RAT BRAIN CORTEX MITOCHONDRIA RELEASE GROUP II SECRETORY PHOSPHOLIPASE A$_2$ UNDER REDUCED MEMBRANE POTENTIAL

Lara Macchioni[#], Lanfranco Corazzi[#],Vincenza Nardicchi[#], Roberta Mannucci[§], Cataldo Arcuri*, Serena Porcellati[#], Tito Sposini*, Rosario Donato* and Gianfrancesco Goracci[#]

From the Department of Internal Medicine, Division of Biochemistry and [§]Laboratory of Image Analysis and

*Department of Experimental Medicine and Biological Sciences, University of Perugia, Italy

CORRESPONDENCE TO:
Prof. Gianfrancesco Goracci,
Department of Internal Medicine,
Division of Biochemistry,
Via del Giochetto,
I-06125 Perugia, Italy.
PHONE: +39-075-585-7420
 FAX: +39-075-585-7420 or 7428
e-mail: goracci@unipg.it

Abbreviations: AA, arachidonic acid; BEL, bromoenol lactone; AACOCF$_3$, arachidonoyltrifluoromethyl ketone; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone; DiOC$_6$(3), 3,3'-diexyloxocarbocyanine iodide; FFA, free fatty acids; FL1, green fluorescence; IGFL, integral fluorescence; IL-1-β, interleukin-1-β; PLA$_2$, phospholipase A$_2$; sPLA$_2$, secreted PLA$_2$; cPLA$_2$, cytosolic Ca^{2+} - dependent PLA$_2$; iPLA$_2$, cytosolic Ca^{2+} - independent PLA$_2$; PT, permeability transition; PTP, permeability transition pore; PUFA, polyunsaturated fatty acids; TNF-α, tumor necrosis factor-α.

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SUMMARY

Activation of brain mitochondrial phospholipase(s) A_2 (PLA_2) might contribute to cell damage and be involved in neurodegeneration. Despite the potential importance of the phenomenon, the number, identities and properties of these enzymes are still unknown. Here, we demonstrate that isolated mitochondria from rat brain cortex, incubated in the absence of respiratory substrates, release a Ca^{2+}-dependent PLA_2 having biochemical properties characteristic to secreted PLA_2 ($sPLA_2$) and immunoreacting with the antibody raised against recombinant type IIA $sPLA_2$ ($sPLA_2$-IIA). Under identical conditions, no release of fumarase in the extramitochondrial medium was observed. The release of $sPLA_2$ from mitochondria decreases when mitochondria are incubated in the presence of respiratory substrates such as ADP, malate and pyruvate which causes an increase of transmembrane potential ($\Delta\psi_m$) determined by cytofluorimetric analysis using $DiOC_6(3)$ as a probe. The treatment of mitochondria with the uncoupler CCCP slightly enhances $sPLA_2$ release. The increase of $sPLA_2$ specific activity following removal of mitochondrial outer membrane indicates that the enzyme is associated with mitoplasts. The mitochondrial localization of the enzyme has been confirmed by electron microscopy in U-251 astrocytoma cells and by confocal laser microscopy in the same cells and in PC-12 cells where, the structurally similar isoform type V-$sPLA_2$, has mainly nuclear localization. In addition to $sPLA_2$ mitochondria contain another phospholipase A_2, Ca^{2+}-independent and sensitive to bromoenol lactone, associated to the outer mitochondrial membrane. We hypothesize that, under reduced respiratory rate, brain mitochondria release $sPLA_2$- IIA that might contribute to cell damage.

RUNNING TITLE: PHOSPHOLIPASE A_2 RELEASE FROM   BRAIN MITOCHONDRIA

INTRODUCTION

Phospholipase A_2 (PLA_2) catalyses the hydrolysis of the ester bond of fatty acids at the *sn-2* position of membrane glycerophospholipids. Since this position is rich of arachidonic acid (AA), great attention has been devoted to the relationship between activation of PLA_2 and the production of eicosanoids (1). However, it is well established that the various isoforms of PLA_2 are involved in many other physiological or pathological processes as the remodeling of membrane phospholipids, the removal of oxidized fatty acids, intra- and extra-cellular signaling, inflammation and tissue repair (2, 3).

Since one of the first biochemical events observed after the onset of brain ischemia is the release of free fatty acids from membrane phospholipids, it has been attributed to PLA_2 a relevant role in the consequent brain damage (4-6). A large body of evidence has been also reported that this class of enzyme is involved in chronic neurodegenerative diseases (7).

Now a day, it is well established that various isoforms of phospholipase A_2 are present in brain tissue (8) and it is conceivable that each of them should have specific roles depending on their cellular and subcellular localization, metabolic state of the cell and response to extracellular stimuli. Three main classes of PLA_2 have been found in brain tissue: cytosolic Ca^{2+}-dependent ($cPLA_2$), cytosolic Ca^{2+}- independent ($iPLA_2$) and secretory ($sPLA_2$) (8,9) but their role in various physiological or pathological events is still largely unclear. Particular attention has been devoted to type IV- $cPLA_2$ because this enzyme shows a great specificity for arachidonic acid and it is very likely involved in the production of eicosanoids following receptor-mediated activation of the enzyme (10). Evidence has been also reported suggesting its involvement in neurodegeneration (7). In fact, $cPLA_2$ appears to be present in reactive glial cells, particularly in regions of neuronal loss, and it is not detectable in those brain regions where neurons do not degenerate or are protected from death (11). However, a

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predominant expression of $cPLA_2$ mRNA in neurons of rat brain has been also reported (12) suggesting its involvement in neurotransmission or other neuronal functions through the generation of lipid mediators as arachidonic acid, eicosanoids and PAF (13-15).

Two Ca^{2+}-independent PLA_2 have been isolated from bovine brain cytosol showing different specificities for 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine and ethanolamine plasmalogens (16). More recently, a 80-kDa type $VI-iPLA_2$ was purified from rat brain (9). This enzyme shows a head-group preference for phosphatidylcholine and its specific activity is 20-50 fold higher than that of type $IV-cPLA_2$ depending on brain areas. Secretory PLA_2, type IIA and type V, are also present in all areas of rat brain and typeIIA-$sPLA_2$ is the predominant enzyme of this class and is associated with the particulate fraction whereas type V $sPLA_2$ is mainly found in the soluble fraction (9).

Secretory PLA_2s have a low molecular weight (13-18 kDa), show a low specificity for fatty acids and require mM Ca^{2+} for full activity. They are widely distributed in animal tissues and extracellular fluids and exert many different functions (17, 18). $sPLA_2$-IIA, one of the several groups of this class, was initially purified from synovial fluid (19) and platelets (20). Enzymes belonging to this group are also present in the venom of various snake species and they have a potent neurotoxic effect (21, 22). Since brain tissue possesses specific presynaptic receptors for $sPLA_2$-IIA from snake venoms, the involvement of an endogenous $sPLA_2$ in neurotransmission seems very likely (23, 22). In fact, exogenous $sPLA_2$ modulates AMPA receptor function (24) and an enzyme, immunochemically identical to $sPLA_2$-IIA, has been detected in synaptic vesicles and secreted upon depolarization or by neurotransmitter stimulation (25).

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Since mediators such as tumor necrosis factor-α (TNF-α) and interleukin-1-β (IL-1β) induce sPLA$_2$ mRNA and enzymatic activity in immortalized astrocyte cell lines (26), the involvement of this group of enzymes in inflammation has been also suggested.

The overall message emerging from these reports indicate that, in general, PLA$_2$(s) are involved in physiological mechanisms but they also participate in the development of neurodegeneration following acute ischemia or as the consequence of chronic neurodegenerative diseases. There is a large body of evidence that, in both cases, mitochondrial functions undergo to substantial alterations which reduce their capability of producing ATP and address neurons toward necrotic or apoptotic cell death (27-31).

The presence of PLA$_2$ activity in brain mitochondria is known since early '70s (32-34), but the identities and the functions of the(se) enzyme(s) are still unclear. More recently, it has been reported that gerbil brain mitochondria contains a PLA$_2$, with an estimated molecular weight of 14 kDa, whose activity increases following ischemia and reperfusion (35). This observation and the detection of sPLA$_2$-IIA in rat brain particulate fraction suggests that brain mitochondria might contain a sPLA$_2$, similar to that isolated from liver mitochondria (36), and it prompted us to verify whether the enzyme could exit from the organelle under impairment of mitochondrial functions, similarly to other mitochondrial proteins such as cytochrome c and caspase-9 (37,38). In this study, we have demonstrated that mitochondria purified from rat brain cortex and mitochondria of neuron-like PC-12 cells and U-251 astrocytoma cells contain an enzyme having biochemical and immunological properties characteristic to sPLA$_2$-IIA. By contrast, in the same cell types, sPLA$_2$-V is not present in mitochondria but it has mainly cytosolic and nuclear localization. The enzyme present in isolated rat brain cortex mitochondria is very likely associated to mitoplasts and it is released

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as the consequence of a reduction in membrane potential. Rat brain cortex mitochondria contain also a Ca^{2+}-independent PLA_2 that appears to be localized on the outer membrane.

MATERIALS AND METHODS

Materials. [^{3}H] oleic acid (10.3 Ci/mmol) was purchased from NEN Research Products (Boston, MA, USA). C. Atrox PLA_2, bovine serum albumin (BSA fraction V), digitonin, trypsin, pronase and dithiothreitol were from Sigma Chemical (St. Louis, MO,USA). Bromoenol lactone (BEL) and arachidonoyltrifluoromethyl ketone (AACOCF$_3$) were purchased from Biomol Research Lab. Inc. (USA) and Calbiochem (San Diego, USA), respectively. Silica Gel 60A plates (Whatman International Ltd, Maidstone, UK) were used for thin-layer chromatography. Human synovial sPLA$_2$ was from Alexis Biochemicals. BioMax-MR2 FILM intensifying screens were from Kodak (Rochester, NY, USA). 3,3'-diexyloxocarbocyanine iodide [DiOC$_6$(3)], Mito-TrackerRed CM-Xros, Alexa 488 and TO-PRO-3 were from Molecular Probes Europe BV, Leiden, The Netherlands). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was from Fluka Chemie AG (Italy). ADP(K$^+$salt), malic and pyruvic acid were purchased from Roche Molecular Biochemicals (Italy). Immunogold Conjugate EM Goat Anti-Mouse IgG (H+L) : 5-nm (British BioCell International).

Preparation of E. coli labeled substrate. Labeled E. coli substrate, generally used for assaying secreted phospholipase A$_2$ (39-41), was prepared as previously reported (42). Briefly, bacteria (E. coli ATCC25922) were grown overnight in triethanolamine medium and resuspended in fresh medium containing [9-10 ^{3}H]oleic acid (10.3 Ci/mmol) complexed with 0.02% bovine serum albumin (fatty acid free). After incubation (3 hours at 37°C), bacteria suspension was autoclaved (20 min at 120 °C and 1 kg/cm^2). After washings, labeled E. coli was sedimented by centrifugation at 15,000 g for 30 min. The specific radioactivity of this

substrate (0.7-3 nCi/nmol phosphatidylethanolamine) was calculated by measuring the radioactivity associated with phosphatidylethanolamine, which represents ≈ 70% of total phospholipids and contained over 90 % of the incorporated labeled oleic acid.

Preparation of rat brain cortex mitochondria. Mitochondria, prepared from rat brain cortex (CD, 2 months old, Charles River, Italy) were purified on sucrose gradient as previously reported (43) and then resuspended in an isotonic medium containing 2 mM HEPES (pH 7.4) and 0.32 M sucrose (S/H buffer). The purity of brain mitochondria was checked by marker assay (44). The specific activity of cytochrome c oxidase (mitochondrial marker enzyme) was 5.7 times higher in mitochondria than in homogenate, whereas NADPH-cytochrome c reductase (microsomal marker enzyme), Na^+, K^+-ATPase (plasma membrane marker) and Arylsulphatase A (lysosomal marker) were less than 5% with respect to the homogenate (45). The integrity of the outer mitochondrial membrane was in the range 90-93%, as calculated by the latency in cytocrome c oxidase assay (45). In some experiments, mitochondria (2-3 mg protein/ml of 0.32 M sucrose and 2 mM HEPES pH 7.4) were incubated with 50 μg of trypsin or pronase at 30 °C, a concentration known to be ineffective on mitochondrial functional parameters (46). After 30 min, aliquots were taken and mitochondria recovered by centrifugation at 10,000g for 10 min. Protein content was determined by the method of Bradford (47) using bovine serum albumin as standard.

Preparation of mitoplasts. Mitoplasts were prepared as previously described (48). Briefly, purified mitochondria were treated with digitonin (0.4 mg/mg protein) for 30 min at 0 C° and then loaded on sucrose gradient for their separation from intact mitochondria.

Cytofluorimetric analysis of mitochondrial membrane potential ($\Delta\Psi_m$). Cytofluorimetric analysis of mitochondrial membrane potential was done as previously reported (48). Briefly, purified mitochondria (0.3 mg protein) were incubated for 10 min, at room temperature, in

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the presence of $DiOC_6(3)$ (1μM), in different respiratory conditions. Suspensions were immediately subjected to cytofluorimetric analysis, using a FACScan flow cytometer (Becton Dickinson Immunocytometry System) equipped with a focused argon laser. For complete depletion of $\Delta\Psi_m$ (positive control), the mitochondria uncoupler CCCP (100 μM) was used. Data were analyzed and stored with the use of a data management system (LYSYS software). $DiOC_6(3)$ green fluorescence (FL1) was plotted on a logarithmic scale vs. the frequency of events. The mean value of the integral of fluorescence (IGFL) was also evaluated.

Determination of phospholipase A₂ activity released from mitochondria. Purified mitochondria were resuspended in S/H buffer (resting state) or, when indicated, in the same buffer containing respiratory substrates or the uncoupler CCCP. The pH of the solutions of ADP, pyruvate and malate were taken to pH 7.4 with KOH before additions. In any case Ca^{2+} was not added and its concentration in S/H buffer, measured with Fura-2 (acid) (42), was 1 μM. Mitochondria were incubated at 37°C and then pelleted at 10,000g for 10 min. Control samples (T_0) were prepared in identical conditions but they were not incubated. A convenient aliquot of the supernatant was taken for PLA₂ assay and incubated at 37°C with [³H]oleate labeled E. coli (approximately 30 nCi per sample) in a total volume of 300 μl containing 125 mM Tris-HCl (pH 7.4), 2mM $CaCl_2$ and 1mg/ml BSA (fatty acid free). The reaction was stopped by the addition of 150 μl 2N HCl and 150 μl of BSA (20 mg/ml). After a period of 20 min at 4°C, labeled bacteria were pelleted at 10,000g for 5 min. The radioactivity of the supernatant, containing free [³H]oleic acid, was determined (49). The activity of PLA₂ was calculated by subtracting blank sample radioactivity from that of samples with extramitochondrial medium.

*Incubation of intact mitochondria or mitoplasts with phospholipase labeled E. coli substrate.*Labeled E. coli substrate was incubated with purified mitochondria resuspended in

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S/H buffer (2-3 mg protein/ml) under the following conditions: A) 125 mM Tris-HCl (pH 7.4) with 2 mM $CaCl_2$; B) 125 mM Tris-HCl (pH 8) with 1 mM EGTA. When indicated mitochondria were preincubated with 5 mM DTT or with 30 μM $AACOCF_3$ or with 50 μM BEL.

Determination of fumarase activity. Fumarase activity was determined spectrophotometrically at 240 nm. The supernatant (4-10 μg protein), recovered after the incubation of mitochondria in S/H buffer, was incubated with 0.5 mM malate for 15 min at 25 °C in 0.35 ml of phosphate buffer (100 mM, pH 7.4) (50).

Western blot analysis. Western blot analysis of proteins, released from rat brain cortex mitochondria, was performed using monoclonal antibody against rat $sPLA_2$ -IIA (generous gift from Dr. H. van den Bosch and Dr. J. Aarsmann, Utrecht) essentially as indicated by van der Helm *et al.* (51). Briefly, mitocondria purified from 10 rat brain cortices, were incubated in S/H buffer for 60 min at 37°C and the incubation medium was subjected to dialyse against 50 mM Na-acetate pH 4.5 in the presence of 0.2 M NaCl at 4°C overnight (52). Samples were concentrated and then subjected to Western blot analysis. Blot was analyzed using Duoscan T1200 (AGFA, Seifert, Germany) scanning densitometer and Quantity One 4.4 software (Bio-Rad, Laboratories, Hercules, CA).

Cell cultures. PC-12 cells were cultured in RPMI 1640 (Euro Clone, UK) supplemented with 10 mM HEPES, 25 mM glucose, 2 mM glutamine, 1mM sodium-pyruvate, 100 U/ml penicillin, 100μg/ml streptomycin, 10%(v/v) horse serum and 5%(v/v) foetal bovine serum, in a H_2O-saturated 5% CO_2 atmosphere at 37°C. Cells were plated on poly(L-lysine)-coated coverglasses (13 mm in diameter) in 4-multiwell plates (3 x 10^4 cell/well) and cultured for 48h.

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U-251 astrocytoma cells were cultured in α-MEM medium supplemented with 10% (v/v) foetal calf serum (Gibco, Grand Island, NY), 100U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone in a H_2O-saturated 5% CO_2 atmosphere at 37 °C. U-251 cells were plated on glass cover slips (13 mm in diameter) in 4-multiwell plates. Cells were seeded at 3 x 10^4 cell/well and cultured for 48h at 37 °C.

Confocal immunofluorescence microscopy. Both PC-12 and U-251 cells, after washings with phosphate buffered saline (PBS), were placed on cover slips and loaded with 500 nM Mito-Tracker Red CM-XRos at 37°C for 15 min. Cells were fixed and permeabilized with a mixture of methanol-acetone (1:1 by vol) at -20 °C for 10 min. When indicated PC-12 cells were fixed with methanol at –20°C for 7 min, rinsed in PBS, and treated with 0.1% (v/v) Triton X-100 in PBS for 10 min at room temperature. Non-specific binding of antibodies or antisera was blocked by a preliminary incubation of fixed cells with 1% (w/v) BSA in PBS (blocking buffer). This was followed by the incubation at room temperature for 1h and 30 min with monoclonal antibody against rat sPLA$_2$-IIA diluted 1:10 in blocking buffer or with polyclonal antibody against sPLA$_2$-V (1:100 in blocking buffer). The cells were washed for 20 min in PBS and incubated with rabbit Ig conjugated to Alexa 488 diluted 1:100 in blocking buffer, at room temperature for 1h. After washing with PBS for 20 min, cells were loaded with 10μM TO-PRO-3, a monomeric cyanine stain for double-stranded DNA giving a blue fluorescence, for 7 min at room temperature. Confocal analysis was performed with a confocal microscope (Bio-Rad MRC 1024, Bio-Rad Laboratories) using an Ar/Kr laser. The fluorescences of Mito-Tracker and Alexa 488 were detected with excitation wavelengths of 568 and 488, respectively, whereas that of TO-PRO-3 was at 647 nm. Images were elaborated with the colocalization module of Imaris software (Bitplane, Zurich, Switzerland) on a SGI Octane workstation.

Electron microscopy. U-251 astrocytoma cells were rapidly frozen in a Cryopress device (Med Vac Inc., St. Louis, USA) by impact onto a liquid helium-cooled copper block. Frozen samples were rapidly transferred into a Balzers FSU 010 cryosubstitution unit and treatedwith: osmium tetroxide in 2% methanol for 8 hours at –90°C, for 8 hours at –60°C and other 8 hours at –30°C. Osmium tetroxide was then removed by two washings with methanol at –30°C. After cryosubstitution, samples were embedded in Lowcryl K4M as described by Carlemalm et al. (53).

Cryosections of U251 cells were treated with Na-metaperiodate for 1 hour at room temperature and then incubated at room temperature for 10 min in PBS containing 3% BSA, 20mM NaN_3 (buffer A). Next, samples were incubated in buffer A containing 1% normal goat serum (NGS) and 100mM glycine for 4h at room temperature. This step was followed by incubation for 3 hours at room temperature and then overnight at 4°C with a monoclonal anti-sPLA$_2$-IIA antibody (1:20 in buffer A + 1% NGS). Sections were washed in buffer A containgng 1% Tween-20 (five times, 1 min each), and incubated for 3 hours at room temperature with goat anti-mouse IgG antibody labelled with 5-nm gold particles (1:80). Section were washed in PBS, post-fixed for 3 min in 2% glutaraldehyde in PBS and counterstained with uranyl acetate. Sections were finally examined by electron microscopy (Philips TEM 208).

Statistical analysis. Statistical significance of the data was evaluated by ANOVA followed by Student's t test or Scheffe's multiple comparison test. Differences were considered statistically significant when p<0.05.

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RESULTS

Release of phospholipase A_2 from rat brain cortex mitochondria: time dependence.

A time dependent and highly significant increase of PLA_2 activity was observed in the extramitochondrial medium after incubation of purified mitochondria in S/H buffer (pH 7.4) (Fig. 1). After 60 min, PLA_2 specific activity in the medium was near 6 times that of non-incubated samples (T_0). A further increase was observed after 90 min but a greater variability of the data was noticed.
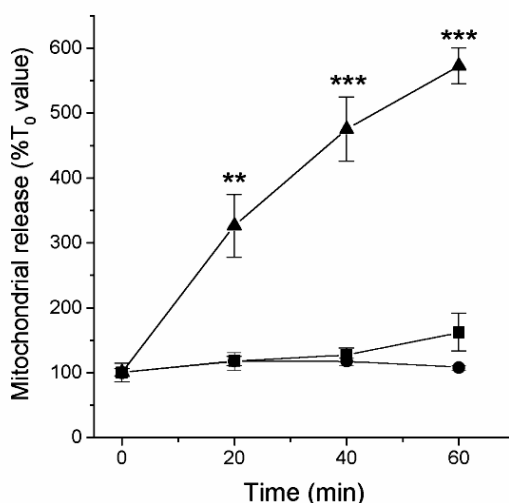
Fig.1. *Release of PLA_2 from mitochondria.*

Mitochondria (0.2-0.3 mg protein) were incubated in S/H buffer (pH 7.4) at 37°C for the indicated time intervals. Mitochondria were pelleted at 10,000g for 10 min and supernatant was assayed for PLA_2 (▲), fumarase (●) or total protein content (■). Results are expressed as percent of values (mean ± SEM) with respect to medium of non incubated mitochondria and are from three independent experiments in duplicate. ** p<0.01; *** p<0.001 vs non incubated samples

The homogenization of brain cortex in the presence of 100 μg/ml heparin reduced PLA_2 activity of the homogenate to 40 % of that measured in control samples (homogenate without

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heparin). Purified mitochondria were then prepared from both homogenates and the release of sPLA$_2$ was determined as described above. No differences were observed in the specific activity of the enzyme released from mitochondria prepared in the presence or in the absence of heparin even if the direct addition of this compound to the assay system reduced PLA$_2$ activity by 70% (data not shown). This indicates that the mitochondrial enzyme was not accessible to heparin during the homogenization of the tissue and during the preparation of mitochondria. Consequently, it had to be associated with the inner mitochondrial compartments in the tissue before the homogenization.
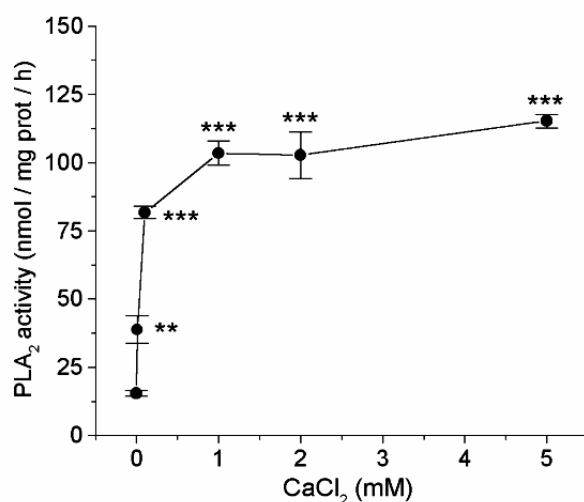
Fig. 2. *Effect of Ca^{2+} concentration on the activity of PLA$_2$ released from mitochondria.*
Mitochondria were incubated in S/H buffer (pH 7.4) at 37°C for 60 min and PLA$_2$ activity was determined in the extramitochondrial medium as in Fig. 1, with EGTA (10 μM) or with increasing Ca^{2+} concentrations. Data are expressed as nmol of released [^{3}H]oleate /mg prot/h and are the mean ± SEM from two independent experiments in triplicate. **p<0.01 or ***p<0.001 vs EGTA.

In order to exclude that the observed phenomenon could be due to an unspecific mitochondrial leakage, the same medium was assayed for fumarase and protein content and

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no significant changes were observed with time up to 90 min. Thus, it is conceivable to conclude that PLA$_2$ is preferentially released from rat brain cortex mitochondria with respect to other mitochondrial proteins.

Properties of the released mitochondrial phospholipase A$_2$. In order to provide a characterization of the released PLA$_2$, we have assayed the effect of Ca^{2+} concentration on its activity. As shown (Fig. 2), the rate of substrate hydrolysis significantly increased with 10 μM Ca^{2+} and was maximal at mM concentration. A relatively low activity (15.5 ± 1.0 nmol/mg protein/h) was measured in the presence of EGTA (10 μM).
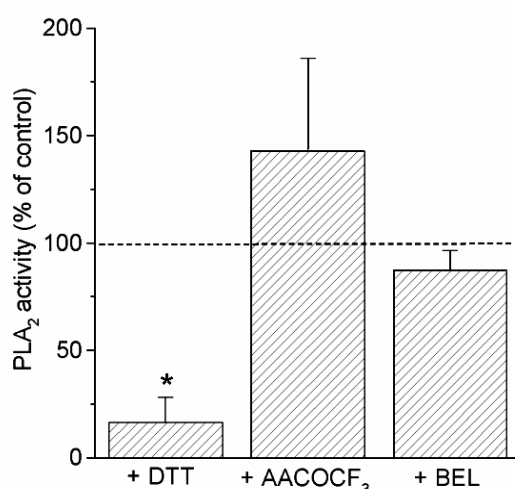

Fig. 3. *Effect of inhibitors on released mitochondrial PLA$_2$ activity.*

Experiments were carried out as in Fig. 1 but the extramitochondrial medium was preincubated with DTT (5 mM for 30 min at 37°C), or AACOCF$_3$ (30 μM for 30 min at 37°C) or BEL (50 μM for 30 min at 40°C) and then PLA$_2$ activity was assayed. Data are mean ± SEM from two experiments in duplicate. * P<0.05 vs control set as 100%.

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A significant reduction of enzyme activity (-80%) was observed in the extramitochondrial medium, with DTT (5 mM), which is an inhibitor of sPLA$_2$ (54). BEL, a specific inhibitor of Ca^{2+}-independent PLA$_2$ (55) and AACOCF$_3$, known to inhibit type IV cytosolic Ca^{2+}-dependent PLA$_2$ (56), were both ineffective (Fig.3).

Western blot analysis of proteins, released upon incubation of mitochondria, revealed that monoclonal antibody against rat liver sPLA$_2$ recognized a protein having a molecular weight ≈ 14 kDa, similar to that of human synovial sPLA$_2$ which did not immunoreacted with the antibody (Fig.4). On the basis of this result, the protein will be referred as type IIA-sPLA$_2$ even if it cannot be completely excluded that the antibody might recognize another isoform of the same group.

From densitometric analysis and from total amount of proteins present in whole mitochondria and in the medium 60 min after incubation in the absence of respiratory substrates, it was possible to estimate that near 50% of the total mitochondrial enzyme immunoreacting with sPLA$_2$ –IIA antibody was released.
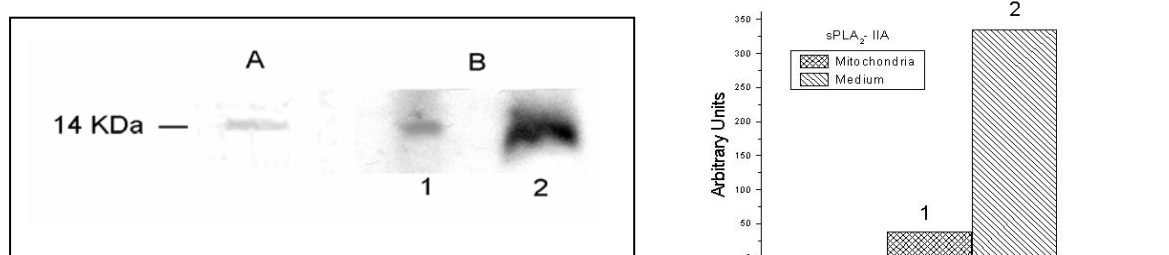
g. 4. Western blot analysis of released mitochondrial proteins.

50 μg of both whole mitochondrial proteins (21 mg total) and of proteins, present in the extramitochondrial medium (1.2 mg total) after incubation as in Fig.1, were subjected to electrophoresis in 15% acrylamide SDS gel and to blot analysis. The blots were probed with monoclonal antibody against PLA$_2$-IIA (1:60 dilution). (Left

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panel) Lane A - 14 kDa sPLA$_2$ (human synovial stained by Ponceau); Lane 1B, mitochondria; lane 2B extramitochondrial medium after incubation in S/H buffer for 60 min. (Right panel) Densitometric analysis.

Effect of respiratory substrates on membrane potential and on the release of sPLA$_2$ from mitochondria. The respiratory activity of purified mitochondria resuspended in S/H buffer (pH 7.4) was evaluated by flow cytometric analysis in different metabolic states. Single-parameter fluorescence histograms of mitochondria stained with the membrane-potential-sensitive DiOC$_6$(3) are reported in Fig. 5. The addition of respiratory substrates caused a shift towards higher fluorescence intensity with respect to that of purified mitochondria resuspended in S/H buffer only (resting state) whereas the treatment with the uncoupler CCCP caused the opposite effect.
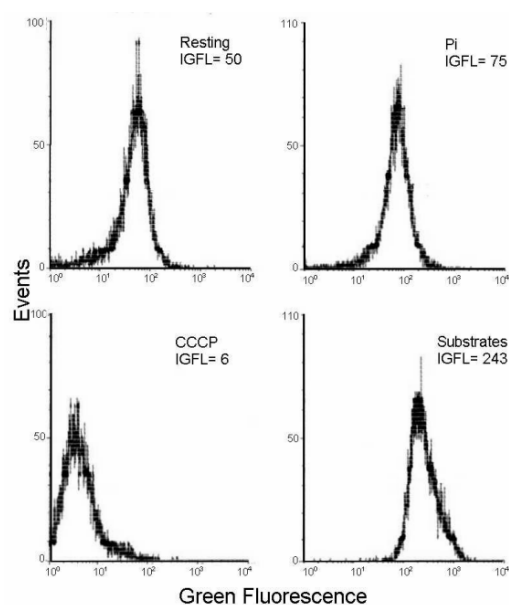
Fig. 5. *Flow cytometric analysis of DiOC$_6$(3) stained mitochondria: frequency histograms.*

Mitochondria (0.3mg protein) were resuspended in S/H buffer (pH 7.4), loaded with DiOC$_6$(3) and incubated at room temperature for 10 min in different respiratory conditions (1 mM K-phosphate; 0.6 mM ADP, 1.5 mM

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pyruvate and 3 mM malate; 100 μM CCCP). The relative number of mitochondria, having the fluorescence intensity indicated in abscissa, is reported in the ordinate axis.

Since the increase of fluorescence correlates with an increase of membrane potential and consequently with the metabolic state of mitochondria, we hypothesized that the reduction of respiratory rate could trigger the release of sPLA$_2$ from mitochondria.

Therefore, PLA$_2$ release in different metabolic conditions was tested. As shown in Fig.6, the addition of respiratory substrates caused near 60 % reduction of the release of mitochondrial sPLA$_2$ whereas a slight increase was observed in the presence of CCCP (100μM) that caused a collapse of $\Delta\Psi_m$ (Fig. 5). Under identical conditions, the addition of CCCP to samples containing ADP-malate-pyruvate partially reversed the effect of respiratory substrates on the release of sPLA$_2$ from mitochondria.
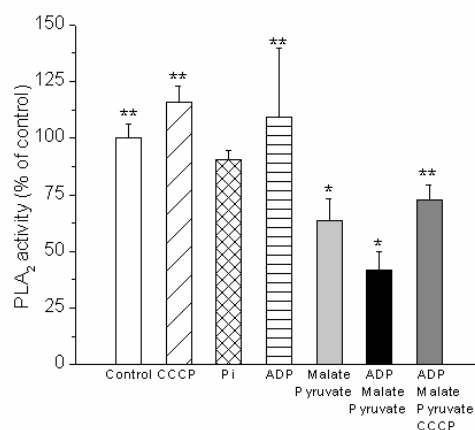

Fig. 6- *Effect of respiratory state on the release of mitochondrial sPLA$_2$.*

Rat brain cortex mitochondria were incubated for 30 min at 37°C in S/H buffer (pH 7.4) (resting state). Respiratory activity of mitochondria was modulated with ADP (0.6 mM), pyruvate (1.5 mM) and malate (3.0 mM) combined as indicated.. When indicated 1mM K-phosphate or 100 μM CCCP was added. After the incubation, mitochondria were pelleted by centrifugation for 10 min at 10,000g and supernatant utilized for

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PLA$_2$ activity assay. Data mean ± SD are expressed as percent of PLA$_2$ activity in resting samples and are from four independent experiments in triplicate. Statistical analysis was performed by ANOVA followed by Scheffe's multiple comparison test. * p<0.05 vs control. ** p<0.05 versus ADP-malate-pyruvate

These observations indicate that a reduction of membrane potential in mitochondria, isolated from rat brain cortex, induces the release of an enzyme having identical biochemical and immunological properties of sPLA$_2$ -IIA.

Incubation of [^{3}H]oleic acid labeled E. coli with mitochondria from rat brain cortex.

We have also assayed the liberation of labeled oleic acid from E. coli substrate by incubating rat brain cortex mitochondria, without respiratory substrates, in S/H buffer with 2 mM Ca^{2+} or in the presence of 1 mM EGTA at different pHs (Fig. 7A). In the presence of Ca^{2+} the maximal rate of the liberation of labeled oleic acid was at pH 7.4 whereas it was at pH 8.0 when Ca^{2+} was substituted by EGTA.
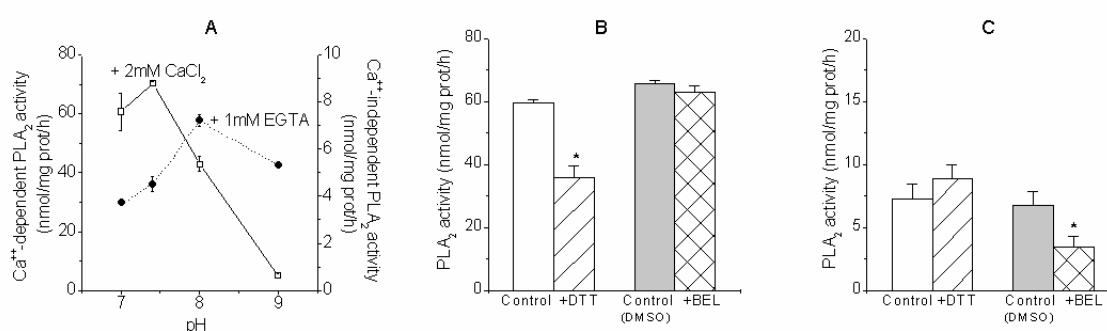

Fig. 7. *Effect of PLA$_2$ inhibitors on the hydrolysis of [^{3}H]oleate E.coli by rat brain cortex mitochondria.*

A) Purified mitochondria (30 μg protein) were incubated in 125 mM Tris-HCl buffer, at various pH values, in the presence of 2 mM Ca^{2+} ( ) or 1 mM EGTA (·); purified mitochondria were preincubated with DTT (5 mM for 30 min at 37°C) or BEL (50 μM for 30 min at 40°C) and then PLA$_2$ was assayed in the presence of 2 mM Ca^{2+} in 125 mM Tris-HCl pH 7.4 (B) or 1mM EGTA in 125 mM Tris-HCl pH 8.0 (C).. Data are expressed

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as nmol of released [³H]oleate/mg prot/h and are the means ± SEM of two experiments in duplicate. * P<0.05 vs control.

The preincubation of mitochondria with 5 mM DTT, reduced the hydrolysis of the substrate in the presence of 2 mM Ca^{2+} (pH 7.4) that was not affected by BEL, known to inhibit Ca^{2+}-independent PLA_2 (Fig. 7B). On the other hand, when PLA_2 activity was assayed at pH 8.0 without Ca^{2+} (Fig. 7C), the treatment with BEL caused a significant decrease of the liberation of labeled oleic acid and was not affected by DTT.

These observations indicate that the direct incubation of purified brain mitochondria with E. coli labeled substrate allows the detection of two distinct PLA_2s, one Ca^{2+}-dependent and the other Ca^{2+}-independent, confirming previous reported data with rat liver mitochondria (57).

To verify the localization of the two enzymes, purified mitochondria were treated with proteases or digitonin and then assayed for PLA_2 activity.

Mitochondria were treated with trypsin or pronase (25 µg/ mg mitochondrial protein) and then assayed for Ca^{2+}-independent or Ca^{2+}-dependent activities. The choice of protease concentration was based on the observation that it did not affect mitochondrial membrane potential, as previously shown (46). The treatment with proteases caused more than 50% decrease in the activity of the Ca^{2+}-independent enzyme whereas it did not affect that of the Ca^{2+}-dependent one (Fig. 8A). The partial hydrolysis of the Ca^{2+}-independent enzyme indicates that it is not fully accessible to proteases.
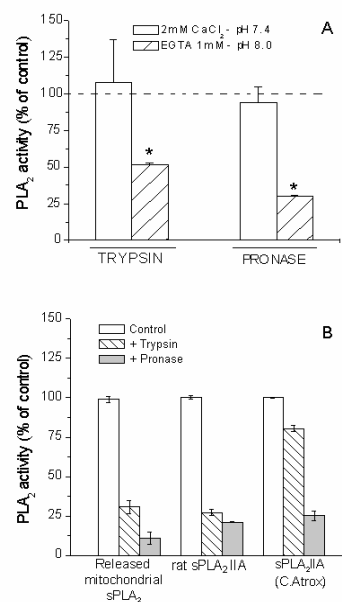
Fig.8. *Effect of trypsin or pronase treatment on mitochondrial PLA$_2$ activities.*

Aliquots of rat brain cortex mitochondria were treated with trypsin or pronase as indicated under Materials and Methods section and then assayed for PLA$_2$ activity in 125mM TrisHCl (pH 7.4) containing 2mM CaCl$_2$ or with 1mM EGTA at pH 8.0. Data are expressed as percent of untreated controls and are the means ± SEM of two experiments in duplicate. *P<0.05 vs control. B) Released mitochondrial sPLA$_2$, rat recombinant sPLA$_2$ - IIA and sPLA$_2$IIA from C. Atrox, were preincubated with trypsin or pronase as indicated under Materials and Methods section and then assayed for PLA$_2$ activity in 125mM Tris-HCl (pH 7.4) containing 2mM CaCl$_2$. In these experiments, lipids of control and treated samples were extracted and the radioactivity of oleic acid released from labeled E. coli substrate was determined after separation by TLC (42). Data are expressed as percent of untreated controls and are the means ± SEM of two experiments in duplicate.

Since, under identical conditions, recombinant sPLA$_2$-IIA (gift from Prof. H. van den Bosch), sPLA$_2$ released from rat brain mitochondria and sPLA$_2$ II-A from Crotalus Atrox venom were inactivated by incubation with proteases (Fig. 8B), it can be concluded that Ca^{2+}-dependent enzyme is not accessible to proteolysis in intact mitochondria.

Similar experiments were performed with digitonin treated mitochondria that allowed the removal of the outer membrane and the isolation of the mitoplasts (48). As shown in Fig. 9A, the specific activity of the Ca^{2+}-dependent PLA$_2$ increased in mitoplasts with respect to intact

mitochondria and it was significantly reduced by the preincubation of mitoplasts with DTT (Fig. 9B).

The 4-fold increase of the specific activity cannot be accounted for only by the removal of the outer membrane proteins but it may reflect an easier release of the enzyme or a better interaction with the labeled substrate. Hydrolysis of the labeled substrate was also observed upon incubation of mitoplasts in the presence of EGTA but to a lesser extent than that measured with purified mitochondria (Fig. 9A).
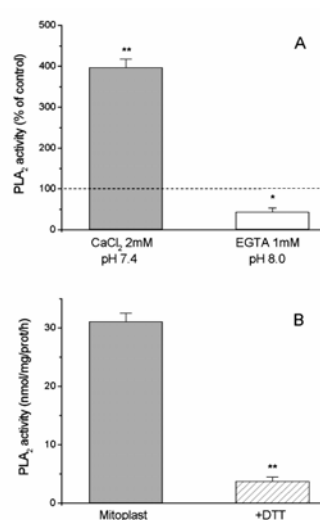

Fig. 9. PLA$_2$ activities in mitoplasts from rat brain cortex.

A) Mitoplasts (30μg protein) were incubated with [³H]oleate E.coli for 1h at 37°C in 125 mM Tris-HCl (pH 7.4) with 2 mM Ca²⁺ or at pH 8.0 with 1mM EGTA. PLA$_2$ activities in mitoplasts are expressed as percent of that measured in mitochondria under identical conditions. B) Mitoplasts were preincubated with or without DTT (as in Fig. 5) and PLA$_2$ activity (nmol/mg protein/h; mean ± SEM) was assayed in 125mM Tris-HCl (pH 7.4) with 2 mM Ca²⁺. Data are from two independent experiments in duplicate. A) * $p < 0.05$ or ** $p < 0.01$ vs mitochondria set as 100%; B) ** $P < 0.01$ vs untreated mitoplasts.

These experiments allowed to detect two distinct PLA$_2$s in rat brain mitochondria: one Ca^{2+}-dependent and associated with mitoplasts, very likely the sPLA$_2$-IIA that is released under certain conditions, and the other one Ca^{2+}-independent and sensitive to BEL present mainly in the outer membrane, which might be the cytosolic iPLA$_2$ previously reported (9) or that found in liver mitochondria (57). In the latter case, we have to suppose that, under our experimental conditions, a change in iPLA$_2$ localization had to take place since this enzyme appears to be present in the inner mitochondrial membrane (57) and it might be responsible for the activity that we have found in mitoplasts in the presence of EGTA (Fig. 9A).

Subcellular localization of sPLA$_2$-IIA and sPLA$_2$-V in PC-12 and U-251 cells.

The presence of sPLA$_2$-IIA in mitochondria was also assessed in neuron-like PC-12 cells which were analyzed by confocal laser microscopy using monoclonal antibody raised against the liver enzyme (51) (Fig. 10 B-green signal) and showed an high degree of colocalization (Fig. 10 C, D) with the red signal of Mito-Tracker which accumulates in active mitochondria (Fig. 10 A) (58). As shown in panel E the percentage of colocalization in various cell layers reached a maximum of 20%. A similar degree of colocalization was obtained when cells were fixed with methanol and acetone, thus avoiding Triton-X100 treatment (Fig. 10 H).

In order to assess the specificity of monoclonal antibody against mitochondrial sPLA$_2$-IIA and to demonstrate that its colocalization with Mito-Tracker dye cannot be the consequence of an artifact of the experimental procedure, experiments with PC-12 cells were repeated comparing the subcellular localization of sPLA$_2$-IIA with that of another secretory PLA$_2$, sPLA$_2$-V, which is also present in rat brain (9). The sequences of the two enzymes have 41% identity as suggested by CLUSTAL W database (ENBL-EBI) (59) and, in various cell types, they are both expressed with an N-terminal sequence typical of secreted enzymes (60,61).

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Both enzymes are highly cationic since their isoelectric points, calculated in Swiss-Prot/TrEMBL, are 9.26 and 8.67 for sPLA$_2$-IIA and sPLA$_2$-V, respectively. Despite the two enzymes have very similar structural properties, their subcellular localization is quite different.
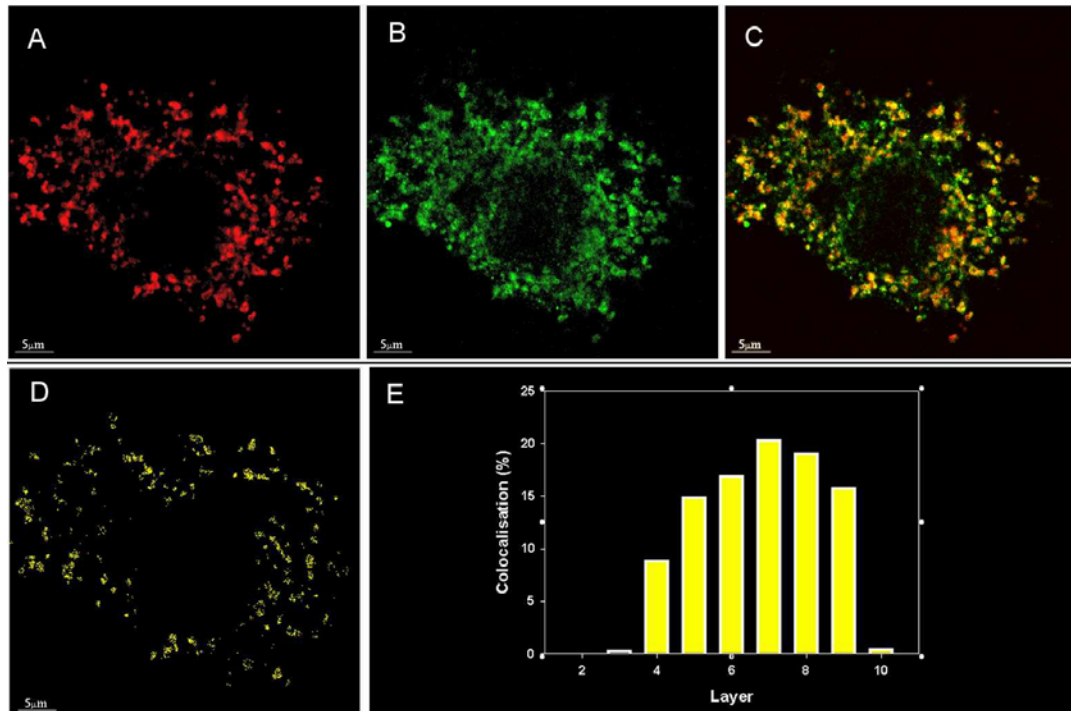

Fig.10. *Immunofluorescence analysis of the anti-sPLA$_2$ –IIA localization by confocal microscopy.* Cells fixed and permeabilized with Methanol/Triton X-100; A) Distribution of Mito-Tracker Red fluorescence; B) Distribution of anti-sPLA$_2$-IIA (Alexa 488, green fluorescence) ; C) Overlay of green and red fluorescence indicating colocalization (yellow areas); D) Colocalization calculated from the two dimensional plot of the frequency of intensity pairs for the green and red fluorescence of a representative layer; E) Percentage of colocalization in each section of the confocal image

In fact, as shown in Fig. 11B, mitochondria are devoid of sPLA$_2$-V since the enzyme does not colocalize with Mito-Tracker (red fluorescence) but it is mainly present in punctate cytoplasmic structures (green fluorescence). Furthermore, it can be observed a certain degree of colocalization in a central section of the cells where sPLA$_2$-V (green fluorescence) colocalizes with the nuclear marker TO-PRO-3 (blue fluorescence). Figs.11C and 11D show

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confocal immunofluorescence analysis of the subcellular distribution of sPLA$_2$-IIA and sPLA$_2$-V in U-251 astrocytoma cells. The choice of a glial cell line was suggested by the consideration that astrocytes are quantitatively predominant in brain cortex and that both sPLA$_2$ are expressed in these cells (62). It is quite evident that, while sPLA$_2$-IIA has a large colocalization with perinuclear mitochondria (C; yellow signal), sPLA$_2$-V colocalize with the nuclear marker (D; green plus blue fluorescence) and it is completely absent in mitochondria. Both isoforms are also present in cytosol.
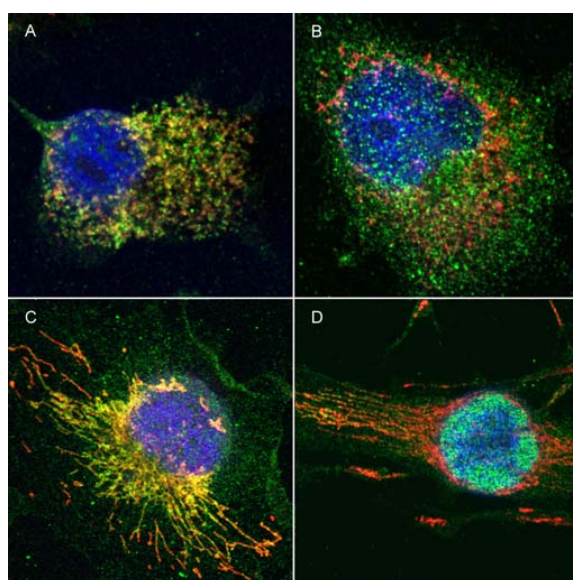

Fig.11. *Immunofluorescence analysis of the anti-sPLA$_2$-IIA and anti-sPLA$_2$-V localization in PC-12 and U-251 astrocytoma cells by confocal microscopy.*

Cells were stained with Mito-tracker (red; mitochondria) and TO-PRO-3 (blue; nuclei), fixed and permeabilized with Methanol/Acetone (1:1) as described in Materials and Methods section; A) Distribution of anti-sPLA$_2$-IIA (Alexa 488, green fluorescence) and colocalization with Mito-Tracker (yellow fluorescence) in PC-12 cells; B) Distribution of anti-sPLA$_2$-V (Alexa 488, green fluorescence) and colocalization with TO-PRO-3 in PC-12 cells (green + blue fluorescence); C) Distribution of anti-sPLA$_2$-IIA (Alexa 488, green fluorescence) and colocalization with Mito-Tracker (yellow fluorecence) in U-251 cells; D) Distribution of anti-sPLA$_2$-V cells (Alexa 488, green fluorescence) and colocalization with TO-PRO-3 (green + blue fluorescence) in U-251. Note the partial colocalization of sPLA$_2$-IIA with mitochondria and absence of the enzyme from nuclei in PC-12 and U-251 cells (A and C, respectively), and the nuclear, besides cytoplamic localization of sPLA$_2$-V in PC-12 and U-251 cells (B and D, respectively), as well as absence of colocalization of sPLA-V with mitochondria.

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The mitochondrial localization of sPLA$_2$-IIA was confirmed by immunoelectron microscopy in U-251 cells (Fig. 12B). Immunogold particles are mainly localized in the inner compartments of mitochondria and this observation is in agreement with the biochemical evidences reported at Figs. 8 and 9. The enzyme is also present in other cellular compartments very likely the endoplasmic reticulum. Control experiments, in which the antibody against type IIA-sPLA$_2$ was omitted, showed no immunogold particles (Fig. 12A).
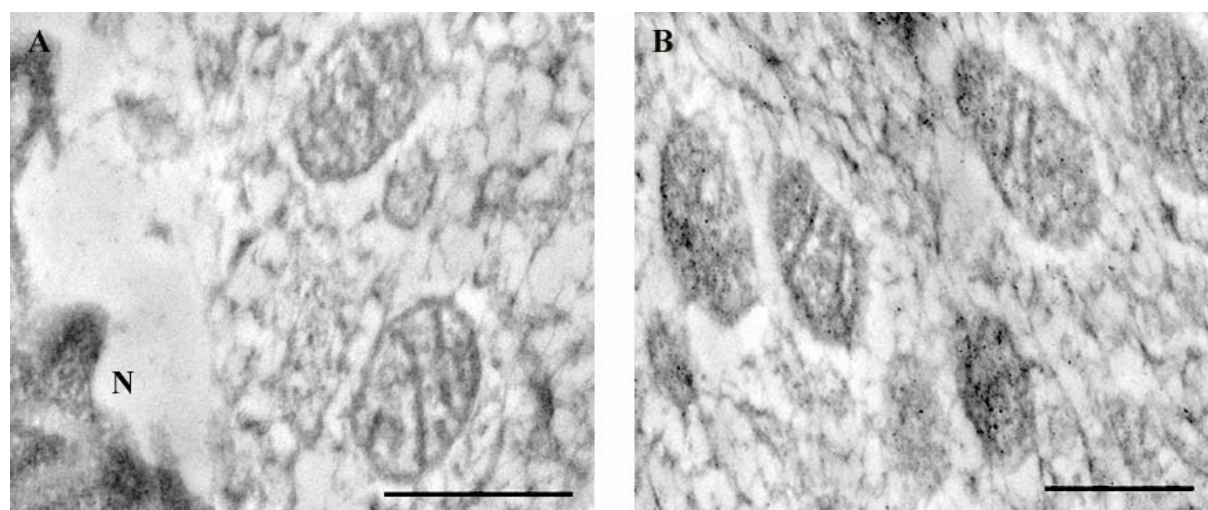
Fig. 12- Subcellular localization of sPLA$_2$-IIA in U-251 astrocytoma cells as investigated by immuno-electron microscopy.
Cryosections of U-251 astrocytoma cells were processed as described in Methods.
A) Control: Cryosection of U-251 cells in which the primary antibody was omitted.
B) Sample treated with anti sPLA-IIA antibody.
Most of the immunogold particles were found within mitochondria (B), while no partcles can be detected in control section (A). Original magnification 38000 x. Bar = 0.5 μm

DISCUSSION

In this study we have demonstrated that rat brain mitochondria possess two PLA$_2$s, at least. One is Ca^{2+}-independent, is sensitive to BEL and appears to be associated with the outer membrane, because its activity is partially lost by the treatments with proteases. Since this enzyme has similar properties to those of the iPLA$_2$ purified from rat brain cytosol and is

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sensitive to BEL (9, 63), we may suppose that it might bind to the mitochondrial membranes under certain circumstances. We cannot exclude that the cytosolic enzyme binds to the outer mitochondrial membrane during tissue homogenization and isolation of mitochondria. We have no further indication on the identity of the mitochondrial iPLA$_2$ but it is worth mentioning that Woelk and Porcellati (33) also reported a Ca^{2+}-insensitive PLA$_2$ using mixed micelles of specifically radiolabelled substrates. More recently, it has been reported that liver mitochondria possess a Ca^{2+}-independent PLA$_2$, which as been identified as BEL sensitive-iPLA$_2$ (57), and it has been proposed that the enzyme might participate in the removal of damaged mitochondria. This enzyme appears to be localized in the inner membrane and then it should not be accessible to proteases.

The other enzyme is very likely sPLA$_2$-IIA on the basis of its biochemical and immunological properties. In this case, it is unlikely that an enzyme, having identical properties but different subcellular localization might account for the activity detected in the mitochondrial fraction. In fact, the treatment of purified mitochondria with proteases did not affect the Ca^{2+}-dependent enzyme (Fig.8) whereas the removal of the outer membrane by digitonin treatment greatly increased its specific activity (Fig.9). This excludes the possibility that sPLA$_2$-IIA, present in other cellular compartments at the time of tissue homogenization, might have contaminated mitochondrial preparations by interacting with components of the outer membrane. On the other hand, it should be also excluded that, during the isolation of mitochondria from brain tissue, sPLA$_2$–IIA might have been unspecifically transported into mitochondria by crossing the outer membrane and then becoming associated with mitoplasts. In fact, the transport of proteins into mitochondria from other cell compartments is a process requiring their recognition at the outer membrane surface and a complex machinery of translocases which are present in the outer membrane (TOM) and in the inner membrane

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(TIM), have specific binding sites and are strictly regulated (64). These considerations are consistent with the mitochondrial localization of sPLA$_2$-IIA in the cells of the nervous tissue, similarly to previously reported data for liver mitochondria (36). The mitochondrial localization of sPLA$_2$-IIA is also supported by the demonstration of the presence of the enzyme, for the first time at the best of our knowledge, in the mitochondria of intact PC-12 cells and U-251 astrocytoma cells, as shown by immunoflourescence colocalization with a specific mitochondrial dye (Figs. 10 and 11). A further and conclusive evidence for the mitochondrial localization of the enzyme has been provided by immunoelectron microscopy of ultracryofixed U-251 astrocytoma cells (Fig. 12).

The results of our immunofluorescence experiments and immunoelectron microscopy have shown that sPLA$_2$-IIA is also associated with cellular components other then mitochondria. It is worth mentioning that previous immunocytochemical studies have localized a type II sPLA$_2$ in granule-like organelles of PC-12 cells and it has been also reported the release of the enzyme in the extracellular medium after stimulation of these cells with epinephrine or KCl (25). These observation further support the hypothesis that sPLA$_2$ may play both intracellular and extracellular roles (3), depending on cell types and/or conditions.

How proteins as sPLA$_2$s, synthesized with an N-terminal signal sequence typical for secretion, could be targeted to intracellular organelles is still unknown. However, it has been proposed that the secreted sPLA$_2$-IIA, lacking the N-terminus signal sequence, could undergo internalization through the association with heparan sulfate chain of glycosylphosphatidilinositol (GPI-anchored HSPG-glypican) (65). The internalized enzyme might then be targeted to intracellular compartments.

The most relevant finding of our study is that isolated mitochondria from rat brain cortex release a PLA$_2$ when incubated in a isotonic medium at pH 7.4 containing $\approx$1 μM Ca^{2+} and no

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respiratory substrates. The addition of respiratory substrates increases membrane potential and reduces the release of the enzyme in the extramitochondrial medium. The released PLA_2 has properties identical to those reported for type IIA secreted enzyme isolated from other cells or fluids (20, 66, 67) and immunoreacts with monoclonal antibody raised against rat liver mitochondrial $sPLA_2$ (68).

A further indication supporting the identification of the enzyme released from mitochondria as a type II secretory PLA_2 derives from the observation that this antibody does not recognize $sPLA_2$-V as also previously reported (51). We cannot exclude that a type II-$sPLA_2s$, other than IIA, might be also present in neural cells and cross-react with the antibody raised against recombinant $sPLA_2$-IIA (51) used in this study. In fact, other type II-$sPLA_2s$ (IIC, IID, IIE and IIF) can be expressed in various mammalian cells (65). For instance, it has been reported that, while type IIA $sPLA_2$ mRNA is present in all rat brain regions and peripheral tissues, type IIC mRNA seems to be specifically expressed in brain (69). However, it seems unlike that the mitochondrial enzyme might be identified as $sPLA_2$-IIC, because the enzyme released from mitochondria is inhibited by heparin (data not shown) whereas type IIC activity is not affected by this proteoglycan (65). Type IID-$sPLA_2$ has been cloned on the basis of a tBLASTn search but Northern analysis has shown that it is not expressed in rat brain (70). Tissue distribution of type IIF isoform, determined by RT-PCR on human adult cDNA panels, and Southern blot, have shown its presence in some human organs but not in brain (71) and, in adult mice, the expression seems to be limited to testis (72). Type IIE-$sPLA_2$ has been also cloned and is expressed in human tissue including brain (73). However, contrary to type IIA, its expression in rat tissues has never been reported thus far. In conclusion, it is reasonable to think, on the light of current knowledge, that brain

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mitochondrial sPLA$_2$ migh be identified as type IIA, which has been isolated from rat and human tissues (36, 66).

Since the removal of the outer mitochondrial membrane increases its specific activity, the enzyme appears to be associated with mitoplasts excluding the possibility of a contamination of a PLA$_2$ from other cellular compartments. Thus, we may conclude that rat brain cortex mitochondria contain sPLA$_2$-IIA, which under reduced respiratory activity is released in the extramitochondrial space. The release of the enzyme is not due to a damage of mitochondrial membranes severe enough to cause the leakage of proteins from matrix since fumarase is not released under the same conditions.

The presence of a secretory PLA$_2$ in mitochondria raises *per se* a number of questions concerning whether or not it may be in the active form in the organelle and induces to speculate on its functions *in vivo*.

Although sPLA$_2$ are fully active at mM Ca^{2+} concentration, a consistent activity can be reached at Ca^{2+} concentration that can be achieved in intracellular compartments (74) and, very likely, in mitochondria. In fact, it is well established that mitochondria play an important role in Ca^{2+} homeostasis and that they can accumulate a large amount of this cation when its cytosolic concentration increases (75, 76). Thus, we may suppose that oscillations of Ca^{2+} concentrations modulate mitochondrial sPLA$_2$ activity and consequently the rate of production of free fatty acids (FFA) and lysophosphoglycerides, which participate in the regulation of some functions of mitochondrial membrane proteins. It is known that FFA modulate cytochrome c oxidase activity (77) and uncouple oxidative phosphorylation (78). FFA also act as physiological modulators of mitochondrial permeability since they cause swelling of the organelle without opening cyclosporin-sensitive mitochondrial permeability transition pores (79).

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Among the FFA produced by the action of PLA_2, great attention has been devoted to the effects of polyunsaturated fatty acids (PUFA) on mitochondrial functions because they appear to play important roles in the decision of cellular life or death. In HL-60 cells, PUFA inhibit their growth and induce apoptosis by releasing cytochrome c from mitochondria by two distinct mechanisms: membrane depolarization-dependent and depolarization-independent (80). Recently, it has been reported that μM arachidonic acid (AA) induce permeability transition (PT) in isolated rat liver mitochondria and in hepatoma cells (81). This effect was erroneously attributed to $cPLA_2$ activation since treatment with aristolochic acid did not prevented PT induced by AA in isolated mitochondria or in intact cells but it prevented PTP opening, cytochrome c release and cell death induced by TNFα (81). However, aristolochic acid is not an inhibitor of either Ca^{2+}-dependent or Ca^{2+}-independent cytosolic PLA_2 (82, 83), but it is considered an inhibitor of $sPLA_2$. Thus, those results support the involvement of this class of enzymes in apoptosis.

The other products of phospholipid hydrolysis by PLA_2, lysophospholipids, also interferes with some mitochondrial functions. For instance, lysophosphatidylcholine modulate the activities of various mitochondrial enzymes causing their increase or decrease depending on its concentration (84). However, at relatively high concentration, lysophosphatidylcholine causes the breakdown of mitochondrial membranes. Lysophosphatidylcholine and other compounds, having similar chemical properties (i.e the lipid mediator PAF and the experimental anticancer drug hexadecylphosphocholine), also influence mitochondrial Ca^{2+} transport and membrane potential. Thus, an impairment of mitochondrial functions due to the accumulation of PLA_2 products is very likely (85,86).

When $\Delta\Psi_m$ decreases as the consequence of reduced respiratory rate, the mitoplast-associated $sPLA_2$ is released in the extramitochondrial space and we may suppose that it

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hydrolyzes membrane phospholipids thereby causing severe cell damage. Although the molecular mechanisms causing the release of sPLA$_2$ from mitoplasts toward the extramitochondrial space are still unknown, our findings suggest that the released enzyme might participate to the cascade of events leading to cell death as the consequence of mitochondrial dysfunction. It is possible that the release of sPLA$_2$ in the extramitochondrial compartment causes additional effects to those induced by the receptor-mediated activation of Ca^{2+}-dependent cPLA$_2$ type IV (87). This might be the case of neuronal degeneration and death induced by excessive glutamate release. *In vivo* and *in vitro* studies have shown that glutamate excitotoxicity is linked to the activation of NMDA receptors, which induces the increase of cytosolic Ca^{2+}, phosphorylation and translocation of cPLA$_2$ from cytosol to membranes and the liberation of fatty acids from phospholipids (88, 89). However, it has been also observed a reduction of $\Delta\Psi_m$ and a failure of energy production in mitochondria of neuronal cells by elevated glutamate concentration (90) and release of pro-apoptotic proteins as cytochrome c (37) and caspase-9 (38). Our results show that, in addition to these proteins, sPLA$_2$ is also released from mitochondria when $\Delta\Psi_m$ is reduced and it might contribute to neuronal cell death.

ACKNOWLEDGMENTS

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Lara Macchioni, Lanfranco Corazzi, Vicenza Nardicchi, Roberta Mannucci, Cataldo Arcuri, Serena Porcellati, Tito Sposini, Rosario Donato and Gianfrancesco Goracci

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