those described by Keller and co workers (Keller et al., 1997a,b). In brief, MTT was dissolved in phosphate buffer saline (PBS) at a concentration of 5 mg/ml. The MTT solution was mixed with synaptosomes (1:10; MTT: synaptosomes, v:v) and allowed to incubate for 2 h at 37 °C. At the end of the incubation with MTT, solubilization buffer (20% SDS, pH 4.7) was added and mixed thoroughly to dissolve the crystals of formazan. The extent of MTT reduction was then measured spectrophotometrically at 570 nm and results were expressed as arbitrary units.

2.7. LDH assay

Lactate dehydrogenase (LDH) leakage was evaluated as a parameter of plasma membrane integrity. LDH assay was performed as previously described in our laboratory (Uranga et al., 2007). Briefly, after the incubation for 5 and 60 min in the presence of either Fe^{2+} or vehicle (co-incubated or not with 2% ethanol or 250 μg/ml D609) Syn were centrifuged at 33,000 × *g* for 20 min at 4 °C. The resulting supernatant was used to determine the activity of LDH, which was measured spectrophotometrically by using an LDH-P UV AA kit following the manufacturer's instructions. Briefly, the rate of conversion of reduced nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide (NAD^+) was followed at 340 nm. Results were expressed as units per litter (U/L).

2.8. Lipid peroxidation assay

Lipid peroxidation was measured using the thiobarbituric acid assay as previously described (Adamczyk et al., 2006). Briefly, after incubation for 5, 30 or 60 min in the presence of either Fe^{2+} or vehicle (co-incubated or not with 250 μg/ml D609), 1 ml of 30% trichloroacetic acid (TCA) were added to 0.5 ml of synaptosomes (1.5 mg protein/ml). Then 0.1 ml of 5N HCl and 1 ml of 0.75% thiobarbituric acid were added. Tubes were capped, the mixtures were heated at 100 °C for 15 min in a boiling water bath and the samples were centrifuged at 1000 × *g* for 10 min. Thiobarbituric acid reactive substances (TBARS) were measured in the supernatant at 535 nm and results were expressed as units of absorbance at 535 nm per mg of protein (Abs 535 nm (arbitrary units)/mg protein).

2.9. Statistical analysis

Statistical analysis was performed using two-way ANOVA followed by Bonferroni's test to compare means. *p*-Values less than 0.05 were considered statistically significant.

3. Results

3.1. PC hydrolysis in synaptosomes exposed to $FeSO_4$

We have previously reported that synaptic endings incubated with $FeSO_4$ (50 μM) constitute an appropriate model for studying the effect of oxidative damage in the nervous system (Uranga et al., 2007). In this work we studied the effect of oxidative stress induced by free iron on synaptic signalling pathways that generate DAG and FFA from PC. For this purpose, PC hydrolysis catalyzed by PC-PLC, PLD and PLA was evaluated in synaptosomes previously exposed to oxidative insult for different periods of time (5, 30 and 60 min). [^{14}C]-DAG generation was stimulated by $FeSO_4$ (50 μM) after 5, 30 and 60 min of incubation by 77, 50 and 65%, respectively, as compared with the control (Fig. 1A). [^{14}C]-FFA generation, on the other hand, was inhibited by 50% after 5 and 60 min of incubation with Fe^{2+} (Fig. 1B).

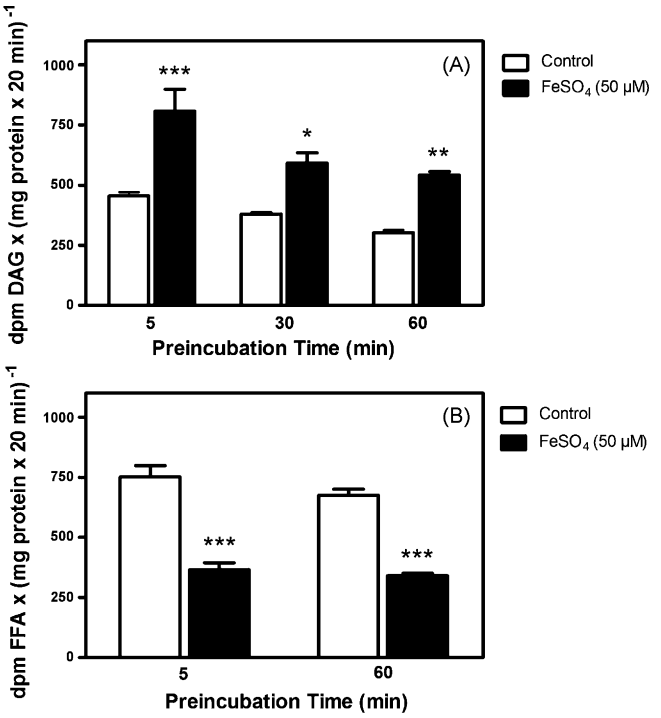

Fig. 1. Effect of $FeSO_4$ on PC hydrolysis in rat cerebral cortex synaptosomes. Rat CC Syn were incubated in Tris base buffer medium (TBM) in the presence of 50 μM $FeSO_4$ or vehicle for 5, 30 and 60 min as described in Section 2. Enzyme reaction was started by adding 100 μl of preincubated synaptosomal suspensions to 100 μl of lipid vesicles containing [^{14}C]-DPPC and non-radiolabeled DPPC to yield 45,000 dpm and 0.125 mM per assay. After 20 min incubation at 37 °C, the enzyme reaction was stopped and lipids were extracted and isolated as described in Section 2. (A) DAG generation from PC. Results were compared to the control condition and the enzyme activity is expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. (B) FFA generation from PC. Results were compared to the control condition and the enzyme activity is expressed as dpm FFA × (mg protein × 20 min)$^{-1}$. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D. ($^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$).

3.2. Participation of PC-PLC and PLD pathways in DAG generation induced by free iron

In order to study the contribution of PC-PLC and PLD pathways to synaptosomal DAG generation, ethanol was included in the enzyme assays. In the presence of primary alcohols, PLD exclusively catalyzes a transphosphatidylation reaction yielding phosphatidylalcohols instead of PA. Thus the generation of phosphatidylalcohols blocks PAP action and allows the measurement of DAG generation exclusively from PC-PLC activity (Mateos et al., 2006). Fig. 2A shows DAG generation in the absence and in the presence of 2% ethanol in synaptosomes incubated with or without $FeSO_4$. DAG generation in the presence of ethanol was stimulated by iron by 65, 43 and 100% with respect to control conditions after 5, 30 and 60 min, respectively, and this generation corresponds to PC-PLC activity (Fig. 2B).

For evaluating the state of the PLD pathway under oxidative conditions, PEth generation (a marker of PLD activity) was measured under the same conditions as those described above. PEth generation increased by 50% in the presence of iron at all the times assayed. The iron-induced PEth generation demonstrates that not only PC-PLC but also PLD is involved in synaptic signalling triggered by the transition metal (Fig. 2C). Fig. 2D shows that in the presence of ethanol, DAG plus PEth levels (expressed as dpm × (mg protein × 20 min)$^{-1}$) correlate to total DAG levels. The relationship between total DAG levels and the sum of DAG plus PEth levels (when the assays were performed in the presence of ethanol) was observed both under control and under oxidative injury conditions.

3.3. Role of lipid-metabolizing pathways on PC hydrolysis

DAG is a potent lipid messenger and as a consequence, cellular levels are strictly controlled by degrading enzymes. For determining the cellular fate of DAG produced from PC, assays were performed in the presence of either a DAG lipase inhibitor (RHC80267), a DAG kinase inhibitor (R59022) or a PIP_2-PLC inhibitor (U73122). Under control conditions DAG lipase, DAG kinase and PIP_2-PLC inhibitors did not modify DAG generation from PC after 5 and 60 min of preincubation. However, in the presence of U73122, DAG levels decreased by 11 and 23% after 5 and 60 min of iron exposure, respectively (Fig. 3).

3.4. Participation of tyrosine phosphorylation in the hydrolysis of PC in cerebral cortex synaptosomes exposed to $FeSO_4$

Tyrosine phosphorylation is an early event that appears in oxidative stress-related processes. We have previously demon-

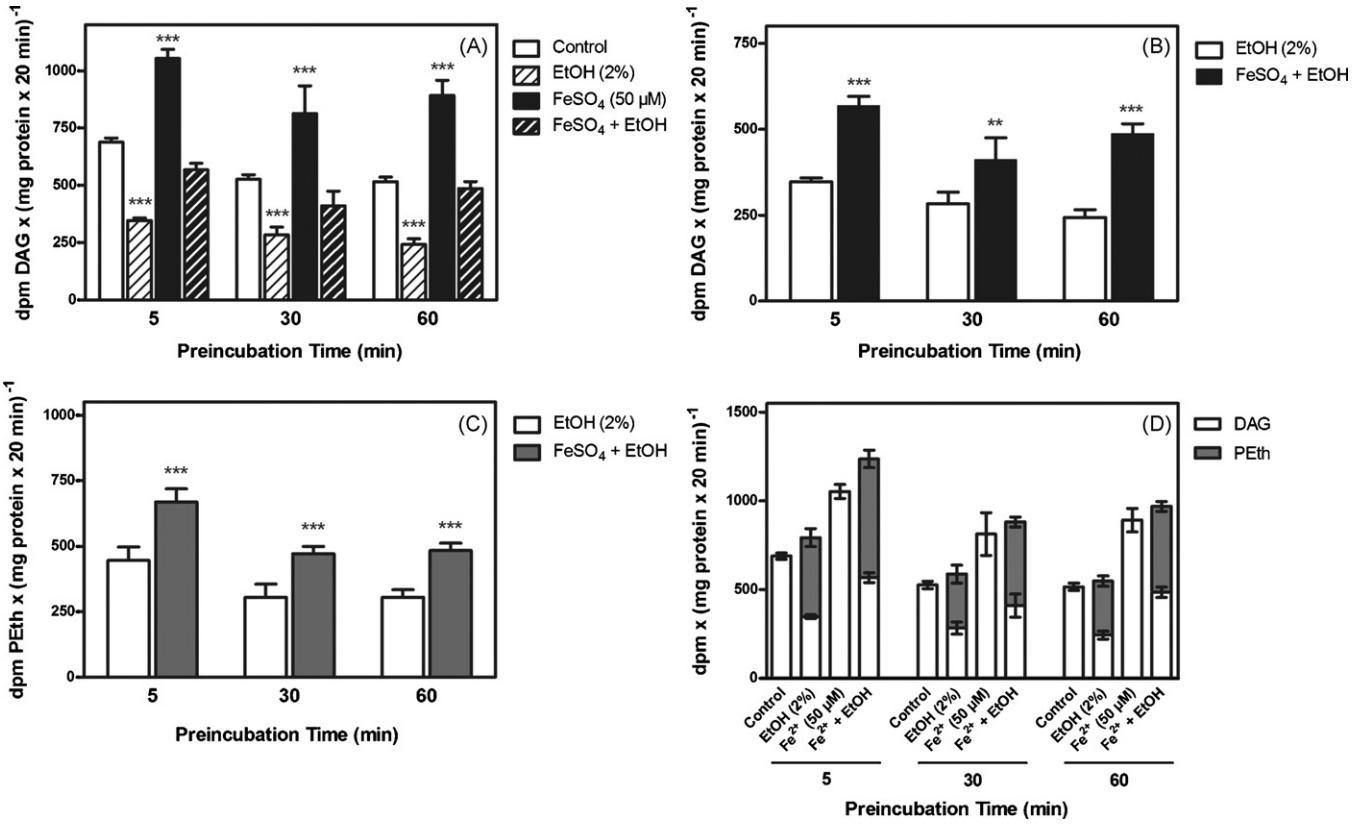

Fig. 2. Contribution of PC-PLC and PLD pathways to DAG generation. (A) DAG generation from PC in the presence or in the absence of ethanol (2%). Synaptosomes were exposed to $FeSO_4$ (50 μM) or vehicle for 5, 30 and 60 min. Enzyme reaction was carried out in the absence or presence of 2% ethanol and lipid vesicles were prepared to yield [^{14}C]-DPPC and cold DPPC to yield 60,000 dpm and 0.125 mM per assay. Results were compared to the control condition and expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. (B) Effect of $FeSO_4$ on PC-PLC activity. Synaptosomes were incubated as described in (A) and DAG generation was measured in the presence of 2% ethanol. Results were compared to the control (2% ethanol) condition and expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. (C) Effect of $FeSO_4$ on PLD activity. Synaptosomes were incubated as described in (B) and PEth production was measured as described in Section 2. Results were compared to the control (2% ethanol) condition and the enzyme activity is expressed as dpm PEth × (mg protein × 20 min)$^{-1}$. (D) Contribution of DAG and PEth levels to total DAG levels. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D. ($^{***}p < 0.001$; $^{**}p < 0.01$).

strated that tyrosine phosphorylation increases in synaptic endings exposed to $FeSO_4$ (Uranga et al., 2007). However, neither herbimycin A nor genistein (two tyrosine kinase inhibitors) were able to modify the stimulation of DAG production from PC elicited by free iron (Fig. 4A and B), nor were any changes observed in PEth production under the same experimental conditions (data not shown).

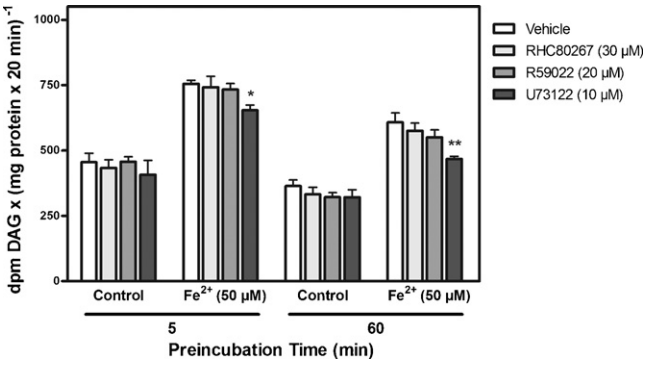

Fig. 3. Influence of lipid metabolizing enzymes on DAG generation from PC. Synaptosomes exposed to 50 μM $FeSO_4$ or vehicle for 5 and 60 min were co-incubated with 30 μM RHC80267, 20 μM R59022 or 10 μM U73122. Enzyme reaction was carried out as described in Fig. 1. Results were compared to the control (vehicle) condition and expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D. ($^{**}p < 0.01$; $^{*}p < 0.05$).

3.5. Involvement of PI3K activity in the generation of DAG induced by oxidative stress

We have recently demonstrated that $FeSO_4$ (50 μM) stimulated PI3K activity in synaptosomal membranes after a short incubation time (Uranga et al., 2007). To determine the crosstalk between PI3K pathway and PC signalling pathways, DAG formation was measured in the presence of $FeSO_4$ (50 μM) and the PI3K inhibitor LY294002 (10 μM). DAG generation induced after 5 min incubation with Fe^{2+} was not influenced by the co-incubation with LY294002 (Fig. 5).

3.6. Evaluation of the deleterious effect of $FeSO_4$

The deleterious effect of Fe^{2+} incubation was evaluated at different levels. Mitochondrial function was measured by MTT reduction. Plasma membrane integrity was evaluated by the leakage of LDH to the external medium and lipid peroxidation by measuring the generation of thiobarbituric acid reactive substances (TBARS). As shown in Fig. 6A, Fe^{2+} induced a significant decrease (14%) in MTT reduction after 60 min exposure. To evaluate the role of PLD and PC-PLC pathways in synaptosomal integrity, MTT reduction was measured in the presence of ethanol (2%) and 250 μg/ml D609 (used as inhibitor of DAG generation pathways, i.e. PC-PLC and sphingomyelin synthase). Ethanol did not affect mitochondrial function in the control or in the presence of free iron, whereas D609 provoked an increase in MTT reduction in all the assayed conditions (Fig. 6A).

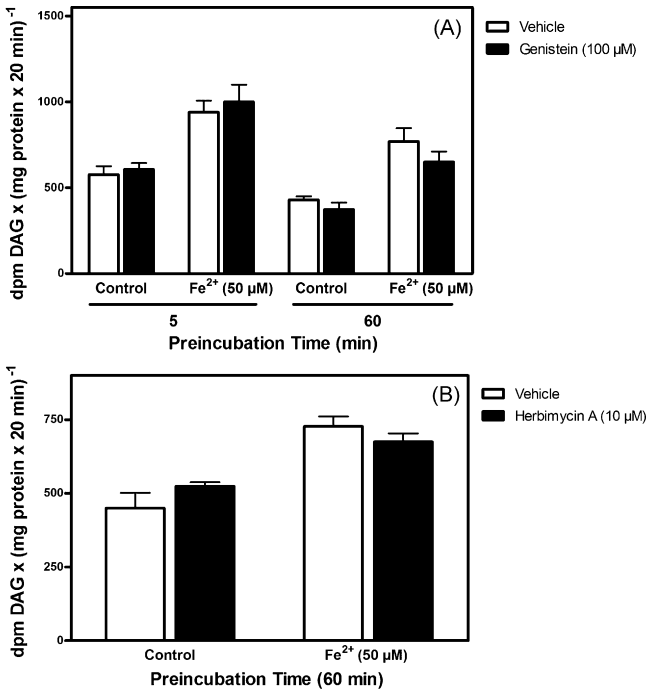

Fig. 4. Role of tyrosine phosphorylation on $FeSO_4$-induced PC hydrolysis. (A) Syn exposed to 50 μM $FeSO_4$ or vehicle were co-incubated with 100 μM genistein for 5 and 60 min. (B) Syn exposed to oxidative condition or to control condition as described above were co-incubated with 10 μM herbimycin A for 60 min. Results were compared to the control (vehicle) condition and the enzyme activity is expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D.

LDH release increased by 28% after 60 min of iron exposure (Fig. 6B). This result demonstrates that the Fe^{2+}-induced oxidative damage disrupts plasma membrane integrity. For evaluating the role of PLD activation induced by iron, LDH measurements were also monitored in the presence of ethanol. The presence of this primary alcohol did not modify LDH measurements after 5 min of incubation. However, after 60 min of preincubation, ethanol increased LDH leakage to the same extent whether in the absence or presence of free iron. Unexpectedly, D609 alone provoked an increase in LDH release with respect to the controls in all the assayed conditions.

TBARS generation was increased by 2.16, 3.8, and 4.6 times after 5, 30 and 60 min of incubation with Fe^{2+}. Such increase was completely abolished in the presence of 250 μg/ml D609 (Fig. 6C).

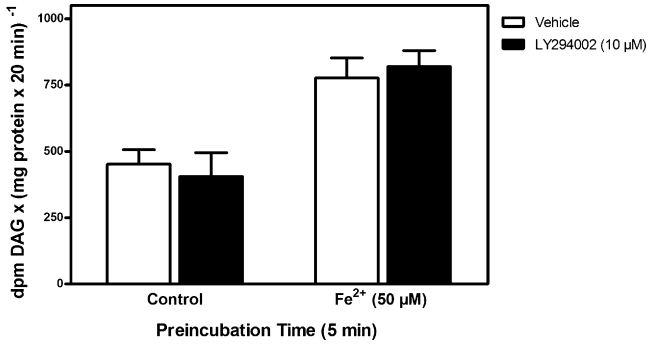

Fig. 5. Involvement of PI3K activity on DAG generation induced by oxidative stress. Rat CC synaptosomes were co-incubated with 10 μM LY294002 and 50 μM $FeSO_4$ or vehicle for 5 min. Enzyme reaction was performed as described in Fig. 1 and results were compared to the control condition and expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D.

3.7. DAG generation from PC in cerebral cortex synaptosomes from aged rats

DAG formation from PC was increased by $FeSO_4$ (50 μM) in synaptosomes from aged rats (28 months old) after 5 and 60 min of incubation. This rise in DAG induced by Fe^{2+} was equivalent to the increase observed in synaptosomes from adult rats (Fig. 7A). The same results were obtained when PC-PLC activity was measured including ethanol 2% in the assay (Fig. 7B). FFA release was inhibited by free iron to the same extent as that reported for adult animals (Fig. 7C). Consequently, $FeSO_4$-induced lipid peroxidation (measured as TBARS generation) to similar levels in Syn from aged animals and from adult rats (Table 1).

4. Discussion

Free iron is highly toxic because of its ability to generate free radicals; ferrous [Fe(II)] iron in particular can generate the highly toxic hydroxyl and superoxide free radicals in the presence of either hydrogen peroxide or molecular oxygen. The effect of free iron is similar to that of β-amyloid peptide (βA), since both are able to induce neuronal damage by promoting membrane lipid peroxidation, impairment of membrane ATPases, glucose and glutamate transport, and mitochondrial function (Butterfield et al., 1999; Andersen, 2004; Berg and Youdim, 2006).

In the present work we show that the generation of the PC-derived lipid messenger DAG is activated in synaptic endings from adult and aged animals exposed to oxidative injury triggered by Fe^{2+} (Fig. 1A). We demonstrate that the generation of this bioactive lipid is due to the activation of PC-PLC and PLD pathways in the synaptic ending (Fig. 2A-D). An increase in DAG generation catalyzed by PC-PLC has been previously associated with reactive oxygen species (ROS) generation during chicken blastodisc differentiation (Zhao et al., 2007). In agreement with these data, the presence of D609, an inhibitor of PC-PLC, has been reported to be involved in neuroprotection mechanisms against oxidative stress in neurons (Perluigi et al., 2006). Moreover, the participation of PLD in oxidative signalling has been described in cardiomyocites and PC12 cells (Banno et al., 2005; Tappia et al., 2006). Our results are in agreement with previous work reporting DAG and PA (measured as PEth) as molecular markers of oxidative stress signalling (Corl et al., 2003; Kim et al., 2004).

The activation of different Ca^{2+}-dependent phospholipase A isozymes producing free arachidonic acid during oxidative stress conditions has been extensively described (Weigel et al., 1997; Sun et al., 2007). However in our experimental model the release of FFA by iron was significantly inhibited (Fig. 1B). These results could be due to the inactivation of a Ca^{2+}-independent PLA2 or possibly the presence of the saturated substrate (DPPC).

We have recently reported that free iron activates the PI3K/Akt pathway in a tyrosine phosphorylation-dependent mechanism in rat CC Syn (Uranga et al., 2007). However, the specific PI3K inhibitor (LY294002) and two tyrosine kinases inhibitors (genistein and herbimycin A), did not affect the levels of DAG in the presence of iron (Figs. 4 and 5). The present findings show that the mechanisms of PC-PLC and PLD activation by free iron seems to be independent of tyrosine phosphorylation and the PI3K/Akt pathway. Furthermore, the results obtained with U73122 suggest the participation of PIP_2-PLC as an upstream effector of PC-PLC and PLD under oxidative injury; however, the inhibition of metabolizing pathways (DAG lipase and DAG kinase) did not alter DAG generation from PC (Fig. 3).

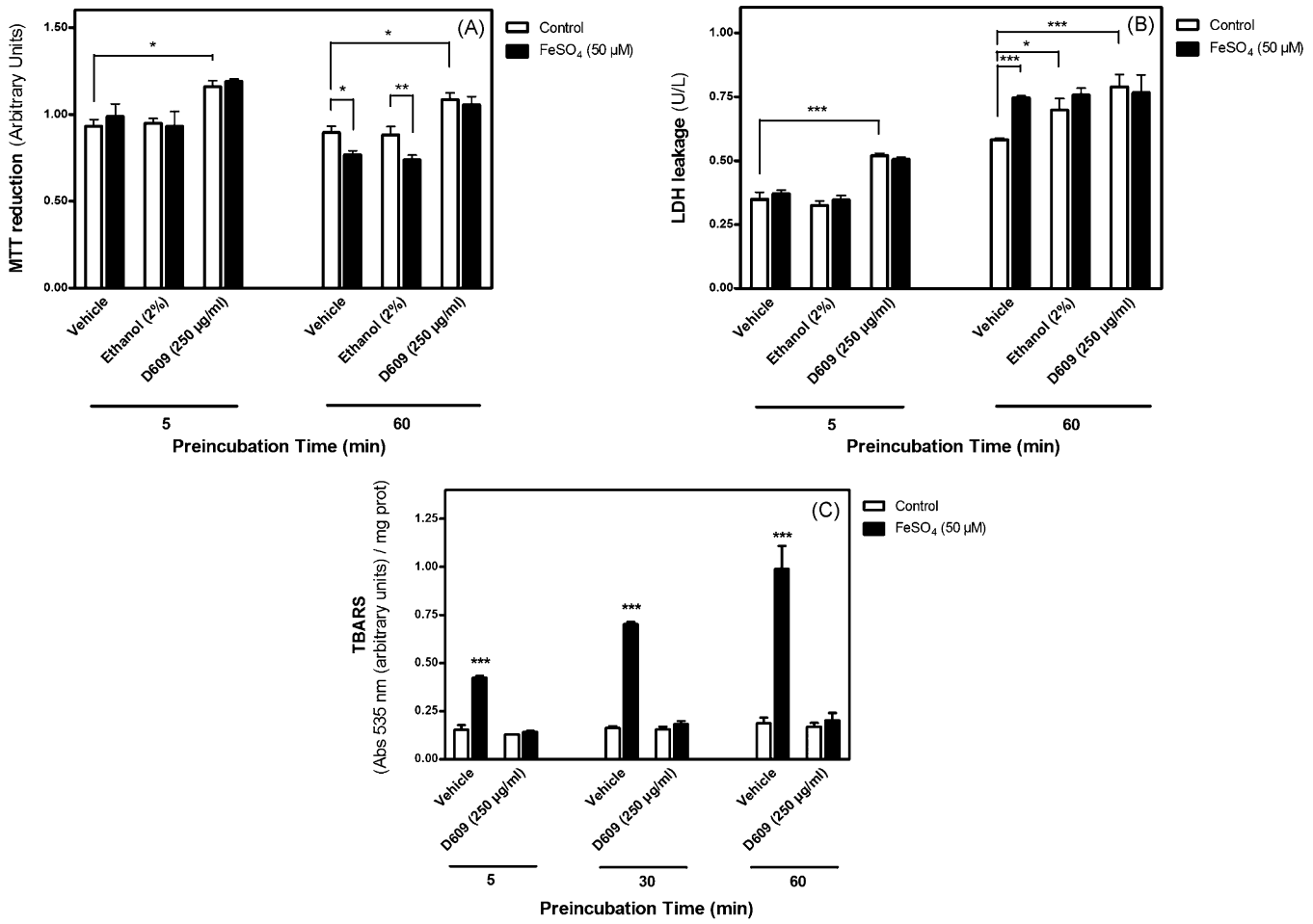

Fig. 6. Effect of $FeSO_4$ on rat CC synaptosomes viability. Synaptosomes exposed to 50 μM $FeSO_4$ or vehicle were co-incubated for 5 and 60 min with 2% ethanol or 250 μg/ml D609. (A) Mitochondrial function under oxidative stress conditions. Mitochondrial function was evaluated by MTT reduction to formazan crystals as described in Section 2. MTT reduction is expressed as arbitrary units. (B) LDH release under oxidative stress conditions. Plasma membrane integrity was monitored by measuring leakage of LDH to the external medium. LDH activity was measured in the external medium as described in Section 2 and is expressed as U/L. (C) TBARS generation under oxidative stress conditions. Syn exposed 50 μM $FeSO_4$ or vehicle were co-incubated for 5, 30 and 60 min with or without 250 μg/ml D609. Lipid peroxidation was evaluated as TBARS generation as described in Section 2. Results are expressed as Abs 535 nm (arbitrary units)/mg protein. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D. and results were compared to the control condition ($^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$).

In synaptosomes exposed to Fe^{2+} there is an increase in lipid peroxidation even after short periods of incubation (Fig. 6C). However, deleterious effects on mitochondrial function and plasma membrane are only observed after longer periods of incubation with the oxidant (Fig. 6A and B). We show here that PC-PLC and PLD activation occur under middle oxidative damage as well as when the deleterious effect of iron is more evident.

D609 inhibits both PC-PLC and sphingomyelin synthase activities. Apart from being an inhibitor of DAG-generating pathways, D609 has been previously proposed as a potent antioxidant and as a membrane fluidizer (Ansari et al., 2006; Moulin et al., 2007). In our experimental model D609 is able to completely prevent lipid peroxidation (measured as TBARS generation) when co-incubated with iron (Fig. 6C). These results demonstrate and confirm the antioxidant properties of this compound. Moreover, D609 was able to prevent mitochondrial damage induced by iron after 60 min (Fig. 6A) but provoked an increase in membrane permeability in all the conditions assayed (Fig. 6B). In view of the many physiological implications of D609 it is difficult to establish the exact role of this compound in synaptosomal viability.

Table 1
Lipid peroxidation induced by iron in synaptosomes from adult and aged animals

TBARS generation Abs 535 nm (arbitrary units)/mg protein						
	5 min preincubation		30 min preincubation		60 min preincubation	
	Control	$FeSO_4$ (50 μM)	Control	$FeSO_4$ (50 μM)	Control	$FeSO_4$ (50 μM)
Adult	0.153 ± 0.025	0.331 ± 0.106	0.162 ± 0.011	0.621 ± 0.087	0.187 ± 0.031	0.866 ± 0.159
Aged	0.146 ± 0.003 (ns)	0.390 ± 0.059 (ns)	0.185 ± 0.017 (ns)	0.651 ± 0.021 (ns)	0.233 ± 0.093 (ns)	0.871 ± 0.009 (ns)

Syn exposed 50 μM $FeSO_4$ or vehicle were incubated for 5, 30 and 60 min. Lipid peroxidation was evaluated as TBARS generation as described in Section 2. Results obtained in aged animals were compared to those obtained in adult animals and are expressed as Abs 535 nm (arbitrary units)/mg protein. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D.

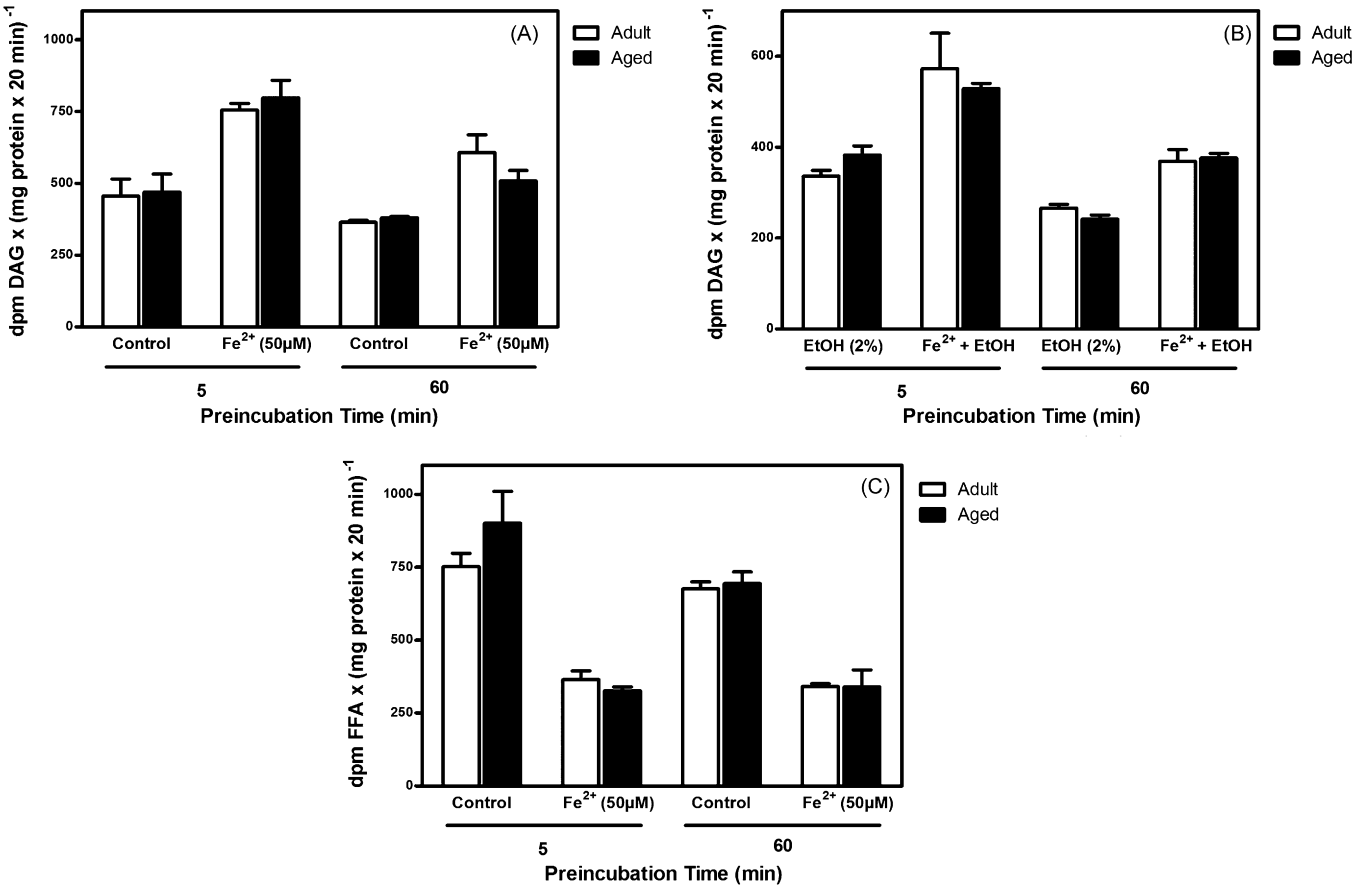

Fig. 7. DAG generation from PC in cerebral cortex synaptosomes from aged rats. Rat CC synaptosomes isolated from adult (4 months old) or aged (28 months old) rats were incubated in the presence of 50 μM $FeSO_4$ or vehicle for 5 and 60 min as described in Fig 1. (A) DAG generation from PC in adult and aged rats. Total DAG generation from PC was measured as described in Fig 1. (B) PC-PLC activity in adult and aged rats. PC-PLC activity was measured in Syn from adult and aged rats by including 2% ethanol in the enzyme reaction mixture as described in Fig 2B. (C) FFA release in adult and aged rats. FFA generation from PC was measured as described in Fig. 1. Results obtained in aged animals were compared to those obtained in adult animals and are expressed as dpm DAG × (mg protein × 20 min)$^{-1}$ or dpm FFA × (mg protein × 20 min)$^{-1}$. Each experiment was performed three times in duplicate, using a pool of three animals on each occasion. Data represent the mean value ± S.D.

Ethanol (used as an inhibitor of PA formation) was not able to prevent mitochondrial damage induced by free iron and as can be observed in Fig. 6B, ethanol is also a membrane fluidizer. It is interesting to note that in the absence of free iron, membrane permeability increases after 60 min of preincubation under all the conditions in which DAG generation is partially inhibited (the presence of ethanol or D609).

In summary, our results indicate that oxidative stress triggered by free iron enhances DAG generation from PC in cerebral cortex synaptosomes from adult and aged rats due to the activation of PC-PLC and PLD pathways. Synaptic endings are considered sites where signal transduction pathways are heavily concentrated and where signalling events exert far-reaching effects on neuronal plasticity and survival (Mattson, 2003). Moreover, PC-derived signalling may play an important role during oxidative stress on neuron cells. Our results constitute the starting point for additional studies aimed at understanding the role of DAG generation from PC during oxidative injury.

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