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Synergy by Secretory Phospholipase A_2 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 A_2 ($sPLA_2$ and $cPLA_2$) 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 [^{3}H]arachidonate metabolism was studied after adding glutamate and $sPLA_2$ from bee venom. $sPLA_2$, at concentrations eliciting low neurotoxicity (≤100 ng/ml), induced a decrease of [^{3}H]arachidonate-phospholipids and preferential reesterification of the fatty acid into triacylglycerols. Free [^{3}H]arachidonic acid accumulated at higher enzyme concentrations, below those exerting highest toxicity. Synergy in neurotoxicity and [^{3}H]arachidonate release was observed when low, nontoxic (10 ng/ml, 0.71 nM), or mildly toxic (25 ng/ml, 1.78 nM) concentrations of $sPLA_2$ were added together with glutamate (80 μM). A similar synergy was observed with the $sPLA_2$ OS2, from Taipan snake venom. The NMDA receptor antagonist MK-801 blocked glutamate effects and partially inhibited $sPLA_2$ OS2 but not $sPLA_2$ from bee venom-induced arachidonic acid release. Thus, the synergy with glutamate and very low concentrations of exogenously added $sPLA_2$ 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 A_2 (PLA_2),[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 PLA_2 activation (2). Although some PLA_2 are calcium-independent (3, 4), most found in the brain are characterized by calcium dependence (4, 5). PLA_2s 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 participate in cell damage (8, 9).

There are secretory and cytosolic PLA_2s ($sPLA_2$ and $cPLA_2$s, respectively). $sPLA_2$ (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). $sPLA_2$s 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, $cPLA_2$ (type IV) has a higher molecular mass (85 kDa), is active at submicromolar Ca^{2+} concentrations, and shows selectivity for *sn*-2-arachidonoyl-PLs (5, 11). $cPLA_2$ is activated by translocation to intracellular and nuclear membranes when there is an agonist-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in the brain (12, 13) as well as in other tissues (4, 14).

Among the neural forms of PLA_2 are (*a*) a calcium-sensitive and arachidonoyl-specific 85-kDa $cPLA_2$ (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 PLA_2 are also present in the brain. The expression of $sPLA_2$ type II is stimulated in the rat brain by ischemia/reperfusion (21) and in cultured astrocytes by inflammatory mediators (22). Moreover, $sPLA_2$ 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, $sPLA_2$ causes activation of Glu release in the rat cerebral cortex (24).

$sPLA_2$ bind to cell surface receptors, the N type and the M type (25–28) identified using $sPLA_2$ purified from snake and bee venoms as ligands. Neurotoxic $sPLA_2$ from Taipan snake venom, OS2, and from bee venom bind to the N-type receptor with high affinity (25, 26). Other $sPLA_2$s such as OS1, also purified from Taipan snake venom, display higher enzymatic activity than the $sPLA_2$s 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 $cPLA_2$ 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 $cPLA_2$ 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 PLA_2-mediated AA release (34–37), which is blocked by the NMDA antagonist MK-801 (38). Recently, the activation of two calcium-dependent $cPLA_2$s (100 and 14 kDa) by Glu was reported (16).

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[1] The abbreviations used are: PLA_2, phospholipase A_2(s); $sPLA_2$, secretory phospholipase A_2(s); $cPLA_2$, cytosolic phospholipase A_2; PAF, platelet-activating factor; AA, arachidonic acid; PL, phospholipid; CHE, cholesterol ester; TAG, triacylglycerol; FFA, free fatty acids; DAG, diacylglycerol; LDH, lactate dehydrogenase.

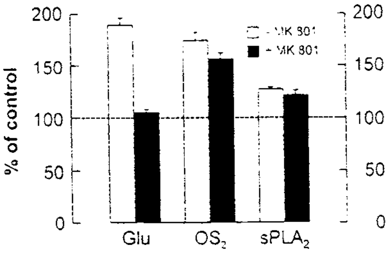

FIG. 2. **MK-801 elicits neuroprotection against Glu but not against sPLA$_2$ from bee venom or OS2.** Rat cortical neurons were treated as described in the legend to Fig. 1 and assayed for LDH release. Values are normalized to percentage of control wells, treated with Locke's solution only; some wells 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 ± 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 sPLA$_2$ ($p < 0.33$) neurotoxicity.

toxic than bee venom sPLA$_2$ at the same concentration (Fig. 2). Furthermore, under conditions where the noncompetitive NMDA antagonist MK-801 blocked 100% of 80 μM Glu toxicity, MK-801 partially blocked OS2, but not bee venom sPLA$_2$-induced toxicity. OS1 did not evoke neuronal death even at 10 μg/ml (LDH percentage above control = 18 ± 9%).

sPLA$_2$ Promotes Arachidonic Acid Release from Phospholipids—[^{3}H]AA-prelabeled neuronal cells were exposed to different concentrations of bee venom sPLA$_2$ for 45 min and further incubated for 20 h (Fig. 3). No differences were observed in total [^{3}H]AA labeling recovered per dish at very low, nontoxic sPLA$_2$ 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 (500–10^3 ng/ml), reflecting cell loss and matching the neurotoxicity assays (Fig. 1). After 20 h the [^{3}H]AA distribution displayed a concentration-dependent loss of [^{3}H]AA-PLs paralleled by an increase in free [^{3}H]AA, [^{3}H]AA-TAG and [^{3}H]AA-DAG. A significant loss in PL labeling was observed even at the lowest sPLA$_2$ concentration (−7%, $p < 0.05$), reaching values 50% lower at the highest toxic concentrations (500–10^3 ng/ml). Up to 100 ng/ml sPLA$_2$, the loss of [^{3}H]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 sPLA$_2$ concentrations, free [^{3}H]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 [^{3}H]AA-TAG labeling plateaued at 500 ng/ml sPLA$_2$. This was paralleled by a large increase in free [^{3}H]AA accumulation, which reached a value 20-fold higher than control. [^{3}H]DAG labeling was very low, displaying the same pattern of changes as free [^{3}H]AA and reaching a 2-fold increase in percentage of labeling at high sPLA$_2$ concentration (500 ng/ml).

Triacylglycerols Are a Finite Reservoir for the Uptake of [^{3}H]AA Released by sPLA$_2$ and Glu—To ascertain if [^{3}H]AA released by bee venom sPLA$_2$ was acylated into TAG and whether or not this correlated with neurotoxicity, the following experiment was performed. The [^{3}H]AA metabolism as affected by a nontoxic concentration of sPLA$_2$ (1 ng/ml) and by a toxic concentration of Glu (80 μM), added individually or combined, was studied at 2 and 20 h after treatment with the agonists (Fig. 4). sPLA$_2$ induced a similar decrease in [^{3}H]AA-PL labeling both at 2 and 20 h. Differences were observed, however, in the distribution of labeling between free [^{3}H]AA and [^{3}H]AA-TAG. Free [^{3}H]AA accumulation was greater at 2 h, decreasing by 20 h concomitantly with an increase in [^{3}H]AA-TAG.

Glu alone triggered a similar loss in [^{3}H]AA-PL compared with sPLA$_2$ by 2 h; however, by 20 h, loss of [^{3}H]AA from PL was 2.8-fold greater than at 2 h. After treatment with Glu alone, free [^{3}H]AA and [^{3}H]AA-TAG varied as a function of time (similar to when sPLA$_2$ was added alone), with higher accumulation of free [^{3}H]AA by 2 h and a preferential reesterification of [^{3}H]AA into TAG by 20 h.

sPLA$_2$ and Glu added together greatly magnified the pattern of [^{3}H]AA changes as a function of time. A synergy on free [^{3}H]AA accumulation was observed due to an apparently less efficient esterification into TAG. By 20 h the level of free [^{3}H]AA reached 1.8–2-fold higher values than when both agonists were individually added. The loss of [^{3}H]AA from PLs was additive, as was the accumulation of [^{3}H]AA-DAG induced at 2 and 20 h.

MK-801 Does Not Block Arachidonic Acid Release Induced by sPLA$_2$ 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 sPLA$_2$ 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 sPLA$_2$ concentrations (Table I), MK-801 did not block the release of [^{3}H]AA from PLs. The phospholipid labeling was decreased by 17% ($p < 0.002$), from 87% in controls to 70% in sPLA$_2$-treated cells. Most of the

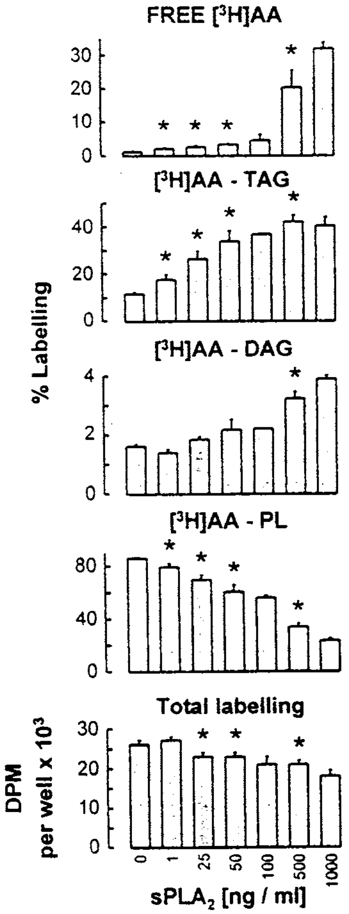

FIG. 3. **Hydrolysis of [^{3}H]arachidonoyl-phospholipids from cortical neurons by sPLA$_2$ from bee venom.** Cortical neuronal cells cultures, labeled overnight with 0.5 μCi/well [^{3}H]AA, were treated for 45 min with increasing concentrations of sPLA$_2$. The percentage of labeling of neuronal free [^{3}H]AA, [^{3}H]AA-TAG, [^{3}H]AA-DAG, and [^{3}H]AA-PLs and total labeling recovered per dish were assessed 20 h later. Mean values ± S.E. from values obtained with 10 different platings are shown. Mean values ± dispersion from the mean are shown for sPLA$_2$ concentrations of 100 ng/ml and 10^3 ng/ml ($n = 2$). An *asterisk* denotes statistically significantly different from control ($p < 0.05$).

[^{3}H]AA released from PL (−11%) was found reesterified into TAG (5 *versus* 16% for control and $sPLA_2$-treated, respectively) and to a lesser extent in CHE (+4%, $p < 0.03$), while free [^{3}H]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 μM), although more toxic than 25 ng/ml bee venom $sPLA_2$ ($sPLA_2$ 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 $sPLA_2$ and Glu were added together to the cells (−30%). Labeling of TAG increased by 24%, and labeling of free [^{3}H]AA increased by 3% ($p < 0.03$). MK-801 pretreatment blocked partially the changes induced by bee venom $sPLA_2$ and Glu, leading to the same profile of lipid labeling induced by $sPLA_2$ alone.

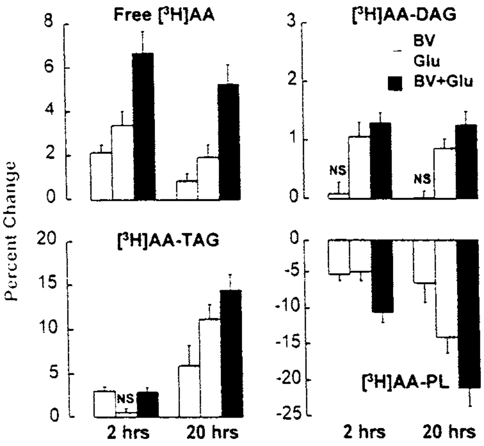

FIG. 4. **$sPLA_2$ and Glu trigger both acute and sustained effects on arachidonic acid metabolism in cortical neurons.** Cortical neuronal cultures prelabeled overnight with [^{3}H]AA (0.5 μCi/well) were treated individually for 45 min with a nontoxic concentration of bee venom $sPLA_2$ (1 ng/ml), Glu (80 μM), 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 ± 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 $sPLA_2$ from snake venom, OS2, added to the cells at the same concentration as $sPLA_2$ from bee venom (25 ng/ml), induced a much greater degradation of [^{3}H]AA-PLs. MK-801, in contrast to the results with bee venom, partially blocked [^{3}H]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 [^{3}H]AA-lipid labeling were observed when the cells were treated with the $sPLA_2$ (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).

$sPLA_2$ Display a Synergy with Glu in [^{3}H]AA Release from Phospholipids—$sPLA_2$ (25 ng/ml) from snake and bee venoms added with Glu displayed synergy leading to a higher [^{3}H]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 $sPLA_2$ (Table I), the synergy with Glu was similar, reaching values for PL hydrolysis 1.4-fold higher for both $sPLA_2$ (Fig. 5*C*). A synergy was also observed in the accumulation of [^{3}H]AA-TAG that increased by 2-fold for bee venom $sPLA_2$ and 1.4-fold for OS2 (Fig. 5*B*). The synergy in free [^{3}H]AA accumulation was much greater with OS2 (3.5-fold) than with $sPLA_2$ from bee venom (2-fold) (Fig. 5*A*), and the synergy of $sPLA_2$ plus Glu was blocked by MK-801 (Table I).

Accumulation of Free [^{3}H]AA in Cortical Neurons Precedes the Toxicity Induced by High Concentrations of Bee Venom $sPLA_2$—Treatment of neuronal cultures with increasing concentrations of $sPLA_2$ resulted in increased neurotoxicity (Fig. 1) and higher degradation of AA-PLs (Fig. 4). Changes in lipid labeling plotted as a function of $sPLA_2$ toxicity are shown in Fig. 6. The accumulation of free [^{3}H]AA was minimal and proportional to increased LDH up to 100%, when $sPLA_2$ toxicity was equal to that of 80 μM Glu (≤100 ng/ml $sPLA_2$). Within this range of neurotoxicity, most of the [^{3}H]AA released from PLs (−30%) was reesterified into TAG. While PLs displayed a gradual loss of [^{3}H]AA up to LDH values of 200% (−50% decrease in PL labeling), accumulation of free [^{3}H]AA peaked between LDH values of 100 and 200%. This increase in free [^{3}H]AA preceded a 4.3-fold increase in LDH release observed for $sPLA_2$ concentrations between 500 ng/ml (217%) and 10^3 ng/ml (937% LDH).

TABLE I

Percentage of labeling of [^{3}H]AA-lipids from neuronal cells in culture 20 h after treatment with $sPLA_2$ and glutamate

Cells were labeled overnight with [^{3}H]AA and then exposed for 45 min to $sPLA_2$ from bee venom (25 ng/ml), OS2 from Taipan snake venom (25 ng/ml), glutamate (80 μM) and/or MK-801 (300 nM). Vales represent percentage distribution of labeling among neutral lipids and phospholipids recovered from cells. Mean values ± S.E. are shown for the number of individual determinations (n) indicated. For samples with $n = 2$, mean values ± S.D. are shown. Asterisks denote values statistically significantly 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 ± 10,723
Glu	$n = 4$	8.2 ± 0.6*	7.3 ± 0.5*	1.3 ± 0.1*	2.3 ± 0.2*	80.8 ± 1.0*	242,010 ± 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 ± 24,050
$sPLA_2$	$n = 4$	9.9 ± 1.2*	15.7 ± 1.3*	2.3 ± 0.1*	2.1 ± 0.3	70.1 ± 2.3*	226,002 ± 11,425*
$sPLA_2$ + MK-801	$n = 4$	7.5 ± 1.5	14.6 ± 2.2*	2.3 ± 0.3*	1.8 ± 0.2	73.8 ± 3.2*	251,838 ± 14,374
$sPLA_2$ + GLU	$n = 4$	7.4 ± 1.1	28.8 ± 4.0*	4.0 ± 0.8*	2.9 ± 0.1*	56.9 ± 4.9*	225,108 ± 15,953
$sPLA_2$ + GLU + MK-801	$n = 4$	7.3 ± 0.7	13.5 ± 1.4*	2.2 ± 0.8*	2.5 ± 0.4	74.4 ± 1.2*	229,122 ± 11,353
OS2	$n = 3$	11.3 ± 1.9*	22.2 ± 3.7*	4.7 ± 0.9*	2.1 ± 0.1*	59.6 ± 6.3*	168,961 ± 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 ± 42,155
OS2 + GLU	$n = 3$	6.0 ± 0.3	32.8 ± 2.6*	15.4 ± 3.3*	3.8 ± 0.2*	41.8 ± 5.8*	173,664 ± 1144*
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 ± 13,950

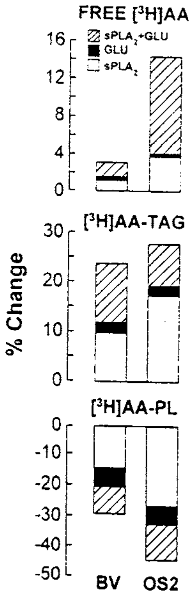

FIG. 5. **Synergistic effect of bee venom sPLA$_2$ plus glutamate on [^{3}H]AA release from phospholipids.** Percentage changes for sPLA$_2$ from bee venom (*BV*) and OS2 from Taipan snake venom (25 ng/ml) and glutamate (80 μM) were calculated from values shown in Table I as described in the legend to Fig. 4. The *open areas* on the *bar graphs* show the changes induced by sPLA$_2$ alone, and the *shaded areas* show changes induced by Glu alone. The additive effects of sPLA$_2$ plus Glu are indicated by the height of *open plus shaded area*; the synergistic effect of sPLA$_2$ and Glu added together is denoted by the *hatched area*, which is *above* and *beyond* the additive areas.

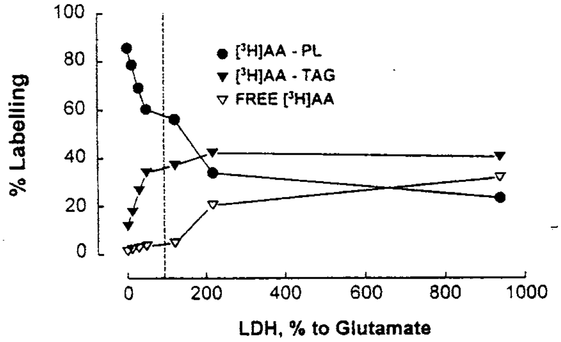

FIG. 6. **sPLA$_2$-induced neurotoxicity correlates with changes in arachidonic acid metabolism.** Plotted values were taken from those shown in Fig. 1 (percentage of LDH release) and Fig. 3 (percentage of lipid labeling) for increasing bee venom sPLA$_2$ concentrations. Cells were treated and data were analyzed as described under "Experimental Procedures." The *vertical dotted line* indicates the percentage of labeling that occurred for sPLA$_2$ concentration (100 ng/ml) with equivalent toxic effect as 80 μM Glu.

DISCUSSION

This study shows that treatment of primary cortical neurons in culture with sPLA$_2$ induces (*a*) a concentration-dependent increase in neurotoxicity; (*b*) sustained activation of [^{3}H]AA mobilization reflected in a gradual loss of [^{3}H]AA from PLs and in an accumulation of free[^{3}H]AA followed by its reesterification into TAG; and (*c*) synergy with Glu (80 μM) for both neurotoxicity and [^{3}H]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 [^{3}H]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 cPLA$_2$ by Glu (16). Moreover, increased cPLA$_2$ activity correlates with Glu neurotoxicity and precedes irreversible neuronal injury (16). It is also possible that, as in mast cells (54), Glu may regulate cPLA$_2$ 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, cPLA$_2$ 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 sPLA$_2$ (100 ng/ml; Fig. 1), the hydrolysis of [^{3}H]AA-PLs 20 h after Glu treatment (−15%; Fig. 4) was half of that generated by 100 ng/ml bee venom sPLA$_2$ (−29%; Fig. 3). These results and the fact that MK-801 blocked Glu neurotoxicity support the notion that mechanisms other than cPLA$_2$ 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 [^{3}H]AA (61).

Bee venom sPLA$_2$-dependent sustained changes in [^{3}H]AA-lipid metabolism (2 and 20 h after adding the enzyme) reveal an active release of [^{3}H]AA from PLs, transient accumulation of free [^{3}H]AA, and reesterification into TAG. A similar effect was observed with Glu, with increased labeling of free [^{3}H]AA by 2 h decreasing by 20 h concomitantly with increased [^{3}H]AA-TAG labeling. Interestingly, free [^{3}H]AA was shunted into TAG even when cells were exposed to very low, nontoxic concentrations of sPLA$_2$ (1–10 ng/ml). Thus, the pathway activated by sPLA$_2$ 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 [^{3}H]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 nonneural 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 sPLA$_2$, [^{3}H]AA released from PLs remained as [^{3}H]AA-TAG. This indicates long lasting metabolic changes, since between 2 and 20 h posttreatment, PLs did not recover basal labeling, and free [^{3}H]AA was shunted into TAG.

The TAG reservoir appears to have a limited capacity to store AA. The maximum was reached at bee venom sPLA$_2$ concentrations between 50 and 100 ng/ml. AA-PL hydrolysis in neuronal cortical cells was much more sensitive to sPLA$_2$ than toxicity, within a range of LDH release similar to that exerted by Glu (Fig. 6). Thus, for sPLA$_2$ concentrations ≤100 ng/ml (toxicity ≤100% to Glu), the bulk of [^{3}H]AA mobilized from PLs was recovered in TAG. Only when TAG reached a 30% increase in labeling above basal level did further degradation of [^{3}H]AA-PLs induced by higher, more toxic $sPLA_2$ concentrations result in preferential accumulation of free [^{3}H]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 $sPLA_2$ is minimized. Moreover, similar mechanisms allow significant mobilization of [^{3}H]AA from PLs without neurotoxic consequences (*i.e.* at 1 ng/ml $sPLA_2$). This supports the potential physiological relevance of $sPLA_2$ actions in promoting the formation of second messenger modulators of synaptic activity.

Neurotoxicity generated by $sPLA_2$ was biphasic with a linear increase up to 500 ng/ml and a sharp increase thereafter (Fig. 1). The estimated EC_{50} 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, $sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$ (25 ng/ml) plus Glu was also paralleled by synergy on PL degradation and 2-fold higher accumulation of free [^{3}H]AA. OS2, which is more toxic than $sPLA_2$ from bee venom (Ref. 25 and present results), displayed a more prominent synergy (3.5-fold) on free [^{3}H]AA accumulation. The results reported here open up several questions for future exploration; *e.g.* is Glu-induced $cPLA_2$ activation potentiating $sPLA_2$-mediated degradation of [^{3}H]AA-PLs and cellular toxicity, or *vice versa*? Recent studies carried out in $P388D_1$ macrophages revealed that PAF-induced AA mobilization involves two different PLA_2s (39) and that activation of $cPLA_2$ favors subsequent $sPLA_2$-induced AA release (40). Also, nerve growth factor, a regulator of mast cell function, has been reported to potentiate $sPLA_2$-induced histamine release (65). These observations suggest that $sPLA_2$ actively hydrolyzes lipids in disorganized membrane areas (66). Further studies combining lower, nontoxic concentrations of Glu and mammalian $sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$ from snake venoms and type III $sPLA_2$ from bee venom. Since the toxicity of OS2 at 25 ng/ml is 2.7-fold higher than that of $sPLA_2$ from bee venom at the same concentration (Fig. 2), the possibility of Glu-NMDA involvement at higher bee venom $sPLA_2$ concentrations on [^{3}H]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 [^{3}H]AA-PL hydrolysis. Also, it is of interest that the profile of [^{3}H]AA lipid labeling for OS2 stimulation in the presence of MK-801 was identical to that generated by bee venom $sPLA_2$ alone and not blocked by MK-801, indicating a similar receptor-mediated component common for both $sPLA_2$. As previously discussed, this could also be the result of $sPLA_2$ interaction with receptors displaying different affinity for the enzymes.

In summary, this study shows that exogenously added $sPLA_2$ and Glu induce sustained changes in neuronal AA-PL metabolism and that $sPLA_2$ plus Glu exerts synergistic mobilization of AA and subsequent neurotoxicity. The present results, taken together with the recent observation that $sPLA_2$ type II in synaptic vesicles is released together with neurotransmitters (23), open up the possibility that glutamatergic neurotransmission involves the corelease of glutamate and $sPLA_2$. Our observations also imply that excitotoxicity may involve not only glutamate, as currently assumed, but may also involve $sPLA_2$ at the synaptic cleft. Further studies will assess if Glu could potentiate endogenous mammalian $sPLA_2$ 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 $sPLA_2$ at the synapse. "Cross-talk" between $cPLA_2$ and $sPLA_2$ has recently been suggested in signal transduction events in macrophages (40), and a complex interplay between Glu-activated $cPLA_2$ and $sPLA_2$ could be envisioned at the synapse. Several of these ideas are currently under investigation in our laboratory.

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