Glutamate stably enhances the activity of two cytosolic forms of phospholipase A₂ in brain cortical cultures

Dae Kyong KIM,* Guy RORDORF,*† Raphael A. NEMENOFF,*‡ Walter J. KOROSHETZ†§ and Joseph V. BONVENTRE*

*Medical and †Neurological Services Massachusetts General Hospital, Boston, MA 02114

and Departments of *Medicine and †Neurology, Harvard Medical School, Boston, MA, U.S.A.

The mechanisms by which glutamatergic neurotransmitters modulate neuronal lipid metabolism are not well established. We have directly measured phospholipase A₂ (PLA₂) enzymic activity in cell-free extracts from cortical neuronal cultures from rat brain and have found that the PLA₂ activity is up-regulated after cells are exposed to glutamate. Brief exposure to a calcium ionophore or phorbol 12-myristate 13-acetate (PMA) stably enhanced PLA₂ activity. Down-regulation of protein kinase C activity partially blocked glutamate's effects. Two Ca²⁺- and pH-dependent forms of PLA₂ were identified in cytosolic extracts. Activation of both forms of PLA₂ was enhanced by prior exposure of the cultures to glutamate. One of the two forms had chromatographic characteristics on heparin–Sephacrose, Mono Q and Superose 12 columns similar to the 100 kDa cytosolic PLA₂

(cPLA₂), and was recognized by an antibody raised to pig spleen cPLA₂. The second form was similar in size to Group-I and -II PLA₂s but differed in chromatographic characteristics. It was not inhibited by dithiothreitol, and did not react with antibodies to pancreatic Group-I PLA₂, features that distinguish it from Group-I and -II PLA₂. In extracts from cells pretreated with glutamate, the activity–Ca²⁺ concentration dose–response relationship of the 13.5 kDa form of PLA₂ was shifted to the left with activation at lower Ca²⁺ concentration as the result of stable modification of the enzyme induced by glutamate. Thus glutamate-induced stable enhancement of PLA₂ activity, by processes involving calcium and protein kinase C activation, is a potential molecular switch probably mediating changes in synaptic function and contributing to excitotoxicity.

INTRODUCTION

Phospholipase A₂ (PLA₂) is a family of enzymes which deacylate phospholipids at the *sn*-2 position, play a central role in the regulation of arachidonic acid (AA) release [1], are rate-limiting for the production of eicosanoids and platelet-activating factor (PAF) and play important physiological and pathophysiological roles in the central nervous system [2]. AA or its metabolites influence the activation of voltage-dependent [3] and ligand-gated [4] channels, modulate second messenger systems [5], gene expression [6] and neurotransmitter uptake [7] and release [8], and enhance the phosphorylation of GAP-43 in nerve-terminal membranes [9]. These and other data suggest that PLA₂ activity and AA release may be important for activity-dependent regulation in the nervous system [10]. In addition, enhanced PLA₂ activity, leading to the production of lysophospholipids, AA, eicosanoids, PAF [11] and reactive oxygen species [12], has also been implicated in destructive cellular processes in various tissues [13] including the central nervous system [14].

Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, causes release of AA from brain tissue [15]. Excessive glutamatergic action may be responsible for increased amounts of AA metabolites implicated in neuropathological conditions. Glutamate might cause AA release by either activating one of several lipases or inhibiting reacylation. AA release might occur secondary to an increase in intracellular [Ca²⁺], with activation of a Ca²⁺-dependent PLA₂; however, PLA₂ may be modulated by mechanisms other than Ca²⁺ [16–18]. AA can also be released by metabolic pathways which do not

involve PLA₂, such as phospholipase C followed by diacylglycerol lipase [17]. The demonstration that release of AA by activation of non-*N*-methyl-D-aspartic acid (NMDA) glutamate receptors in striatal cultures requires both membrane depolarization and activation of the metabotropic glutamate receptor [15] suggests that glutamate's effects on AA release involve mechanisms more complex than increases in cytosolic free Ca²⁺ concentration alone.

Multiple forms of PLA₂ have been described [1,19] differing in their biochemical characteristics, intracellular localization [13] and tissue distribution. In many, but not all [20,21] cases, PLA₂ activity is Ca²⁺-dependent. We previously found that PLA₂ activity was stably up-regulated in gerbil brain exposed to ischaemia/reperfusion [22]. We now report that the activities of the cytosolic forms of PLA₂ in cell-free extracts prepared from rat neuronal/glia cultures are stably enhanced by prior exposure of the cultures to glutamate. We characterize two forms of PLA₂ in brain cortical cultures that are activated by glutamate. This stable enhancement of PLA₂ activity may play an important role in physiological and pathophysiological processes associated with glutamatergic neurotransmission.

EXPERIMENTAL

Tissue culture and agonist addition

Mixed cortical cell cultures containing both neuronal and glial elements were prepared by dissociating neurons from the cortex of 1- or 2-day-old Long Evans rat pups as previously described

Abbreviations used: PLA₂, phospholipase A₂; AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 2-[1-¹⁴C]AA-PC, 1-stearoyl-2-[1-¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine; 2-[1-¹⁴C]AA-PE, 1-acyl-2-[1-¹⁴C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine; NMDA, *N*-methyl-D-aspartic acid; PAF, platelet-activating factor; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; LDH, lactate dehydrogenase; TBS, Tris-buffered saline; cPLA₂, cytosolic PLA₂; DMSO, dimethyl sulphoxide.

‡ Present address: Division of Renal Disease and Hypertension, University of Colorado School of Medicine, Denver, CO 80262, U.S.A.

§ To whom correspondence should be addressed at: Kennedy 915, Department of Neurology, Massachusetts General Hospital, Fruit Street, Boston, MA 02114.

[23]. Dissociated cortical cells were plated at a density of about 2×10^6 per 35 mm-diam. dish on poly-L-lysine coated dishes in Earles minimal essential medium (MEM) supplemented with 5% (v/v) rat serum, 0.5 mM glutamine and 21 mM glucose. Non-neuronal cell division was halted by addition of uridine-deoxyuridine ($10 \mu\text{M}$) when cultures were 80% confluent. One-half of the medium was replaced weekly. 14- and 16-day-old cultures were used for experiments. Glial cultures were grown by the same dissociation method except that cells were plated into tissue-culture flasks and allowed to attain confluence. After 2 weeks cells are detached with trypsin and EGTA, washed, treated with trypsin inhibitor and transferred to poly-L-lysine-treated 35-mm-diam. plates. A thin uniform glial layer forms in 1 week and no neurons are present.

Glutamate was added to the cultures for 20 min. Extracts were then prepared as described below. Phorbol 12-myristate 13-acetate (PMA) (100 nM final concentration, unless otherwise specified), phorbol 12-monoacetate (100 nM), an inactive form of phorbol ester, or the calcium ionophore A23187 ($1 \mu\text{M}$ final concentration, unless otherwise specified) were added to the medium for 30 min. In some cases kynurenic acid (1 mM) and MgCl_2 (final concentration 11.3 mM) were added for 2 h after glutamate was removed in order to evaluate reversibility of the glutamate effect. To down-regulate protein kinase C (PKC) activity, PMA ($16 \mu\text{M}$) was added to the medium for 16 h prior to glutamate addition [24,25]. Lactate dehydrogenase (LDH) release into the medium was assayed by the method of Walker et al. [26]. We have found that LDH release correlates well with neuronal cell counts as markers of cell viability [23].

Preparation of cell extracts

Mixed cortical cell cultures or pure glial cultures were rinsed three times and scraped into 1.2 ml of homogenization buffer containing 50 mM Hepes, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, and the protease inhibitors pepstatin ($20 \mu\text{M}$), leupeptin ($20 \mu\text{M}$), trasylol (1000 kallikrein-inactivating unit/ml) and phenylmethanesulphonyl chloride (0.1 mM) at pH 7.5. Cells were homogenized with 25 strokes in a tight Dounce homogenizer. The homogenate was spun at 100000 g for 60 min to remove nuclei, unbroken cells, mitochondria and microsomes. The resulting supernatant was designated the 'extract'. The protein concentration of cell extracts was determined by the Bradford method [28]. A different homogenization buffer was used when supernatants were chromatographed on heparin-Sepharose columns (see below).

To make membrane extracts the neuronal cultures were homogenized as above and subjected to ultracentrifugation at 100000 g for 60 min. The resulting pellets were sonicated in the same buffer for 15 s in an ice bath at 40 W output and 40% duty cycle (Sonicator, Ultrasonic Processor; Heat Systems-Ultrasonics Inc.). The lysates were centrifuged again as above and the resulting pellets were resuspended by brief sonication. Samples of 14 μg of protein were assayed for PLA_2 activity as described below.

Extract fractionation

Superose 12 gel-filtration FPLC

Cell extracts (500 μl) or a concentrate (approximately 200 μl) of active fractions from other columns were loaded on to a 24 ml Superose 12 gel-filtration FPLC column (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM Hepes, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl at pH 7.4. Flow rate was 0.5 ml/min. Fractions (1 ml) were collected and assayed for

PLA_2 activity and protein. The column was calibrated using the following standards: BSA (66 kDa), ovalbumin (45 kDa), pig pancreatic PLA_2 (13.5 kDa) and vitamin B_{12} (1.35 kDa).

Heparin-Sepharose CL-6B column chromatography

Neurons and human monocytic U937 cells were harvested in homogenizing buffer consisting of 50 mM Tris/HCl, 1 mM EDTA and 0.12 M NaCl, pH 9.0, and sonicated for 20 s in an ice bath at 40 W output and 40% duty cycle (Sonicator, Ultrasonic Processor; Heat Systems-Ultrasonics Inc.). The lysates were centrifuged for 1 h at 100000 g. The resulting supernatants were diluted 1:1 with buffer A (50 mM Tris/HCl, 1 mM EDTA, pH 7.4) and loaded on to a heparin-Sepharose CL-6B (Pharmacia, LKB Biotechnology Inc.) column (0.5 cm $\times$ 4 cm) pre-equilibrated with buffer A. Activity was eluted with buffer A (non-binding fraction) at 1 ml/min for 10 min. Additional activity was then eluted with a linearly increasing gradient of NaCl concentration in buffer A.

Mono Q ion-exchange chromatography

The heparin non-binding fractions were loaded on to Mono Q (HR 5/5, Pharmacia LKB Biotechnology Inc., Sweden) pre-equilibrated with buffer A. The column was eluted at a flow rate of 1 ml/min, with a 20 ml linear gradient of 0–1.0 M NaCl, and 1 ml fractions were collected. Aliquots (20 μl) of each fraction were assayed at 37 °C for 30 min for PLA_2 activity as described below.

PLA_2 assay

PLA_2 was assayed using two methods differing primarily in preparation of the substrate and the technique used for separation of liberated AA from phospholipid. In a particular set of experiments the same method was employed to measure PLA_2 activity in all experimental conditions. One method was modified slightly from that previously described [29]. 1-Stearoyl-2-[1- ^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (2-[1- ^{14}C]AA-PC), and 1-acyl-2-[1- ^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine (2-[1- ^{14}C]AA-PE) were used as substrates. Since cytosolic activity was higher with 2-[1- ^{14}C]AA-PE this was the substrate used unless otherwise specified in the paper. Substrates were dried down with an N_2 gas stream and resuspended in dimethyl sulphoxide (DMSO). Extracts from control and treated cultures were matched for protein. Substrate (2 μl ; final assay substrate concentration 15 μM), was pipetted into an Eppendorf microfuge tube. Reactions were initiated by the addition of 34 μl of cortical cell extract and 3 mM Ca^{2+} (1 mM in excess of the combined EDTA and EGTA concentrations) at a physiological pH of 7.5. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped with a quench solution containing 2% acetic acid in ethanol. Separation of substrate from released free fatty acid was performed using silica-gel TLC plates (Whatman, Springfield Mill, U.K.). Aliquots (50 μl) of the reaction mix were spotted on to the plates and developed with the organic phase of ethyl acetate, iso-octane, water and acetic acid (55:75:100:8, by vol.). Blank samples were run containing extract buffer. The activity was evaluated directly on the TLC plates using a Berthold Automatic TLC-Linear Analyser (Berthold, Germany).

PLA_2 activity was also assayed using sonicated liposomes [30], containing 2-[1- ^{14}C]AA-PE. The standard PLA_2 assay buffer (200 μl) contained 75 mM Tris/HCl, 5 mM CaCl_2 , 1 mg/ml BSA and 0.5 nmol of the phospholipid (approximately 65000 d.p.m.) at pH 7.5. The reaction was carried out at 37 °C for 30 min and

was stopped by adding 1.25 ml of Dole's reagent, 78 % isopropyl alcohol, 20 % *n*-heptane, 2 % 0.5 M H₂SO₄ in water [31]. Free fatty acid was extracted in the following manner. An aliquot (0.55 ml) of water was added and the sample was vortexed and centrifuged for 5 min. Then 0.75 ml of the upper phase was transferred to a new tube to which 50 mg of silica gel and 0.75 ml of *n*-heptane was added. The samples were vortexed and centrifuged again for 5 min each. A sample (1.4 ml) of supernatant was then counted in a liquid scintillation counter. PLA₂ activity was expressed as pmol of arachidonic acid released per min per mg of protein.

Immunoprecipitation of PLA₂

Immunoprecipitation of PLA₂ was performed as described previously [30]. Rabbit antiserum raised against 100 kDa PLA₂ [30], rabbit antiserum to pancreatic Group-I PLA₂ (UBI, Lake Placid, NY, U.S.A.), or control rabbit serum (Sigma, St. Louis, MO, U.S.A.) was mixed with Protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc., Sweden; 2:1, v/v) pre-equilibrated with 20 mM Tris/HCl (pH 7.5) buffer, containing 5 mg/ml BSA for 24 h at 4 °C on an orbital shaker. The antibody we have raised against cPLA₂ does not recognize the 14 kDa forms of PLA₂ [30], nor does the antibody to Group-I PLA₂ recognize the 100 kDa form of the enzyme. The beads were then washed six times with 20 vol. of 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 50 mg/ml BSA. To estimate specific interaction of rabbit antiserum with the 100 kDa PLA₂, 50 µl of washed beads were incubated, for various times at 4 °C, with 500 µg of protein from the 100000 g supernatants of neurons or an aliquot (500 µl) of heparin non-binding fractions of neuronal extracts. The samples were centrifuged and the supernatants were assayed for PLA₂ activity. For the specific binding of antiserum to Group-I PLA₂, the sample was collected from the heparin-binding fractions. A sample which served as a positive control was prepared from heparin-binding fractions of 100000 g supernatants of rat platelets (Group-II PLA₂). An aliquot (50 µl) of washed beads was added to each sample (500 µl). Incubation was performed for 12 h at 4 °C with constant shaking. The samples were centrifuged and the supernatants were assayed for PLA₂ activity.

Immunoblotting

Proteins were separated by SDS/10%-PAGE [32] and then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, U.S.A.) in 25 mM Tris/HCl (pH 8.3), containing 190 mM glycine and 20 % methanol. Non-specific binding of antisera to nitrocellulose membranes was prevented by pre-incubation of the nitrocellulose in 3 % (w/v) BSA in Tris-buffered saline (TBS) (24.8 mM Tris/HCl, 137 mM NaCl, 2.7 mM KCl, pH 8.0) containing 0.1 % NaN₃ for 1 h at room temperature. The blocked nitrocellulose membranes were incubated with antisera (diluted 1:4000) for 6 h at room temperature with constant shaking. Unbound antibodies were removed with three washes of TBS, and the sites of antibody binding were detected by using peroxidase-conjugated goat anti-(rabbit IgG) (Bio-Rad) and the chromogenic substrate, 4-chloro-1-naphthol.

Partial purification of platelet group-II PLA₂

Group-II PLA₂ was partially purified from 100000 g supernatant of rat platelets by heparin-Sepharose CL-6B column chromatography as described previously [33].

Ca²⁺ dependency of PLA₂ activity

PLA₂ activity was measured in samples diluted 1:8 with buffers containing various amounts of CaCl₂ using the DMSO-suspended substrate as described above. Free Ca²⁺ concentration of each sample was determined. Ca²⁺ concentrations below 1 µM were measured with fura-2 free acid, using a dual-wavelength spectrofluorometer (Deltascan Spectrofluorometer, Photon Technology Inc., Princeton, NJ, U.S.A.) [34]. For concentrations greater than 1 µM the free Ca²⁺ concentration was determined with a Ca²⁺-selective electrode [34].

Materials

2-[1-¹⁴C]AA-PE (55.1 mCi/mmol), and 2-[1-¹⁴C]AA-PC (56.7 mCi/mmol) were purchased from Amersham. Protein A-Sepharose, heparin-Sepharose CL-6B, Mono Q and Superose 12 columns were obtained from Pharmacia LKB Biotechnology Inc., Sweden. Group-I pig pancreatic PLA₂ was obtained from Sigma. All other chemicals were of the highest purity available from commercial sources. Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Melville, NY, U.S.A.) with BSA as the standard.

Statistics

Values are presented as means ± S.E.M. Data were compared using analysis of variance or Student's *t* test as appropriate. A *P* value of < 0.05 was considered statistically significant.

RESULTS

PLA₂ activity is stably enhanced in neurons exposed to glutamate

PLA₂ specific activity in neuronal cultures was greater with phosphatidylethanolamine (PE) than phosphatidylcholine (PC) as substrate. With either PC or PE as substrate, the PLA₂ specific activity of cytosolic extracts from cultures containing both neurons and a confluent glial monolayer was greater than that measured in confluent cultures of glial cells (14.4 ± 1.5 versus 6.1 ± 0.3 pmol/mg per min with PE as substrate), indicating that most of the PLA₂ activity measured in mixed cultures was neuronal in origin.

Glutamate produced a dose-dependent increase in PLA₂ activity in neural cultures with maximal activation observed at a glutamate concentration of 125 µM (Figure 1). Glutamate did

Figure 1 Effect of glutamate on PLA₂ activity in cortical cultures

PLA₂ enzymic activity in cortical culture extracts, determined using PE as substrate, was enhanced at concentrations of glutamate equal to or greater than 125 µM. Experiments were performed on sister cultures from a single plating and repeated in three separate cell platings. A representative curve is shown.

Table 1 PLA₂ activity in extracts of cortical cultures exposed to glutamate, a calcium ionophore or a phorbol myristate acetate (PMA)

Glutamate, A23187 and PMA enhanced PLA₂ activity. At the concentrations used, the effects of A23187 and PMA on PLA₂ activity were not additive. Treatment of the cultures with the inactive phorbol ester (phorbol 12-monoacetate), which has no effect on PKC, did not enhance PLA₂ activity. 2-[1-¹⁴C]AA-PE was used as substrate. Experiments were performed on five separate platings of cells. *P < 0.005 compared with vehicle-treated control values.

	PLA ₂ activity (pmol/min per mg)
Control	27.6 ± 3.8
Glutamate (500 μM)	63.2 ± 5.5*
A23187 (1 μM)	53.2 ± 3.8*
PMA (100 nM)	81.2 ± 4.4*
Phorbol 12-monoacetate (100 nM)	25.6 ± 4.0
A23187 (1 μM) + PMA (100 nM)	79.1 ± 5.4*

not affect PLA₂ activity in cytosolic extracts from cultures containing only glial cells (see below). The glutamate concentration in the synaptic cleft giving rise to an excitatory post-synaptic current has been estimated to be greater than 1 mM [35] and glutamate levels may remain elevated for prolonged periods of time in pathophysiological states.

To evaluate whether the effect of glutamate on PLA₂ activity was reversible we treated cells for 20 min with 150 μM glutamate, at which point we washed away the glutamate and added blockers of neurotransmission, kynurenic acid (1 mM) and MgCl₂ (final concentration 11.3 mM) [36]. Removal of glutamate and addition of blockers for 2 h resulted in a return of PLA₂ levels to baseline, demonstrating reversibility of the glutamate effect (results not shown).

Since glutamate causes neuronal death in these cortical cultures at doses similar to those that enhance PLA₂ activity [23] we evaluated whether enhancement of PLA₂ activity occurred prior to the loss of membrane integrity. Although exposures to 150 μM glutamate for only 3 min were found to enhance PLA₂ activity (W. J. Koroshetz and J. V. Bonventre, unpublished work), there was no increase in LDH activity in the medium even after 20 min of exposure to 150 μM glutamate. Neuronal counts were performed in identical microscopic fields before glutamate exposure and compared with counts 2 h after washing out the glutamate and adding blockers of neurotransmission [23]. In one plating more than 95 %, and in another 58 %, of the neurons survived for a least 2 h after a 20 min exposure to 150 μM glutamate. These results indicate that the increase in PLA₂ activity seen very early after glutamate exposure precedes irreversible cell injury.

Membrane fraction PLA₂ activity was lower than cytosolic activity and was not decreased after 150 μM glutamate treatment for 20 min nor changed after glutamate removal and exposure to blockers of neurotransmission. Thus the increase in cytosolic activity could not be explained by a translocation of activity from membrane to cytosolic fractions.

The calcium ionophore, A23187, and phorbol ester, PMA, enhance the activity of soluble PLA₂

Activation of neuronal glutamate receptors increases intracellular [Ca²⁺], PKC activity, and phosphorylation of neuronal proteins [37]. Glutamate's effect on PLA₂ activity was mimicked by treating cultures with the PKC activator PMA at 100 nM (Table 1), but not the inactive compound, phorbol 12-monoacetate, H-7 [1-(5-isoquinolinylsulphonyl)-2-methylpiperazine], a PKC

Table 2 Effect of PKC down-regulation on glutamate's ability to increase PLA₂ activity

After 16 h of exposure to 16 μM PMA, PLA₂ activity was indistinguishable from baseline levels. Acute exposure to PMA failed to stimulate PLA₂ activity and glutamate's effect on PLA₂ activity was significantly reduced in PKC down-regulated cells. Experiments were performed on five separate platings of cells. *P < 0.05; **P < 0.005 compared with vehicle-treated control values.

Pretreatment	Conditions	PLA ₂ activity (pmol/min per mg)
—	Control	28.1 ± 3.0
—	PMA	79.2 ± 4.9**
—	Glutamate	67.1 ± 3.5**
PMA pretreated	Control	26.4 ± 4.8
PMA pretreated	PMA	31.8 ± 5.0
PMA pretreated	Glutamate	45.0 ± 3.9*

Table 3 Effects of glutamate, A23187 and PMA on glial PLA₂ activity

Extracts were prepared from cortical cultures that were passaged and contained a confluent flat layer of glial cells but no neurons. PMA, but not glutamate or A23187, enhanced PLA₂ activity in cultures of glial cells. Data were obtained from three experiments. 2-[1-¹⁴C]AA-PE was used as substrate. *P < 0.05 compared with vehicle-treated control values.

	PLA ₂ activity (pmol/min per mg)
Control	10.2 ± 2.0
Glutamate (500 μM)	11.4 ± 1.9
A23187 (1 μM)	10.8 ± 2.0
PMA (100 nM)	17.0 ± 2.0*

inhibitor, could not be used because it caused marked changes in the underlying glial morphology. The calcium ionophore, A23187 (1 μM), also enhanced PLA₂ activity (Table 1) but this increase was significantly less than that observed with PMA. Raising the PMA concentration to 500 nM or raising the ionophore concentration to 5 μM were without any further effect (results not shown). When PMA (100 nM) and A23187 (1 μM) were added together, PLA₂ activity was equal to that measured with PMA alone. No cell death was evident at the time the cells were harvested after treatment with PMA or A23187.

Pretreatment of cultures with 16 μM PMA for 16 h down-regulates PKC, but does not alter glutamate-induced membrane currents, phosphoinositol metabolism, or ⁴⁵Ca²⁺ uptake [25]. The PLA₂ activity in cytosolic extracts from PMA-pretreated cells was not elevated and did not increase when the cultures were exposed acutely to 100 nM PMA (Table 2). In the PKC down-regulated cells the glutamate stimulation of PLA₂ activity was only approximately one-half of that measured in PMA-treated cells in which PKC was not down-regulated.

PLA₂ activity had an alkaline pH optimum of 8.5 (data not shown). The pH dependency of PLA₂ activity in extracts of glutamate-treated cultures was indistinguishable from that measured in extracts of control cultures.

In pure glial cultures, PMA (100 nM) significantly stimulated glial PLA₂ activity, but glutamate or A23187 (at 500 μM and 1 μM respectively) had no effect on PLA₂ activity (Table 3). When samples of glial cell extracts were fractionated by Superose 12 gel-filtration chromatography two peaks of activity were found, migrating with the same characteristics on Superose 12

Figure 2 Superose 12 FPLC gel-filtration chromatography of cortical cell extracts from control and glutamate-treated cultures

Cortical brain culture extracts (0.5 ml) were applied to a 24-ml column and eluted at 0.5 ml/min. Fractions (1 ml) were collected and PLA₂ activity was measured using 2-[1-¹⁴C]AA-PC (PC; not shown) and 2-[1-¹⁴C]AA-PE (PE). The column was calibrated using the following standards (arrows): BSA (66 kDa), ovalbumin (45 kDa), pig pancreatic PLA₂ (13.5 kDa) and, not shown, vitamin B₁₂ (1.35 kDa). Two peaks of activity (fractions 13 and 17) were found with both substrates. Glutamate enhances the activity of both forms of the enzyme (fractions 13 and 17). Total activity of both forms was significantly increased by exposure to glutamate with either PE or PC as substrate (PE data is shown). The recovery is close to 100% in both conditions. Error bars represent S.E.M.s in experiments on four platings. *P < 0.05; **P < 0.005 compared with control fraction PLA₂ activity.

Figure 3 Heparin-Sepharose chromatographic separation of cytosolic PLA₂ activity

There are two major peaks of PLA₂ activity in soluble fractions from control and glutamate-treated brain cortical cultures, one in the flow-through fractions (heparin non-binding), and one that elutes with a peak at 0.8 M NaCl (heparin-binding). There is increased activity in each of the two peaks in the extracts from glutamate-treated cells as compared with control cells. For comparison a 100 000 *g* supernatant of rat platelets was also loaded on to this column and eluted under the same conditions as neuronal PLA₂. Platelet Group-II activity eluted at a higher NaCl concentration than did the neural heparin-binding activity. 2-[1-¹⁴C]AA-PE was used as substrate for PLA₂ activity.

chromatography as the forms present in mixed neuronal/glial cultures (see below, data not shown).

Characteristics of two forms of cytosolic PLA₂ (cPLA₂) whose activities are enhanced by glutamate

Two forms of PLA₂ activity were noted when cytosolic extracts from control and glutamate-treated cells were fractionated by gel-filtration chromatography (Figure 2). A similar elution pattern was seen using either PE or PC as substrate. A larger-

Table 4 Immunoprecipitation of heparin non-binding activity with anti-(pig spleen 100 kDa PLA₂) serum

Fifty microlitres of washed Protein A-beads, which had been preincubated with control serum or anti-cPLA₂ serum, were incubated for 6 h at 4 °C with an aliquot (500 µl) of heparin non-binding fractions of cortical brain cultures or partially purified pig spleen cPLA₂. The incubation medium was then centrifuged at 10 000 *g* at 4 °C and the supernatants assayed for PLA₂ activity.

	PLA ₂ activity in supernatants (c.p.m.)	
	Normal serum	Anti-(100 kDa PLA ₂) serum
Heparin-non-binding fractions from cortical cultures	2760	345
Pig spleen cPLA ₂	3200	250

molecular-mass form migrated with an apparent molecular mass of approx. 66 kDa, similar to the migration pattern of a form, with a molecular mass of 100 kDa by SDS/PAGE, cPLA₂, that we have characterized in rat kidney [38], pig spleen and other tissues [30]. A smaller form had an estimated molecular mass of 13.5 kDa. Both activities were increased by glutamate exposure.

When 100 000 *g* supernatants of control and glutamate-treated culture lysates were fractionated by heparin-Sepharose column chromatography, there were two major peaks of activity; one in the non-binding fraction and one eluting at a NaCl concentration of approx. 0.8 M NaCl (Figure 3). Glutamate enhanced the PLA₂ activity of each of the two forms of PLA₂ (results not shown). Rat platelet PLA₂ eluted at a higher NaCl concentration than did the heparin-bound cortical form of PLA₂, indicating that this neural form was distinct from the platelet Group-II form of PLA₂.

The activity that did not bind to heparin was then loaded on to a Mono Q anion-exchange column and eluted with a linear gradient of NaCl. The activity eluted in a single peak at 0.5 M NaCl in a position identical to the elution position of cPLA₂ from U937 cell extracts, which we subsequently fractionated on the same column. When the fractions from the Mono Q column, containing PLA₂ activity, were collected, concentrated to ~ 200 µl, and loaded on to a Superose 12 gel-filtration column, the major peak of activity migrated in a pattern comparable with that of cPLA₂. Thus, by these chromatographic criteria the larger form of PLA₂ present in the cortical cultures, eluting in fraction 13 from the Sepharose 12 gel-filtration column, was the 100 kDa PLA₂. To confirm that this cortical form was cPLA₂ we performed immunodepletion and Western blot analysis with an antibody raised to the pig spleen 100 kDa PLA₂ [30]. As demonstrated in Table 4, incubation of the heparin non-binding fractions with the antibody resulted in a marked drop in PLA₂ activity that was similar to that observed when this antibody was added to partially purified pig spleen PLA₂. A Western blot of the 100 000 *g* neuronal culture supernatant probed with the antibody resulted in a single band identical in position to that observed in supernatants of LLC-PK₁ cells overexpressing cPLA₂ (Figure 4). Clearly these data indicate that one of the forms of neuronal culture PLA₂ is cPLA₂, or a very closely related form.

When the fractions containing the heparin-binding form of PLA₂ activity (Figure 3) were loaded on to a Superose 12 FPLC column, the activity eluted at fraction 17 (Figure 5), the same fraction where the small-molecular-mass (approx. 13.5 kDa)

Figure 4 Western blot analysis of PLA₂ from cortical cultures using anti-(pig spleen 100 kDa PLA₂) serum

Proteins in amounts of 100 µg (lanes A and B), or 200 µg (lane C) were prepared from 100 000 *g* supernatants of cultured renal epithelial (LLC-PK₁) cells (lane A), cPLA₂-overexpressing LLC-PK₁ cells (lane B) and rat neurons (lane C), respectively, loaded on SDS/PAGE gel, transferred to nitrocellulose and immunoblotted with antiserum raised to the pig spleen 100 kDa PLA₂. In the transfected cells and neurons there was a prominent band at 100 kDa which was absent in the non-transfected LLC-PK₁ cell line.

form of PLA₂ eluted when the extracts were added directly on to the column (Figure 2). Thus this activity is the same size as Group-I and -II forms of PLA₂. We then further evaluated whether this smaller-molecular-mass brain cortical culture PLA₂ was a Group-I or -II PLA₂. As discussed previously the cortical PLA₂ activity eluted from the heparin–Sephadex column at a lower NaCl concentration than did the platelet Group-II PLA₂. The dithiothreitol sensitivity of the cortical form and its reactivity with antiserum to pancreatic PLA₂, which reacts with both Groups I and II, were also examined. Dithiothreitol inhibits Group-I and -II PLA₂ but not cPLA₂. As shown in Table 5, the activity in the heparin-binding fractions from cortical cultures was not inhibited by dithiothreitol, whereas both pig pancreatic Group-I and rat platelet Group-II PLA₂ activities were inhibited by dithiothreitol. As expected, the activity in the heparin non-binding fraction (the 100 kDa PLA₂) was not inhibited by dithiothreitol.

Figure 5 Superose 12 FPLC gel-filtration chromatography of extract fractions which bind to a Heparin–Sephadex CL-6B column and contain PLA₂ activity

The active heparin-binding fractions were pooled and concentrated to ~200 µl using Centricon 10 (Amicon). The concentrate was injected into a Superose 12 FPLC column. The peak of activity elutes at an estimated molecular mass of 13.7 kDa. 2-[1-¹⁴C]AA-PE was used as substrate for PLA₂ activity.

Table 5 Dithiothreitol has no inhibitory effect on either form of rat cortical culture PLA₂ activity

The sensitivities of PLA₂ activities contained in either 100 000 *g* supernatants, heparin non-binding or binding fractions to inhibition by dithiothreitol were compared with the sensitivity of purified pig pancreatic Group-I PLA₂ and platelet Group-II PLA₂. The PLA₂ enzyme preparations were pre-incubated at 37 °C for 30 min in 75 mM Tris/HCl, 5 mM CaCl₂ with or without 5 mM dithiothreitol. After adding 2-[1-¹⁴C]AA-PE as substrate, the samples were further incubated for 30 min at 37 °C and assayed for PLA₂ activity. Dithiothreitol inhibits Group-I and -II PLA₂s but not the cortical culture heparin-binding (low-molecular-mass) and non-binding (high-molecular-mass) forms.

	PLA ₂ activity in supernatants (c.p.m.)	
	No addition	Dithiothreitol (5 mM)
100 000 <i>g</i> supernatants	1970	2030
Heparin non-binding fractions from cortical cultures	1350	1400
Heparin binding fractions from cortical cultures	745	876
Pig spleen cPLA ₂	1870	1954
Pig pancreatic Group-I PLA ₂	2490	345
Rat platelet Group-II PLA ₂	2570	120

Table 6 Anti-pancreatic Group-I PLA₂ antibody does not recognize the small-molecular-mass cortical culture PLA₂

Washed Protein A-beads (50 µl), with attached anti-(pancreatic PLA₂) antibody, was added to 500 µl of heparin-binding fractions containing PLA₂ activity. As a positive control Group-II PLA₂ was prepared from heparin-binding fractions of 100 000 *g* supernatants of rat platelets. Incubation was performed for 12 h at 4 °C with constant shaking. The samples were centrifuged and the supernatants were assayed for PLA₂ activity using 2-[1-¹⁴C]AA-PE as substrate.

	PLA ₂ activity in supernatants (c.p.m.)	
	Normal serum	Anti-pancreatic PLA ₂ antibody
Heparin-binding fractions from cortical cultures	1670	1540
Rat platelet Group-II PLA ₂	3200	250

Antibody raised to pancreatic PLA₂ did not immunoprecipitate activity from the heparin-binding fraction whereas activity was immunoprecipitated from suspensions of partially purified platelet group-II PLA₂ (Table 6). The antibody to pancreatic PLA₂ also did not recognize the 13.5 kDa PLA₂ on Western blots (results not shown). These results indicate that the small-molecular-mass PLA₂ in cortical cultures is distinct from similar sized forms previously described in platelets, pancreas and other organs.

Glutamate affects the Ca²⁺-dependency of PLA₂ but not its pH dependency

The activities of both forms of PLA₂ were dependent upon the Ca²⁺ concentration in the buffer (Figures 6a and 6b). The activity of the larger-molecular-mass form (fraction 13 from the Superose 12 column) obtained from control cultures was increased significantly when Ca²⁺ concentration was increased from 100 nM to 750 nM and reached a plateau value when [Ca²⁺] was raised to 100 µM (shown with PE as substrate in Figure 6a). Ca²⁺

Figure 6 Calcium dependency of neuronal cytosolic PLA₂ activity

(a) PLA₂ activity of the larger-molecular-mass form (fraction 13 on Figure 2) as a function of [Ca²⁺]. Cell extracts were prepared from cultures exposed to 500 μ M glutamate and from control sister cultures and fractionated by Superose 12 FPLC gel-filtration chromatography. The activity of the larger form of the enzyme increased when [Ca²⁺] was increased over the physiological range (0.1–1.0 μ M). PLA₂ activity at these Ca²⁺ concentrations was significantly enhanced by exposure of the cells to 500 μ M glutamate for 20 min. 2-[1-¹⁴C]AA-PE was the substrate. Error bars represent S.E.M.s in experiments on three platings. (b) PLA₂ activity of the smaller form (fraction 17 on Figure 2) as a function of [Ca²⁺]. The difference between the PLA₂ activity in cultures exposed to 500 μ M glutamate for 20 min and control sister cultures was greater for the smaller-molecular-mass form of the enzyme fractionated on a Superose 12 FPLC column. Glutamate stimulation of cells induced a Ca²⁺-dependent increase in cytosolic PLA₂ activity at significantly lower Ca²⁺ concentrations (1 μ M) than observed in control cultures (5 μ M). 2-[1-¹⁴C]AA-PE was the substrate. Error bars represent S.E.M.s on experiments on three platings. In some cases, where the error bars are not visible, the standard error was smaller than the radius of the circle delineating the mean value.

activation of the enzymic activity of the higher-molecular-mass form was potentiated by prior glutamate exposure.

The activity of the smaller-molecular-mass form (fraction 17 from the Superose 12 column) was also Ca²⁺-dependent; however, in extracts from control cells, PLA₂ activity did not progressively increase until the [Ca²⁺] was increased above 1 μ M (Figure 6b). In extracts derived from glutamate-treated cells the smaller-molecular-mass form (fraction 17) of the enzyme was activated at lower Ca²⁺ concentrations (Figure 6b). In these extracts PLA₂ activity increased when [Ca²⁺] was increased above 100 nM. Furthermore the activity at maximal [Ca²⁺] was increased by 175% when compared with fractions from control cells (Figure 6b).

DISCUSSION

Our results indicate that the excitatory neurotransmitter, glutamate, reversibly enhances the Ca²⁺-dependent PLA₂ enzymic activity in rat brain cortical cultures. Cytosolic extracts of cortical cultures exposed to glutamate have increased PLA₂ activity that survives cell homogenization and centrifugation. This effect differs from a simple activation of PLA₂ by Ca²⁺, as has been proposed in studies [15,39] where AA release has been used as an

indicator of PLA₂ activity in neurons. Phosphorylation may account for the glutamate-induced stable increase in PLA₂ activity. Since the phorbol ester, PMA, increases PLA₂ activity to an extent equivalent to that of glutamate and glutamate's effect was partially blocked by a longer duration pre-exposure to PMA, a treatment known to down-regulate PKC [25], PKC is probably involved in glutamate's activation of PLA₂. The stable increase in PLA₂ activity observed in cultures exposed to A23187 may also be related to Ca²⁺ activation of PKC. Dumuis et al. demonstrated that activation of the metabotropic receptor which leads to activation of PKC [40] was necessary for AA release by non-NMDA glutamate agonists [15]. PKC and p42 MAP kinase (a kinase downstream of PKC in a signalling cascade) can directly activate cPLA₂ [41,42]. A direct G-protein-coupled activation process [31,43] could also contribute to glutamate's effect on PLA₂ activity. A Ca²⁺-mediated translocation of PLA₂ activity from the membrane to the cytosol is unlikely to explain the increased cytosolic activity measured after glutamate exposure since our studies demonstrate that membrane activity is low and glutamate exposure also increases membrane activity.

Glutamate did not affect PLA₂ activity in pure glial cultures. This is consistent with the observation that NMDA increases release of AA from cultures of rat hippocampal neurons but not from astrocytes [44]. Thus, barring an indirect effect on glial PLA₂ by the presence of neurons, the effect of glutamate on mixed neuronal/glial cultures is probably due to activation of neuronal glutamate receptors.

Two forms of PLA₂ migrated on Superose 12 gel-filtration columns in a pattern similar to the forms we described in kidney [13] and gerbil brain [22]. Activation of both forms of PLA₂ was enhanced by prior exposure of the culture to glutamate. The larger form had chromatographic characteristics identical to the cPLA₂ [30,38,46], which migrates at 100 kDa by SDS/PAGE. This form was recognized by a cPLA₂ antibody [30]. The second, smaller form was similar in size to Group-I and -II forms isolated from other tissues but was eluted at a different NaCl concentration from a heparin-Sepharose column than did platelet Group-II PLA₂, was not inhibited by dithiothreitol, and did not react with an antibody to pancreatic Group-I PLA₂, distinguishing it from Group-I and -II PLA₂s.

In cortical cultures both forms of cytosolic PLA₂ were Ca²⁺-dependent. As seen in Figure 6(a) the high-molecular-mass form of PLA₂ becomes activated at Ca²⁺ concentrations observed in neurons after growth factor stimulation [47], glutamate exposure [48], membrane depolarization [47] and tetanic stimulation [50]. In contrast, in extracts from non-stimulated cultures, Ca²⁺ concentrations as high as 1 μ M did not activate the low-molecular-mass form of PLA₂. However, after stimulation of the cultures with 75–100 μ M glutamate for 20 min, the threshold [Ca²⁺] necessary for activation of the low-molecular-mass form of PLA₂ decreased and significant activation was seen at 600 nM. A shift of PLA₂'s enzyme activity into this range of [Ca²⁺] might well influence *in vivo* physiological processes dependent upon PLA₂-mediated AA release. In addition to this effect of shifting PLA₂'s Ca²⁺ sensitivity, pretreatment of the culture with glutamate also doubled the maximal enzyme activity of both forms measured at high Ca²⁺ concentrations.

To our knowledge this demonstration of an increase in the Ca²⁺-sensitivity of PLA₂, occurring as a result of stimulation of intact cells with an agonist, is unique. This change in Ca²⁺ sensitivity and increased enzyme activity at all activating Ca²⁺ concentrations may underly the potentiation of agonist-induced AA or eicosanoid release by PKC activation in a variety of cell types while PKC activation alone, without an agonist-induced increase in cytosolic [Ca²⁺], has little effect on such release

[15,51,52]. Therefore, the importance of the observation is likely to be more general than the effects of glutamate in the nervous system.

The consequences of a glutamate-induced transition to a higher PLA₂ activity state are potentially quite important. AA, and the eicosanoids derived from AA, are chemical messengers which can leave the post-synaptic cell, diffuse across the synaptic cleft and modulate excitatory neurotransmission. PLA₂ activity may have both pre- and post-synaptic influences on glutamatergic transmission [14]. AA may be involved in the induction of long-term potentiation [53]. AA enhances phosphorylation of GAP-43, a major phosphoprotein of the presynaptic membrane that is believed to be important in the development and plasticity of synaptic connections [9].

In addition to enhancing excitatory neurotransmission, PLA₂ and its products, free fatty acids, PAF and lysophospholipids, may contribute to neuronal injury in the central nervous system by damaging membranes, impairing mitochondrial energetics [54,55], and increasing oxidative stress [56]. We have previously reported an increase in brain PLA₂ activity with ischaemia [22]. We (D. K. Kim, G. Rordorf, R. A. Nemenoff, W. J. Koroshetz and J. V. Bonventre, unpublished work) and others [57] have found that nordihydroguaric acid, which we have found to inhibit cPLA₂ [30], protects cultured cortical neurons from glutamate toxicity.

In conclusion we have demonstrated that at least two soluble forms of Ca²⁺-dependent PLA_s are present in cortical cultures from the rat brain. Both forms are activated by glutamate. Glutamatergic-mediated stable enhancement in the activities of PLA_s could serve to increase the release of AA or eicosanoids after a robust excitatory input. This effect is partially mediated via PKC and is due, at least in part, to an increase in the Ca²⁺ sensitivity of one of the forms of PLA₂. Glutamate-enhanced PLA₂ activity could serve to modulate synaptic function and/or contribute to brain injury in those pathological states linked to excitotoxicity.

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