

Synergy by Secretory Phospholipase A₂ and Glutamate on Inducing Cell Death and Sustained Arachidonic Acid Metabolic Changes in Primary Cortical Neuronal Cultures*

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Secretory and cytosolic phospholipases A₂ (sPLA₂ and cPLA₂) may contribute to the release of arachidonic acid and other bioactive lipids, which are modulators of synaptic function. In primary cortical neuron cultures, neurotoxic cell death and [³H]arachidonate metabolism was studied after adding glutamate and sPLA₂ from bee venom. sPLA₂, at concentrations eliciting low neurotoxicity (100 ng/ml), induced a decrease of [³H]arachidonate-phospholipids and preferential reesterification of the fatty acid into triacylglycerols. Free [³H]arachidonic acid accumulated at higher enzyme concentrations, below those exerting highest toxicity. Synergy in neurotoxicity and [³H]arachidonate release was observed when low, nontoxic (10 ng/ml, 0.71 nM), or mildly toxic (25 ng/ml, 1.78 nM) concentrations of sPLA₂ were added together with glutamate (80 μM). A similar synergy was observed with the sPLA₂ OS2, from Taipan snake venom. The NMDA receptor antagonist MK-801 blocked glutamate effects and partially inhibited sPLA₂ OS2 but not sPLA₂ from bee venom-induced arachidonic acid release. Thus, the synergy with glutamate and very low concentrations of exogenously added sPLA₂ suggests a potential role for this enzyme in the modulation of glutamatergic synaptic function and of excitotoxicity.

Membrane unsaturated fatty acid turnover and the synthesis of bioactive lipids are modulated by phospholipases A₂ (PLA₂),¹ ubiquitous mammalian enzymes that catalyze the hydrolysis of *sn*-2-acyl ester bonds of phospholipids (PLs) (1). Arachidonic acid (AA), eicosanoids, and platelet-activating factor (PAF) are bioactive lipids generated through PLA₂ activation (2). Although some PLA₂ are calcium-independent (3, 4), most found in the brain are characterized by calcium dependence (4, 5). PLA₂s are overstimulated in the brain during seizures and ischemia (6–8) as a consequence of increased calcium influx and/or intracellular calcium mobilization, which, in turn, results in the accumulation of bioactive lipids that par-

ticipate in cell damage (8, 9).

There are secretory and cytosolic PLA₂s (sPLA₂ and cPLA₂s, respectively). sPLA₂ (14 kDa) are active at submillimolar concentrations of calcium and do not display selectivity for unsaturated fatty acids at the *sn*-2-position of PLs (4, 5). sPLA₂s are found in pancreatic secretions (type I), platelets, neurons, mast cells, snake venoms, inflammatory exudates (type II), and bee venom (type III) (4, 5, 10). In contrast, cPLA₂ (type IV) has a higher molecular mass (85 kDa), is active at submicromolar Ca²⁺ concentrations, and shows selectivity for *sn*-2-arachidonoyl-PLs (5, 11). cPLA₂ is activated by translocation to intracellular and nuclear membranes when there is an agonist-induced increase in intracellular calcium concentration ([Ca²⁺]_i) in the brain (12, 13) as well as in other tissues (4, 14).

Among the neural forms of PLA₂ are (a) a calcium-sensitive and arachidonoyl-specific 85-kDa cPLA₂ (12, 15, 16), highly expressed in astrocytes (17), other cytosolic calcium-dependent forms (12, 16), and calcium-independent forms (3, 18, 19); and (b) membrane-bound forms (15), including a very high molecular mass (180-kDa) form from human temporal cortex (20). Secretory PLA₂ are also present in the brain. The expression of sPLA₂ type II is stimulated in the rat brain by ischemia/reperfusion (21) and in cultured astrocytes by inflammatory mediators (22). Moreover, sPLA₂ type II is stored in synaptic vesicles and released by depolarization or neurotransmitter stimulation, and its secretion is coupled with the activation of catecholamine release (23). Furthermore, sPLA₂ causes activation of Glu release in the rat cerebral cortex (24).

sPLA₂ bind to cell surface receptors, the N type and the M type (25–28) identified using sPLA₂ purified from snake and bee venoms as ligands. Neurotoxic sPLA₂ from Taipan snake venom, OS2, and from bee venom bind to the N-type receptor with high affinity (25, 26). Other sPLA₂s such as OS1, also purified from Taipan snake venom, display higher enzymatic activity than the sPLA₂s OS2 and bee venom (2.7- and 7-fold higher, respectively) (25). Although OS1 binds with high affinity to M-type receptors (26–28), it does not bind to N-type receptors (25) and is therefore non-neurotoxic.

Activation of cPLA₂ mediates the formation of modulators of synaptic transmission such as free AA (8), eicosanoids (29, 30), and PAF (31). Ischemia and seizures promote a rapid increase in brain free AA (6, 7, 32, 33), oxygenated metabolites of AA, and free radicals, all of which are potent neuronal injury mediators (for review, see Ref. 8). A sustained activation of cPLA₂ has been reported after ischemia/reperfusion (13, 15). Glu, which causes excitotoxic neuronal damage, increases calcium influx through NMDA receptors in postsynaptic neurons, leading to PLA₂-mediated AA release (34–37), which is blocked by the NMDA antagonist MK-801 (38). Recently, the activation of two calcium-dependent cPLA₂s (100 and 14 kDa) by Glu was reported (16).

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¹ The abbreviations used are: PLA₂, phospholipase A₂(s); sPLA₂, secretory phospholipase A₂(s); cPLA₂, cytosolic phospholipase A₂; PAF, platelet-activating factor; AA, arachidonic acid; PL, phospholipid; CHE, cholesterol ester; TAG, triacylglycerol; FFA, free fatty acids; DAG, diacylglycerol; LDH, lactate dehydrogenase.

Other PLA₂s are modulators of membrane PL metabolism and/or generate membrane fusogenic molecules, *i.e.* free fatty acids and lyso-PLs. However, non-AA-specific-sPLA₂s contribute, together with the cPLA₂, to modulate AA metabolism under physiological conditions and in primed conditions (5, 39–42). sPLA₂ venoms have long been known to be neurotoxic (10, 43). For example, sPLA₂ from *Naja mocambique*, as well as the PLA₂ activator melittin, have previously been shown to promote neural injury, *in vitro* and *in vivo* (44). The present study has tested the hypothesis that sPLA₂ potentiates neurotoxicity by Glu by briefly exposing rat cortical neuronal cultures to this neurotransmitter in the presence of and in the absence of sPLA₂. This hypothesis is supported in part by the observation that sPLA₂ is released at synapses from vesicles that also store Glu (23). sPLA₂ from bee venom and Taipan snake venom OS2, ligands of the N-type sPLA₂ receptor, and OS1 from Taipan snake venom, a ligand of the M-type sPLA₂ receptor, have been used (25–28). Moreover, sustained changes in neuronal [³H]AA metabolism under these conditions have been observed. Our study has addressed the following: (a) the effect of Glu-activated cPLA₂ and exogenously added sPLA₂ on [³H]AA release from neuronal PLs; (b) the action of MK-801 on agonist-induced AA changes and neurotoxicity; (c) the neurotoxic potential of exogenously added sPLA₂ (OS2 and bee venom sPLA₂); and (d) the neurotoxic effect of the excitatory neurotransmitter Glu and sPLA₂ when added to the cells simultaneously.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-³H]Arachidonic acid ([³H]AA, 185 Ci/mmol) was purchased from DuPont NEN. Silicagel GHL TLC plates were from Analtech, Inc. (Newark, DE). Bovine serum albumin fatty acid-free and lipid standards were from Sigma. Organic solvents (high pressure liquid chromatography grade) were from EM Science. sPLA₂ from bee venom and OS1 and OS2 from Taipan snake venom were purified as described previously (25).

Primary Neuronal Cultures—Cortical neuronal cultures were established from 15-day-old rat embryos as described previously (46, 47). Cells were plated in poly-L-lysine-coated dishes at a density of 4×10^5 cells/well in 48-well plates for toxicity experiments and 7.5×10^5 cells/well in 24-well plates for lipid analysis. The cells were cultured in neuronal culture medium (NCM) containing 10% fetal calf serum and 10% horse serum in a 5% CO₂ incubator at 37 °C. Astrocyte proliferation was prevented by adding cytosine arabinoside (10^{-5} M) at day 4 after plating, left on for 3 days, and then replaced with minimum essential medium containing 10% horse serum. Cells were used for experiments at 14–21 days *in vitro*. The percentage of neuronal versus glial cells remained at approximately 80 versus 20%, respectively, as previously reported (48).

Neuronal Toxicity Assay—Lactate dehydrogenase (LDH) release was used to quantitatively assess cell injury. Cells were treated as described previously (47), and LDH release was measured 20 h after exposure to sPLA₂ and/or Glu. Briefly, cells were exposed for 45 min at room temperature to highly purified sPLA₂ from bee venom and snake venom (OS1 or OS2) and/or Glu (80 μM) in Locke's solution without magnesium. Locke's solution was exchanged with minimum essential medium without phenol red, and the cultures were returned to the CO₂ incubator for 20 h at 37 °C. Using similar cortical neuron culture, this procedure results in minimal LDH release before 12 h and maximal release by 20 h (49). The LDH release was assayed using the Sigma kit with the aid of a DU series 640 Beckman spectrophotometer equipped with a graphic video display.

[³H]Arachidonic Acid Metabolism and Lipid Analysis—Cells were labeled overnight with [³H]AA (0.5 μCi/well, specific activity 184.6 Ci/mmol) in minimum essential medium supplemented with 0.2% fatty acid-free bovine serum albumin. 18–20 h later, the medium of cell cultures was replaced with minimum essential medium. The cells were exposed to Glu and/or sPLA₂ as described previously and returned to the CO₂ incubator for 2 and 20 h at 37 °C. The supernatants were removed, and 750 μl of methanol was added to each well. The cells were scraped and transferred into a glass tube, and chloroform was added in the following proportion: chloroform:methanol, 2:1 (v/v). Lipids were extracted by sonication for 30 min, and the lipid extracts were washed

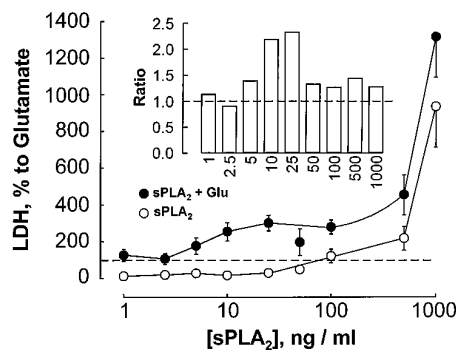


FIG. 1. Secretory phospholipase A₂ from bee venom and Glu elicit synergy of neurotoxicity. Rat cortical neurons were treated for 45 min at room temperature in Locke's solution with Glu (80 μM) and with increasing concentrations of sPLA₂ from 1 ng/ml (71 pM) to 10³ ng/ml (71 nM) in the presence or absence of Glu (80 μM). The media were assayed for LDH release 20 h later. LDH released by Glu in each plating was considered as 100%, and all other values were normalized to a percentage of the Glu-LDH value. Mean values ± S.D. from at least *n* = 10 individual determinations are shown. Values of LDH for 25 ng/ml sPLA (130%) or higher were significantly above Glu alone (*p* < 0.05). The dotted line indicates a Glu value (100%) attained at 100 ng/ml sPLA₂ concentration. *Inset*, plotted values are the ratio between percentage of LDH release induced by sPLA₂ added together with Glu divided by the sum of the percentage LDH values obtained with sPLA₂ and Glu (100%) assessed individually in the same plating. A ratio of 1 (dotted line) indicates that the effects are additive.

following the procedure of Folch *et al.* (50). Aliquots in duplicate were taken to determine total [³H]AA incorporation by liquid scintillation counting.

Neutral lipids and total PLs were isolated by monodimensional TLC on precoated, 0.25-mm-thick, silica gel GHL plates using hexane:diethyl ether:acetic acid, 40:60:1.3 (v/v/v), as a developing solvent. For the results presented in Table I, the composition of the chromatographic solvent was changed to 60:40:1.3 (v/v/v) in order to isolate cholesterol ester (CHE) from triacylglycerols (TAG) that run together in the first system. The results indicated that changes observed in previous experiments in the CHE plus TAG fraction were due mainly to TAG. Lipid standards were added to the samples as a carrier and were spotted in a parallel line on the TLC plate to individualize each lipid band. TLC plates were developed with iodine, lipid bands were scraped, and the radioactivity was determined in a Beckman scintillation counter.

Statistical Analysis—The significance of the data was evaluated with Student's *t* test for unpaired data. Statistical values were considered significantly different when *p* ≤ 0.05.

RESULTS

Potent Neurotoxicity Elicited by sPLA₂—Neurotoxicity of sPLA₂ from bee venom, added alone or with Glu (80 μM) to primary neuronal cortical cultures, was studied by measuring LDH release (Fig. 1). A neurotoxic concentration of Glu (47) resulted in 92 ± 7% increase in LDH release compared with control cells (*n* = 54 from 10 different platings). No significant toxicity was observed with bee venom sPLA₂ concentrations up to 10 ng/ml (Fig. 1A). At higher concentrations up to 10³ ng/ml, the neurotoxicity displayed by sPLA₂ was biphasic. First, there was a dose-dependent increase in LDH release (up to 500 ng/ml, EC₅₀ = 7.1 nM), followed by a sharp 4.3-fold increase in LDH at 10³ ng/ml (EC₅₀ = 57 nM). The sPLA₂ neurotoxicity at 100 ng/ml (7.1 nM) was similar to that of 80 μM Glu (Fig. 1). An additive neurotoxic effect was observed when cells were exposed simultaneously to 80 μM Glu and low (1–5 ng/ml) or high (50–10³ ng/ml) sPLA₂ concentrations (Fig. 1, *inset*). The combination of sPLA₂ in a concentration range of 10–25 ng/ml with Glu led to a significant synergy on LDH release, resulting in values 2.3–2.4-fold higher than those of the additive effects of sPLA₂ and Glu.

The sPLA₂ OS2 purified from snake venom was found to be more potent as a neurotoxin than the sPLA₂ from bee venom. For example, at 25 ng/ml OS2 was approximately 2.7-fold more

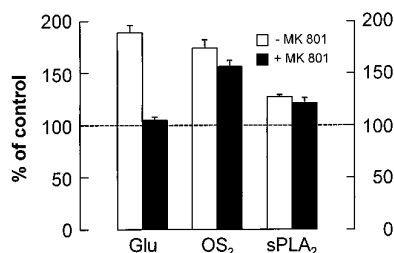


FIG. 2. MK-801 elicits neuroprotection against Glu but not against sPLA₂ from bee venom or OS₂. Rat cortical neurons were treated as described in the legend to Fig. 1 and assayed for LDH release. Values are normalized to percentage of control wells, treated with Locke's solution only; some wells were treated with MK-801 (300 nM) for 10 min prior to the addition of toxins or Glu and remained in the wells during treatment. Mean values are shown \pm S.E. from a representative set of culture plates derived from the same plating, treated and assayed on the same day. The dotted line (100%) indicates control, (nontoxic) levels for LDH. MK-801 blocks Glu ($p < 0.001$) by 100% but does not affect OS₂ ($p < 0.11$) and bee venom sPLA₂ ($p < 0.33$) neurotoxicity.

toxic than bee venom sPLA₂ at the same concentration (Fig. 2). Furthermore, under conditions where the noncompetitive NMDA antagonist MK-801 blocked 100% of 80 μ M Glu toxicity, MK-801 partially blocked OS₂, but not bee venom sPLA₂-induced toxicity. OS₁ did not evoke neuronal death even at 10 μ g/ml (LDH percentage above control = $18 \pm 9\%$).

sPLA₂ Promotes Arachidonic Acid Release from Phospholipids—[³H]AA-prelabeled neuronal cells were exposed to different concentrations of bee venom sPLA₂ for 45 min and further incubated for 20 h (Fig. 3). No differences were observed in total [³H]AA labeling recovered per dish at very low, nontoxic sPLA₂ concentration (1 ng/ml). At higher concentrations (25–50 ng/ml), the recovery was decreased by 10% and by 20–30% at more toxic concentrations (500–10³ ng/ml), reflecting cell loss and matching the neurotoxicity assays (Fig. 1). After 20 h the [³H]AA distribution displayed a concentration-dependent loss of [³H]AA-PLs paralleled by an increase in free [³H]AA, [³H]AA-TAG and [³H]AA-DAG. A significant loss in PL labeling was observed even at the lowest sPLA₂ concentration (–7%, $p < 0.05$), reaching values 50% lower at the highest toxic concentrations (500–10³ ng/ml). Up to 100 ng/ml sPLA₂, the loss of [³H]AA from phospholipids (–29%) was paralleled by its active reesterification into TAG, which showed a 25% increase above the control value. Within this range of sPLA₂ concentration, free [³H]AA showed a small yet significant gradual increase, reaching values 2- and 4.5-fold higher than controls at 1 ng/ml and 100 ng/ml, respectively. The [³H]AA-TAG labeling plateaued at 500 ng/ml sPLA₂. This was paralleled by a large increase in free [³H]AA accumulation, which reached a value 20-fold higher than control. [³H]DAG labeling was very low, displaying the same pattern of changes as free [³H]AA and reaching a 2-fold increase in percentage of labeling at high sPLA₂ concentration (500 ng/ml).

Triacylglycerols Are a Finite Reservoir for the Uptake of [³H]AA Released by sPLA₂ and Glu—To ascertain if [³H]AA released by bee venom sPLA₂ was acylated into TAG and whether or not this correlated with neurotoxicity, the following experiment was performed. The [³H]AA metabolism as affected by a nontoxic concentration of sPLA₂ (1 ng/ml) and by a toxic concentration of Glu (80 μ M), added individually or combined, was studied at 2 and 20 h after treatment with the agonists (Fig. 4). sPLA₂ induced a similar decrease in [³H]AA-PL labeling both at 2 and 20 h. Differences were observed, however, in the distribution of labeling between free [³H]AA and [³H]AA-TAG. Free [³H]AA accumulation was greater at 2 h, decreasing by 20 h concomitantly with an increase in [³H]AA-TAG.

Glu alone triggered a similar loss in [³H]AA-PL compared

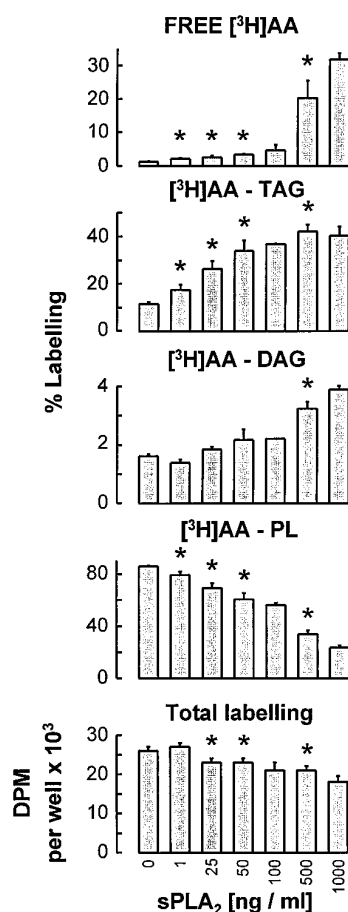


FIG. 3. Hydrolysis of [³H]arachidonoyl-phospholipids from cortical neurons by sPLA₂ from bee venom. Cortical neuronal cells cultures, labeled overnight with 0.5 μ Ci/well [³H]AA, were treated for 45 min with increasing concentrations of sPLA₂. The percentage of labeling of neuronal free [³H]AA, [³H]AA-TAG, [³H]AA-DAG, and [³H]AA-PLs and total labeling recovered per dish were assessed 20 h later. Mean values \pm S.E. from values obtained with 10 different platings are shown. Mean values \pm dispersion from the mean are shown for sPLA₂ concentrations of 100 ng/ml and 10³ ng/ml ($n = 2$). An asterisk denotes statistically significantly different from control ($p < 0.05$).

with sPLA₂ by 2 h; however, by 20 h, loss of [³H]AA from PL was 2.8-fold greater than at 2 h. After treatment with Glu alone, free [³H]AA and [³H]AA-TAG varied as a function of time (similar to when sPLA₂ was added alone), with higher accumulation of free [³H]AA by 2 h and a preferential reesterification of [³H]AA into TAG by 20 h.

sPLA₂ and Glu added together greatly magnified the pattern of [³H]AA changes as a function of time. A synergy on free [³H]AA accumulation was observed due to an apparently less efficient esterification into TAG. By 20 h the level of free [³H]AA reached 1.8–2-fold higher values than when both agonists were individually added. The loss of [³H]AA from PLs was additive, as was the accumulation of [³H]AA-DAG induced at 2 and 20 h.

MK-801 Does Not Block Arachidonic Acid Release Induced by sPLA₂ from Bee Venom but Partially Blocks the Effect of OS₂ from Snake Venom—The involvement of NMDA receptors on AA release from PLs induced by sPLA₂ and Glu was investigated by preincubating cells with 300 nM MK-801 for 10 min prior to adding the agonists, followed by lipid analysis 20 h later. Both at low, nontoxic (1 ng/ml) (data not shown) and at higher (25 ng/ml) bee venom sPLA₂ concentrations (Table I), MK-801 did not block the release of [³H]AA from PLs. The phospholipid labeling was decreased by 17% ($p < 0.002$), from 87% in controls to 70% in sPLA₂-treated cells. Most of the

[³H]AA released from PL (+11%) was found reesterified into TAG (5 versus 16% for control and sPLA₂-treated, respectively) and to a lesser extent in CHE (+4%, $p < 0.03$), while free [³H]AA labeling was doubled (from 1 to 2%, $p < 0.03$). MK-801 pretreatment did not alter the profile of lipid labeling, *i.e.* the decrease in PLs and the parallel increase in TAG and free AA labeling.

Glu (80 μ M), although more toxic than 25 ng/ml bee venom sPLA₂ (sPLA₂ toxicity 29% compared with Glu; Fig. 1), induced only a 6% ($p < 0.002$) decrease in PL labeling concomitantly with increased labeling of TAG (+2%, $p < 0.004$), CHE (+2%, $p < 0.02$), and FFA (+0.4%, $p < 0.03$). MK-801 pretreatment blocked by 100% Glu-induced PL degradation and other lipid changes. Higher degradation of PLs was observed when bee venom sPLA₂ and Glu were added together to the cells (−30%). Labeling of TAG increased by 24%, and labeling of free [³H]AA increased by 3% ($p < 0.03$). MK-801 pretreatment blocked partially the changes induced by bee venom sPLA₂ and Glu, leading to the same profile of lipid labeling induced by sPLA₂ alone.

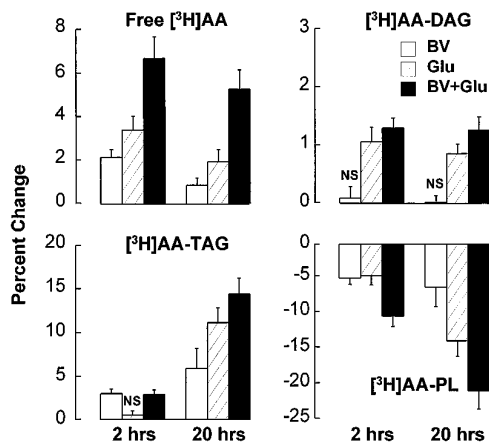


FIG. 4. sPLA₂ and Glu trigger both acute and sustained effects on arachidonic acid metabolism in cortical neurons. Cortical neuronal cultures prelabeled overnight with [³H]AA (0.5 μ Ci/well) were treated individually for 45 min with a nontoxic concentration of bee venom sPLA₂ (1 ng/ml), Glu (80 μ M), or both agonists together. Cells were harvested 2 and 20 h later and the percentage distribution of lipid labeling was determined. Percent change represents the percentage of labeling in stimulated cells minus the percentage of labeling of individual lipids in controls. Mean values \pm S.E. from at least $n = 5$ determinations for 2 h and $n = 11$ for 20 h are shown. All percent change values are significantly different from controls ($p < 0.05$) except for those shown as NS (not significantly different).

The sPLA₂ from snake venom, OS2, added to the cells at the same concentration as sPLA₂ from bee venom (25 ng/ml), induced a much greater degradation of [³H]AA-PLs. MK-801, in contrast to the results with bee venom, partially blocked [³H]AA-PL hydrolysis induced by OS2 when added alone or together with Glu (Table I). Moreover, the total labeling recovered per well treated with OS2 and OS2 plus Glu was decreased by 35%, indicating a massive loss of cells. The DPM/well obtained when the cells were pretreated with MK-801 was similar to controls. Minimal changes in [³H]AA-lipid labeling were observed when the cells were treated with the sPLA₂ (25 ng/ml) from snake venom OS1 (data not shown), which does not bind to neuronal membranes and which was found to be non-neurotoxic (see above).

sPLA₂ Display a Synergy with Glu in [³H]AA Release from Phospholipids—sPLA₂ (25 ng/ml) from snake and bee venoms added with Glu displayed synergy leading to a higher [³H]AA-PL degradation than the sum of the effect of the individual agonists (Table I, Fig. 5). Although the toxicity and PL hydrolysis induced by OS2 was much greater than that of bee venom sPLA₂ (Table I), the synergy with Glu was similar, reaching values for PL hydrolysis 1.4-fold higher for both sPLA₂ (Fig. 5C). A synergy was also observed in the accumulation of [³H]AA-TAG that increased by 2-fold for bee venom sPLA₂ and 1.4-fold for OS2 (Fig. 5B). The synergy in free [³H]AA accumulation was much greater with OS2 (3.5-fold) than with sPLA₂ from bee venom (2-fold) (Fig. 5A), and the synergy of sPLA₂ plus Glu was blocked by MK-801 (Table I).

Accumulation of Free [³H]AA in Cortical Neurons Precedes the Toxicity Induced by High Concentrations of Bee Venom sPLA₂—Treatment of neuronal cultures with increasing concentrations of sPLA₂ resulted in increased neurotoxicity (Fig. 1) and higher degradation of AA-PLs (Fig. 4). Changes in lipid labeling plotted as a function of sPLA₂ toxicity are shown in Fig. 6. The accumulation of free [³H]AA was minimal and proportional to increased LDH up to 100%, when sPLA₂ toxicity was equal to that of 80 μ M Glu (≤ 100 ng/ml sPLA₂). Within this range of neurotoxicity, most of the [³H]AA released from PLs (−30%) was reesterified into TAG. While PLs displayed a gradual loss of [³H]AA up to LDH values of 200% (−50% decrease in PL labeling), accumulation of free [³H]AA peaked between LDH values of 100 and 200%. This increase in free [³H]AA preceded a 4.3-fold increase in LDH release observed for sPLA₂ concentrations between 500 ng/ml (217%) and 10³ ng/ml (937% LDH).

TABLE I

Percentage of labeling of [³H]AA-lipids from neuronal cells in culture 20 h after treatment with sPLA₂ and glutamate

Cells were labeled overnight with [³H]AA and then exposed for 45 min to sPLA₂ from bee venom (25 ng/ml), OS2 from Taipan snake venom (25 ng/ml), glutamate (80 μ M) and/or MK-801 (300 nM). Values represent percentage distribution of labeling among neutral lipids and phospholipids recovered from cells. Mean values \pm S.E. are shown for the number of individual determinations (n) indicated. For samples with $n = 2$, mean values \pm S.D. are shown. Asterisks denote values statistically different from control (Student's t test, $p < 0.05$).

Condition		Labeling					Total
		CHE	TAG	FFA	DAG	PL	
		%	%	%	%	%	dpm/well
Control	$n = 8$	5.8 ± 0.8	4.9 ± 0.5	1.0 ± 0.1	1.7 ± 0.1	86.6 ± 1.3	$261,084 \pm 10,723$
Glu	$n = 4$	$8.2 \pm 0.6^*$	$7.3 \pm 0.5^*$	$1.3 \pm 0.1^*$	$2.3 \pm 0.2^*$	$80.8 \pm 1.0^*$	$242,010 \pm 15,687$
Glu + MK-801	$n = 4$	6.7 ± 0.6	4.7 ± 0.1	1.0 ± 0.0	1.5 ± 0.2	86.1 ± 0.6	$261,186 \pm 24,050$
sPLA ₂	$n = 4$	$9.9 \pm 1.2^*$	$15.7 \pm 1.3^*$	$2.3 \pm 0.1^*$	2.1 ± 0.3	$70.1 \pm 2.3^*$	$226,002 \pm 11,425^*$
sPLA ₂ + MK-801	$n = 4$	7.5 ± 1.5	$14.6 \pm 2.2^*$	$2.3 \pm 0.3^*$	1.8 ± 0.2	$73.8 \pm 3.2^*$	$251,838 \pm 14,374$
sPLA ₂ + GLU	$n = 4$	7.4 ± 1.1	$28.8 \pm 4.0^*$	$4.0 \pm 0.8^*$	$2.9 \pm 0.1^*$	$56.9 \pm 4.9^*$	$225,108 \pm 15,953$
sPLA ₂ + GLU + MK-801	$n = 4$	7.3 ± 0.7	$13.5 \pm 1.4^*$	$2.2 \pm 0.8^*$	2.5 ± 0.4	$74.4 \pm 1.2^*$	$229,122 \pm 11,353$
OS2	$n = 3$	$11.3 \pm 1.9^*$	$22.2 \pm 3.7^*$	$4.7 \pm 0.9^*$	$2.1 \pm 0.1^*$	$59.6 \pm 6.3^*$	$168,961 \pm 50,967^*$
OS2 + MK-801	$n = 2$	6.2 ± 2.0	16.6 ± 4.1	2.3 ± 0.9	2.3 ± 0.1	72.6 ± 6.9	$224,448 \pm 42,155$
OS2 + GLU	$n = 3$	6.0 ± 0.3	$32.8 \pm 2.6^*$	$15.4 \pm 3.3^*$	$3.8 \pm 0.2^*$	$41.8 \pm 5.8^*$	$173,664 \pm 11,44^*$
OS2 + GLU + MK-801	$n = 2$	5.0 ± 0.5	14.8 ± 3.0	2.5 ± 0.6	2.1 ± 0.0	75.5 ± 4.1	$233,448 \pm 13,950$

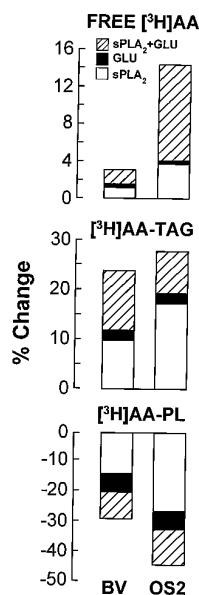


FIG. 5. Synergistic effect of bee venom sPLA₂ plus glutamate on [³H]AA release from phospholipids. Percentage changes for sPLA₂ from bee venom (BV) and OS2 from Taipan snake venom (25 ng/ml) and glutamate (80 μM) were calculated from values shown in Table I as described in the legend to Fig. 4. The open areas on the bar graphs show the changes induced by sPLA₂ alone, and the shaded areas show changes induced by Glu alone. The additive effects of sPLA₂ plus Glu are indicated by the height of open plus shaded area; the synergistic effect of sPLA₂ and Glu added together is denoted by the hatched area, which is above and beyond the additive areas.

DISCUSSION

This study shows that treatment of primary cortical neurons in culture with sPLA₂ induces (a) a concentration-dependent increase in neurotoxicity; (b) sustained activation of [³H]AA mobilization reflected in a gradual loss of [³H]AA from PLs and in an accumulation of free [³H]AA followed by its reesterification into TAG; and (c) synergy with Glu (80 μM) for both neurotoxicity and [³H]AA-PL hydrolysis.

Neurotoxicity and sustained changes in AA metabolism, triggered by 45-min exposure of primary cortical neurons to Glu were blocked by the NMDA receptor antagonist MK-801 (Fig. 2, Table I) in agreement with previous studies (34–37, 51–53). Moreover, the release of [³H]AA from PLs was observed 2 h after the treatment of neuronal cultures with Glu, and even greater release was observed 20 h later (Fig. 4). Long lasting changes in AA metabolism may be the result of calcium and protein kinase C-mediated, sustained activation of neuronal cPLA₂ by Glu (16). Moreover, increased cPLA₂ activity correlates with Glu neurotoxicity and precedes irreversible neuronal injury (16). It is also possible that, as in mast cells (54), Glu may regulate cPLA₂ activity at early time points by protein kinase C-mitogen activated protein kinase phosphorylation and later by enhanced expression of the enzyme. Modulation of gene expression and increased protein synthesis are involved in long term cellular responses as in neuronal plasticity or delayed neuronal death. In fact, cPLA₂ activation by NMDA-glutamatergic synaptic activity may lead to the formation of PAF, a potent bioactive lipid, which, in turn, mediates the induction of early response genes and subsequent gene cascades (2, 55–57). PAF could also potentiate excitotoxicity by enhancing Glu release (58, 59).

Although the toxicity of Glu (80 μM) was similar to that induced by bee venom sPLA₂ (100 ng/ml; Fig. 1), the hydrolysis of [³H]AA-PLs 20 h after Glu treatment (–15%; Fig. 4) was half of that generated by 100 ng/ml bee venom sPLA₂ (–29%; Fig. 3). These results and the fact that MK-801 blocked Glu neuro-

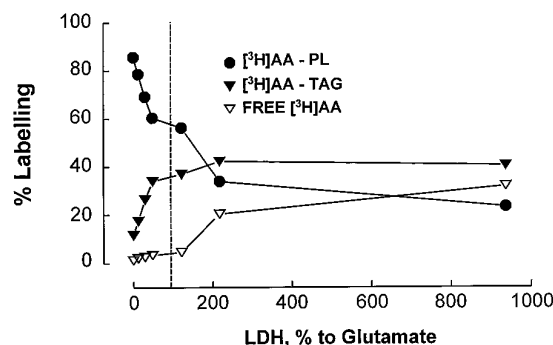


FIG. 6. sPLA₂-induced neurotoxicity correlates with changes in arachidonic acid metabolism. Plotted values were taken from those shown in Fig. 1 (percentage of LDH release) and Fig. 3 (percentage of lipid labeling) for increasing bee venom sPLA₂ concentrations. Cells were treated and data were analyzed as described under "Experimental Procedures." The vertical dotted line indicates the percentage of labeling that occurred for sPLA₂ concentration (100 ng/ml) with equivalent toxic effect as 80 μM Glu.

toxicity support the notion that mechanisms other than cPLA₂ activation mediated by Glu-activated NMDA-gated calcium channels contribute to its neurotoxic action (8). Glu may also activate metabotropic receptors that, in turn, activate phospholipase C with the release of AA-DAG, a potent activator of protein kinase C (60). Sequential degradation of AA-DAG by diacylglycerol lipases and monoacylglycerol lipases contribute also to increased free [³H]AA (61).

Bee venom sPLA₂-dependent sustained changes in [³H]AA-lipid metabolism (2 and 20 h after adding the enzyme) reveal an active release of [³H]AA from PLs, transient accumulation of free [³H]AA, and reesterification into TAG. A similar effect was observed with Glu, with increased labeling of free [³H]AA by 2 h decreasing by 20 h concomitantly with increased [³H]AA-TAG labeling. Interestingly, free [³H]AA was shunted into TAG even when cells were exposed to very low, nontoxic concentrations of sPLA₂ (1–10 ng/ml). Thus, the pathway activated by sPLA₂ may be physiologically relevant, withholding AA from its conversion to eicosanoids and from exerting effects of its own. AA is a modulator of synaptic function and potentiates Glu-NMDA neurotransmission, leading to excitotoxic damage (8). Free AA can be further metabolized to eicosanoids, potent modulators of synaptic function (29, 30), which, when overproduced, become injury mediators (8). TAG may also be a transient reservoir of AA when there is activation of degradative pathways, protecting the cells from the loss of this essential fatty acid. In fact, part of the [³H]AA released during repeated seizures from neuronal membrane PLs in rat brain is shunted into TAG (7). This pathway was also activated in retina by experimental detachment (62), where another polyunsaturated fatty acid, docosahexaenoate (22:6n-3), is actively esterified into TAG. A reversible accumulation of AA-TAG occurs in non-neuronal cells cultured in the presence of high concentrations of FFA (63, 64). In the present study, even 20 h after transient cell stimulation with nontoxic concentrations of sPLA₂, [³H]AA released from PLs remained as [³H]AA-TAG. This indicates long lasting metabolic changes, since between 2 and 20 h post-treatment, PLs did not recover basal labeling, and free [³H]AA was shunted into TAG.

The TAG reservoir appears to have a limited capacity to store AA. The maximum was reached at bee venom sPLA₂ concentrations between 50 and 100 ng/ml. AA-PL hydrolysis in neuronal cortical cells was much more sensitive to sPLA₂ than toxicity, within a range of LDH release similar to that exerted by Glu (Fig. 6). Thus, for sPLA₂ concentrations ≤100 ng/ml (toxicity ≤100% to Glu), the bulk of [³H]AA mobilized from PLs

was recovered in TAG. Only when TAG reached a 30% increase in labeling above basal level did further degradation of [³H]AA-PLs induced by higher, more toxic sPLA₂ concentrations result in preferential accumulation of free [³H]AA. Taken together these results suggest that as long as the cells are able to shunt AA to TAG, they are protected from accumulation of free AA, and the neurotoxicity of sPLA₂ is minimized. Moreover, similar mechanisms allow significant mobilization of [³H]AA from PLs without neurotoxic consequences (*i.e.* at 1 ng/ml sPLA₂). This supports the potential physiological relevance of sPLA₂ actions in promoting the formation of second messenger modulators of synaptic activity.

Neurotoxicity generated by sPLA₂ was biphasic with a linear increase up to 500 ng/ml and a sharp increase thereafter (Fig. 1). The estimated EC₅₀ for the two components (7.1 nM and 56.8 nM, respectively) is consistent with the two high affinity binding sites for OS2 in synaptic membranes (25). Moreover, sPLA₂ from bee venom competes with OS2 for both binding sites (25). At present there is no information regarding the location of these receptors in the same or different cells or at the pre- and/or post-synaptic level.

Bee venom sPLA₂ at nontoxic (10 ng/ml) and mildly toxic (25 ng/ml) concentrations, when added together with Glu, displayed synergy in neurotoxicity (Fig. 1). Moreover, a 2.3-fold higher toxicity induced by sPLA₂ (25 ng/ml) plus Glu was also paralleled by synergy on PL degradation and 2-fold higher accumulation of free [³H]AA. OS2, which is more toxic than sPLA₂ from bee venom (Ref. 25 and present results), displayed a more prominent synergy (3.5-fold) on free [³H]AA accumulation. The results reported here open up several questions for future exploration; *e.g.* is Glu-induced cPLA₂ activation potentiating sPLA₂-mediated degradation of [³H]AA-PLs and cellular toxicity, or *vice versa*? Recent studies carried out in P388D₁ macrophages revealed that PAF-induced AA mobilization involves two different PLA₂s (39) and that activation of cPLA₂ favors subsequent sPLA₂-induced AA release (40). Also, nerve growth factor, a regulator of mast cell function, has been reported to potentiate sPLA₂-induced histamine release (65). These observations suggest that sPLA₂ actively hydrolyzes lipids in disorganized membrane areas (66). Further studies combining lower, nontoxic concentrations of Glu and mammalian sPLA₂ type II, present together with Glu in synaptic vesicles (23), may further elucidate the involvement of both agonists in AA mobilization during glutamatergic synaptic activity.

Up to 25 ng/ml sPLA₂ from bee venom induced long lasting changes in AA-PL hydrolysis but did not involve the NMDA-glutamate pathway, since changes in AA metabolism and neurotoxicity were not blocked when sPLA₂ stimulation occurred in the presence of MK-801 (Fig. 2, Table I). Moreover, MK-801 partially blocked OS2 effect on AA-PL hydrolysis (Table I). This effect could be related to the origin/structure of the type II sPLA₂ from snake venoms and type III sPLA₂ from bee venom. Since the toxicity of OS2 at 25 ng/ml is 2.7-fold higher than that of sPLA₂ from bee venom at the same concentration (Fig. 2), the possibility of Glu-NMDA involvement at higher bee venom sPLA₂ concentrations on [³H]AA-PL hydrolysis cannot be ruled out. Nevertheless, the present results suggest that, at least for OS2, stimulation of Glu release at presynaptic endings followed by its interaction with NMDA receptors may be involved in the acute effects of OS2 resulting in a sustained [³H]AA-PL hydrolysis. Also, it is of interest that the profile of [³H]AA lipid labeling for OS2 stimulation in the presence of MK-801 was identical to that generated by bee venom sPLA₂ alone and not blocked by MK-801, indicating a similar receptor-mediated component common for both sPLA₂. As previously discussed, this could also be the result of sPLA₂ interaction with receptors

displaying different affinity for the enzymes.

In summary, this study shows that exogenously added sPLA₂ and Glu induce sustained changes in neuronal AA-PL metabolism and that sPLA₂ plus Glu exerts synergistic mobilization of AA and subsequent neurotoxicity. The present results, taken together with the recent observation that sPLA₂ type II in synaptic vesicles is released together with neurotransmitters (23), open up the possibility that glutamatergic neurotransmission involves the corelease of glutamate and sPLA₂. Our observations also imply that excitotoxicity may involve not only glutamate, as currently assumed, but may also involve sPLA₂ at the synaptic cleft. Further studies will assess if Glu could potentiate endogenous mammalian sPLA₂ actions that could, in turn, stimulate further Glu release. In this connection it is relevant that the synthesis of PAF, a retrograde messenger of long term potentiation (58), may be enhanced by sPLA₂ at the synapse. "Cross-talk" between cPLA₂ and sPLA₂ has recently been suggested in signal transduction events in macrophages (40), and a complex interplay between Glu-activated cPLA₂ and sPLA₂ could be envisioned at the synapse. Several of these ideas are currently under investigation in our laboratory.

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