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# Human group IIA secretory phospholipase $A_2$ potentiates $Ca^{2+}$ influx through L-type voltage-sensitive $Ca^{2+}$ channels in cultured rat cortical neurons

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**Abstract**

Mammalian group IIA secretory phospholipase $A_2$ ($sPLA_2$-IIA) generates prostaglandin $D_2$ ($PGD_2$) and triggers apoptosis in cortical neurons. However, mechanisms of $PGD_2$ generation and apoptosis have not yet been established. Therefore, we examined how second messengers are involved in the $sPLA_2$-IIA-induced neuronal apoptosis in primary cultures of rat cortical neurons. $sPLA_2$-IIA potentiated a marked influx of $Ca^{2+}$ into neurons before apoptosis. A calcium chelator and a blocker of the L-type voltage-sensitive $Ca^{2+}$ channel (L-VSCC) prevented neurons from $sPLA_2$-IIA-induced neuronal cell death in a concentration-dependent manner. Furthermore, the L-VSCC blocker ameliorated $sPLA_2$-IIA-induced morphologic alterations and apoptotic features such as condensed chromatin and fragmented DNA. Other blockers of VSCCs such as N type and P/Q types did not affect the neurotoxicity of $sPLA_2$-IIA. Blockers of L-VSCC significantly suppressed $sPLA_2$-IIA-enhanced $Ca^{2+}$ influx into neurons. Moreover, reactive oxygen species (ROS) were generated prior to apoptosis. Radical scavengers reduced not only ROS generation, but also the $sPLA_2$-IIA-induced $Ca^{2+}$ influx and apoptosis. In conclusion, we demonstrated that $sPLA_2$-IIA potentiates the influx of $Ca^{2+}$ into neurons via L-VSCC. Furthermore, the present study suggested that eicosanoids and ROS generated during arachidonic acid oxidative metabolism are involved in $sPLA_2$-IIA-induced apoptosis in cooperation with $Ca^{2+}$.

**Keywords:** apoptosis, $Ca^{2+}$ influx, group IIA secretory phospholipase $A_2$, L-type voltage-sensitive $Ca^{2+}$ channel, nimodipine, reactive oxygen species.

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Secretory phospholipase $A_2$s ($sPLA_2$s) are a growing family of enzymes that cleave the *sn*-2 fatty acyl ester bond of glycerophospholipids to yield a fatty acid and a lysophospholipid (Vadas and Pruzanski 1986; Arita *et al*. 1989). $sPLA_2$s have several common characteristics, including a relatively low molecular mass (13–18 kDa), the presence of six to eight disulfide bonds, an absolute catalytic requirement for millimolar concentration of $Ca^{2+}$, and a broad specificity for phospholipids with different polar head groups and fatty acid chains (Tishchfield 1997; Lambeau and Lazdunski 1999). Among $sPLA_2$s, group IIA $sPLA_2$ ($sPLA_2$-IIA) is thought to be one of the key enzymes in the pathogenesis of inflammatory disease, because its local and systemic levels are elevated in diseases, such as acute lung injury (Furue *et al*. 1999; Koike *et al*. 2000). An $sPLA_2$ inhibitor was reported to suppress lung injury induced by oleic acid and intestinal ischemia reperfusion (Furue *et al*. 1999; Koike *et al*. 2000).

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*Abbreviations used*: AD, Alzheimer's disease; AMPA, α-amino-2-hydroxy-5-methyl-4-isoxazolepropionic acid; Aβ, amyloid β protein; AP-7, 2-amino-4-phosphonobutyrate; BAPTA-AM, acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetra-acetic acid; CPP, 3-[(+/–)-2-carboxypiperazine-4-yl]propyl-1-phosphonate; $[Ca^{2+}]i$, concentration of intracellular $Ca^{2+}$; DCFDA, 2′,7′-dichlorofluorescin diacetate; DiCl, dichloro kynurenic acid; $IC_{50}$, concentration giving 50% inhibition: L-VSCC, L-type VSCC; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye; NBQX, 2,3-dihydroxy-6-nitro-7sulfamoylbenzo(f)quinoxaline; N-VSCC, N-type VSCC; PCP, phencyclidine; PG, prostaglandin; $PLA_2$, phospholipase $A_2$; P/Q-VSCC, P/Q-type VSCC; ROS, reactive oxygen species; $sPLA_2$, secretory $PLA_2$; $sPLA_2$-IB, group IB $sPLA_2$; $sPLA_2$-IIA, group IIA $sPLA_2$; TUNEL, TdT-mediated dUTP-biotin nick-end labeling; VSCC, voltage-sensitive calcium channel.

 

$sPLA_2$-IIA gene expression is induced in the ischemic brain (Lauritzen *et al*. 1994). It is strictly localized to the hippocampus and the cerebral cortex, in which neurons are vulnerable to ischemia (Smith *et al*. 1984). Recently, we have found that $sPLA_2$-IIA activity in the cerebral cortex was increased after ischemia, and was significantly suppressed by a potent $sPLA_2$-specific inhibitor, indoxam (Yagami *et al*. 2002a). Indoxam prevented the neurodegeneration in the penumbra after ischemia, in which apoptosis is caused (Umemura *et al*. 1995). $sPLA_2$-IIA caused cell death in primary cultures of rat cortical neurons. Morphologic and ultrastructural characteristics of neuronal cell death by $sPLA_2$-IIA were apoptotic, as evidenced by condensed chromatin and fragmented DNA. Prior to apoptosis, $sPLA_2$-IIA liberated free fatty acids, including arachidonic acid, and generated prostaglandin $D_2$ ($PGD_2$) from neurons. Indoxam not only suppressed arachidonic acid release and $PGD_2$ generation, but also prevented neurons from $sPLA_2$-IIA-induced neuronal cell death (Yagami *et al*. 2002a).

The injury and death of neurons that occur as the result of both acute insults (e.g. stroke) and chronic neurodegenerative disorders [e.g. Alzheimer's disease (AD)] appear to involve disturbances in cellular calcium homeostasis and reactive oxygen species (ROS) metabolism (Jesberger and Richardson 1991; Mattson *et al*. 1993; Choi 1995). Dysregulation of calcium and ROS metabolism can be initiated by activation of receptors for neurotoxins such as glutamate and amyloid β protein (Aβ). Calcium enters the cytoplasm through voltage-dependent and ligand-gated channels in the plasma membrane (Tsien *et al*. 1991). *N*-methyl-D-aspartate (NMDA) receptors (Lipton and Rosenberg 1994) and the L-type voltage-sensitive $Ca^{2+}$ channel (L-VSCC; Ueda *et al*. 1997a) were prominently involved in glutamate- and Aβ-induced neuronal cell death, respectively. Glutamate causes neuronal cell death via necrosis and apoptosis (Nicotera and Lipton 1999), whereas Aβ does so via apoptosis (Ueda *et al*. 1996). Furthermore, neurotoxic non-mammalian $sPLA_2$ triggers influx of $Ca^{2+}$ into neurons (Clapp *et al*. 1995). Taken together, these studies suggest that the excess influx of $Ca^{2+}$ into neurons might be involved in the mammalian $sPLA_2$-IIA-induced neuronal cell death.

In the present study, we ascertained whether $sPLA_2$-IIA dysregulates $Ca^{2+}$ homeostasis in rat cortical neurons. Here, we provide the first evidence that $sPLA_2$-IIA potentiates the $Ca^{2+}$ influx through L-VSCC, thereby inducing neurotoxicity.

## Materials and methods

### Materials

Nimodipine was synthesized at the Shionogi Research Laboratories (Osaka, Japan; Shimizu *et al*. 1990). Human $sPLA_2$-IIA was prepared as described previously (Kramer *et al*. 1989). Arabinosylcytosine C, acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetra-acetic acid (BAPTA-AM), poly-L-lysine, vitamin E, probucol, butylated hydroxytoluene, chlorpromazine, D-(–)-2-amino-4-phosphonobutyrate (AP-7), CPP (3-((+/–)-2-carboxypiperazin-4-yl)propyl-1-phosphonate), DiCl (dichloro kynurenic acid), NBQX [2,3-dihydroxy-6-nitro-7sulfamoylbenzo-(f)quinoxaline], and PCP (phencyclidine) were purchased from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium, Leibovitz's L-15 medium, trypsin, deoxyribonuclease I, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco (Grand Island, NY, USA). Hoechst 33258 fluorescent dye was purchased from Molecular Probes (Eugene, OR, USA). 2′,7′-Dichlorofluorescin diacetate (DCFDA) was purchased from Kodak (Tokyo, Japan). ω-Agatoxin GVIA, ω-conotoxin GVIA, and ω-conotoxin MVIIC were purchased from the Peptide Institute (Osaka, Japan).

### Measurements of $sPLA_2$-IIA activity

$PLA_2$ activity was measured with a phosphatidyl glycerol/sodium cholate mixed micelle assay (PG/Chol assay; Tojo *et al*. 1993). The specific activity of $sPLA_2$-IIA was 765.87 μmol/min/mg protein. The final activity of $sPLA_2$-IIA (1 μM) was 10.7 μmol/min/mL in the culture medium.

### Animals

The following experimental procedures used were approved by the Institutional Animal Care and Use Committee at the Discovery Research Laboratories of Shionogi and Co. Ltd, and all efforts were made to minimize the number of animals used and their suffering. Pregnant Sprague–Dawley rats were used. The rats were housed individually in macrolon cages with free access to food and water and maintained on a 12-h light–dark cycle, at 25°C room temperature. All experiments were carried out according to the guidelines of the European Community's Council for Animal Experiments.

### Tissue cultures

Neuronal cell cultures were prepared from cerebral cortices of day-19 Sprague–Dawley rat embryos as previously reported (Yagami *et al.* 2002c). Cerebral cortices were dissociated in isotonic buffer with 4 mg/mL trypsin and 0.4 mg/mL deoxyribonuclease I (Ueda *et al.* 1994). Cells were plated at a density of $2.5 \times 10^5$ cells/$cm^2$ on poly-L-lysine-coated dishes in conditioning medium, Leibovitz's L-15 medium supplemented with 5% fetal bovine serum, and 5% horse serum at 37°C in 5% $CO_2$ and 9% $O_2$. Cultures were treated with 0.1 μM arabinosylcytosine C on day 1 and used for experiments on day 2 after plating.

### Analysis of neuronal survival

Neurons ($2.5 \times 10^5$ cells/$cm^2$) were treated with $sPLA_2$-IIA in the presence or absence of various compounds at 37°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT) reduction assay reflecting mitochondrial succinate dehydrogenase activity was employed for assessment of neurotoxicity of $sPLA_2$-IIA, as previously reported (Yagami *et al*. 2002d).

### Measurement of $PGD_2$

$PGD_2$ was measured as previously reported (Yagami *et al*. 2002b). Supernatants of culture medium (1 mL) were mixed homogenously

with cold ethanol (4 mL). The mixture was centrifuged at 1500 *g* at 4°C for 10 min for removal of the particulate matter. Supernatants were diluted with an appropriate volume of distilled water to yield a final concentration of 10% ethanol, and the pH was adjusted to 3.5–4.0. Samples were loaded onto reversed-phase (C18) Sep-Pack cartridges, which had been prepared by washing with ethanol followed by distilled water. Samples were washed onto the Sep-Pak with 15 mL of 10% aqueous ethanol, followed by 15 mL of petroleum ether. Samples were extracted with 5 mL of methyl formate. The methyl formate effluents were pooled and evaporated with a heating module and dissolved in radioimmunoassay (RIA) buffer (50 mM phosphate buffer, pH 7.3, with 0.1% gelatin and 0.1% azide). $PGD_2$ was measured with their respective RIA kits (in duplicate/sample).

### Fluoromicroscopic analysis

Condensation of chromatin was assessed as previously described (Yagami *et al*. 2001b). Neurons ($2.5 \times 10^5$ cells/cm$^2$) were treated with 1 μM sPLA$_2$-IIA in the presence or absence of 10 μM indoxam or 10 μM nimodipine at 37°C for 48 h. The culture medium was exchanged with phosphate-buffered saline (PBS) containing 10 μM Hoechst 33258 fluorescent dye. Cells were incubated for 10 min at 37°C in the dark and washed with PBS. Stained nuclei were categorized as follows: (i) nuclei with homogeneously stained chromatin; (ii) nuclei with condensed chromatin, crescent-shaped areas of condensed chromatin often located near the periphery of the nucleus; and (iii) fragmented nuclei, more than two condensed micronuclei within the area of a neuron.

### *In situ* labeling of nuclear DNA fragments

Neurons ($2.5 \times 10^5$ cells/cm$^2$) were treated with 1 μM sPLA$_2$-IIA in the presence or absence of 10 μM indoxam or 10 μM nimodipine at 37°C for 48 h. As previously reported (Yagami *et al*. 2003a), neurons were stained by the TUNEL technique (TdT-mediated dUTP-biotin nick end-labeling). Apoptotic cells could be distinguished morphologically from necrotic cells by the presence of condensed brown nuclei.

### Measurement of $Ca^{2+}$ influx

$Ca^{2+}$ influx into cultured cells was measured as previously described (Yagami *et al*. 2003b). Neurons ($2.5 \times 10^5$ cells/cm$^2$) were treated with 1 μM sPLA$_2$-IIA in the presence or absence of various compounds at 37°C. After treatment, neurons were pre-incubated for 5 min at 37°C with basal saline containing 145 mM NaCl, 10 mM Tris–HCl (pH 7.4), 0.4 mM $KH_2PO_4$, 1.2 mM $MgCl_2$, 3.1 mM KCl, 10 mM glucose and 0.5 mM $CaCl_2$. The cells were then exposed to basal saline containing $^{45}CaCl_2$ (200 kBq/mL). $Ca^{2+}$ uptake was terminated after 10 s of incubation by washing twice with basal saline without glucose, and then the cells were solubilized with 1 mL sodium dodecyl sulfate (0.5%) and deoxycholate (0.05%). Samples were mixed with scintillation fluid (Picofluor 40; Perkin Elmer Life Science Products, Boston, MA, USA) and quantified by a liquid scintillation counter. Data are given as percentages of corresponding vehicle-treated values.

### Measurement of ROS

Intracellular ROS were measured by DCFDA assay (Ueda *et al*. 1997a). Neurons ($2.5 \times 10^5$ cells/cm$^2$) were treated with 1 μM sPLA$_2$-IIA in the presence or absence of various compounds at 37°C. Neurons were loaded with 1 mM DCFDA for 20 min 15 h later, and then intracellular reactive oxygen species were measured. Data are given as percentages of DCFDA fluorescence of corresponding vehicle-treated values.

### Statistical analysis

Data are given as means ± SEM ($n = 4$). All experiments were carried out at least twice with comparable results. Presented results were representative of all experiments. Data were analyzed statistically with Student's non-paired *t*-test for comparison with the control group. Data on various inhibitors and blockers groups were analyzed statistically by use of two-way ANOVA followed by Dunnett's test for comparison with the sPLA$_2$-IIA group as previously reported (Yagami *et al*. 2001a). Concentration giving 50% inhibition ($IC_{50}$) values were calculated by Microsoft Excel Fit as previously reported (Asakura *et al*. 1999).

## Results

### Effects of a calcium chelator on sPLA$_2$-IIA-induced neuronal cell death

Primary cultures of dissociated cortical neurons were exposed to sPLA$_2$-IIA, and neuronal cell death was quantified 48 h later (Fig. 1). sPLA$_2$-IIA at doses less than 0.1 μM did not reduce neuronal cell survival. Increasing concentrations of sPLA$_2$-IIA above 0.1 μM resulted in a significant increase in neuronal cell death in a dose-dependent manner, with a half-maximal concentration of 1.1 μM (Fig. 1a). A calcium chelator, BAPTA-AM, attenuated the neurotoxicity of sPLA$_2$-IIA in a concentration-dependent manner (Fig. 1b). These results suggested that calcium mobilization might contribute to the sPLA$_2$-IIA-induced neuronal cell death.

### Effect of sPLA$_2$-IIA on the influx of $Ca^{2+}$ into neurons

sPLA$_2$-IIA triggered neuronal cell death in a time-dependent manner after 24 h (Fig. 2a). Prior to cell death, the enzyme liberated arachidonic acid and generated $PGD_2$, which possesses neurotoxicity (Yagami *et al*. 2002a). $PGD_2$ was not generated within 10 h, but was produced at 15 h after sPLA$_2$-IIA treatment (Fig. 2b). On the other hand, sPLA$_2$-IIA increased the influx of $Ca^{2+}$ at 6 h, reached a peak at 18 h, and then elevated the level of $Ca^{2+}$ influx persistently (Fig. 2c). Thus, sPLA$_2$-IIA potentiated the influx of $Ca^{2+}$ into neurons, generated $PGD_2$, and caused neuronal cell death.

### Effects of a sPLA$_2$-IIA inhibitor and glutamate receptor blockers on sPLA$_2$-IIA-induced neuronal cell death

Indoxam, an indolizine derivative, was created as a novel sPLA$_2$ inhibitor by the Shionogi Research Laboratories (Hagishita *et al*. 1996). Indoxam inhibited the enzymatic activity of sPLA$_2$-IIA ($IC_{50} = 1.5$ nM; Yokota *et al*. 1999).

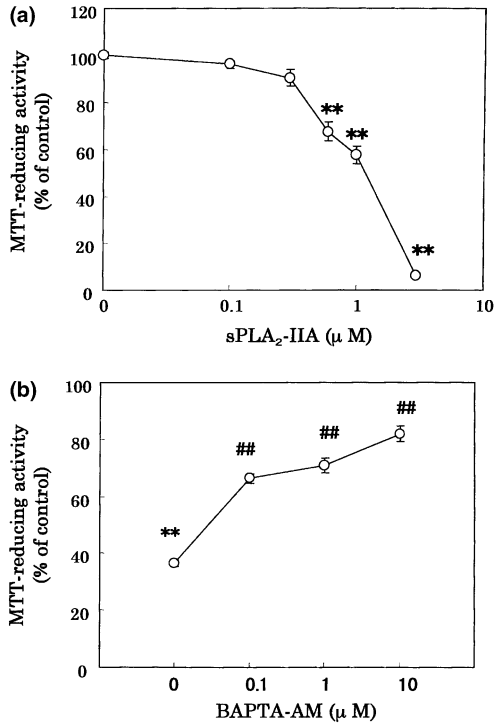


**Fig. 1** Effect of a $Ca^{2+}$ chelator on $sPLA_2$-IIA-induced neuronal cell death. (a) Neurotoxicity of $sPLA_2$-IIA: cortical cultures were treated with of $sPLA_2$-IIA at the indicated concentrations. Control value of MTT-reducing activity was 0.41 ± 0.02. MTT-reducing activity was determined 48 h later. (b) Effect of $Ca^{2+}$ chelator: cortical cultures were treated with 1 μM $sPLA_2$-IIA in the presence of BAPTA-AM at the indicated concentrations. MTT-reducing activity was determined 48 h later. Control value of MTT-reducing activity was 0.33 ± 0.02. The cell density of control cultures was 2.5 ± 0.2 × $10^5$ cells/$cm^2$. Data are expressed as means ± SEM ($n = 4$). $^{**}p < 0.01$, compared with control (no addition of $sPLA_2$-IIA), $\#\#p < 0.01$, compared with $sPLA_2$-IIA alone by ANOVA followed by Dunnett's test.

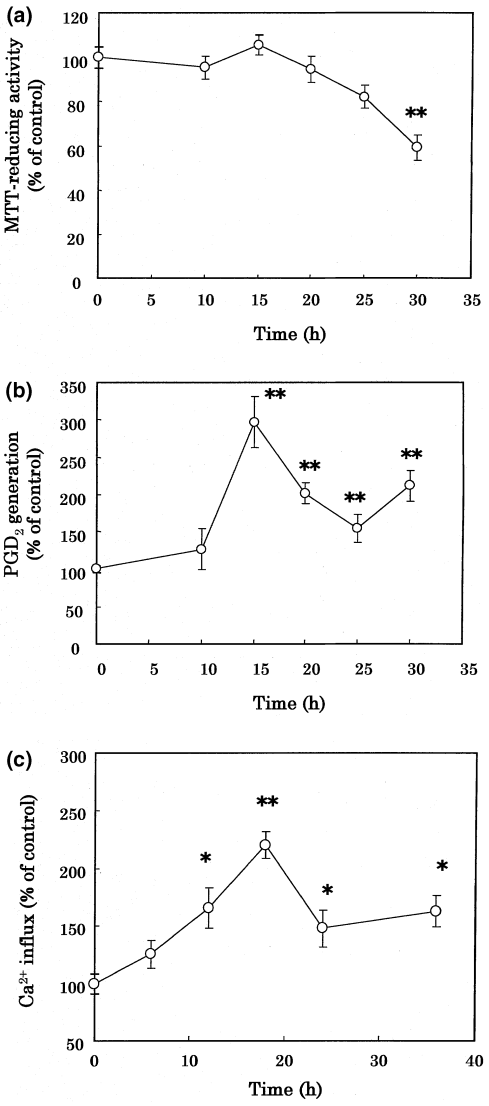


**Fig. 2** Generation of $PGD_2$ and influx of $Ca^{2+}$ during $sPLA_2$-IIA-induced neuronal cell death. Cortical cultures were treated with 1 μM $sPLA_2$-IIA. MTT-reducing activity (a), $PGD_2$ (b), and $Ca^{2+}$ influx (c) were measured at the indicated time points after $sPLA_2$-IIA treatment. Control value of MTT-reducing activity was 0.39 ± 0.02. Control value of $PGD_2$ was 64 ± 9 pg/mL. Control value of $Ca^{2+}$ influx was 5216 ± 449 cpm. The cell density of control cultures was 2.5 ± 0.2 × $10^5$ cells/$cm^2$. Data are expressed as means ± SEM ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control (time = 0 h) by ANOVA followed by Dunnett's test.

The inhibitor exhibited protective effects against the neurotoxicity of $sPLA_2$-IIA (Fig. 3a). Indoxam significantly prevented neurons from undergoing $sPLA_2$-IIA-induced cell death as previously reported (Yagami *et al.* 2002a).

In cortical neurons, neuronal cell death is induced synergistically by non-mammalian $sPLA_2$ and glutamate (Kolko *et al.* 1996). To ascertain whether glutamate affects the neurotoxicity of $sPLA_2$-IIA, we examined the effect of glutamate receptor blockers on $sPLA_2$-IIA-induced neuronal cell death. MK-801 (a non-competitive NMDA receptor blocker), AP-7 (a competitive NMDA receptor blocker), PCP (a non-competitive NMDA receptor blocker), CPP (a competitive NMDA receptor blocker), DiCl (a non-competitive NMDA receptor blocker), and NBQX [a competitive α-amino-2-hydroxy-5-methyl-4-isoxazolepropronic acid (AMPA)/kinate receptor blocker] did not attenuate the neurotoxicity of $sPLA_2$-IIA (Fig. 3a). It is unlikely that glutamate was involved in the neurotoxicity of $sPLA_2$-IIA.

## Effects of VSCC blocker on $sPLA_2$-IIA-induced neuronal cell death

Besides glutamate receptors, L-VSCC blockers are associated with neurotoxicity (Ueda *et al.* 1997a). To ascertain whether L-VSCC blockers affect neurotoxicity of $sPLA_2$-IIA, we evaluated effects of VSCC blockers on $sPLA_2$-IIA-induced neuronal cell death. Neither an N-type

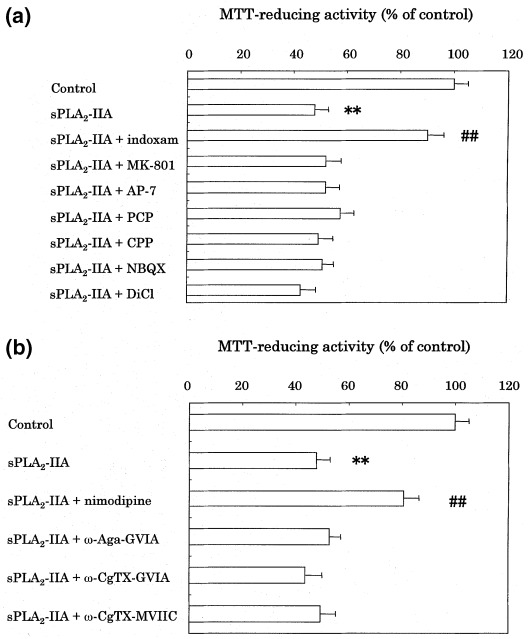


**Fig. 3** Effects of calcium channel blockers on sPLA$_2$-IIA-induced neuronal cell death. (a) Glutamate receptor blockers: neurons were treated with 1 μM sPLA$_2$-IIA in the absence or presence of 10 μM indoxam or 30 μM various glutamate receptor blockers. Control value of MTT-reducing activity was 0.36 ± 0.02. MTT-reducing activity was measured 48 h later. Data are expressed as means ± SEM ($n = 4$). Comparisons were made by ANOVA followed by Dunnett's test. $^{**}p < 0.01$, compared with control. $^{\#\#}p < 0.01$, compared with sPLA$_2$-IIA alone. (b) VSCC blockers: Neurons were treated with 1 μM sPLA$_2$-IIA in the absence or presence of 10 μM indoxam or 3 μM VSCC inhibitors. MTT-reducing activity was measured 48 h later. Control value of MTT-reducing activity was 0.36 ± 0.02. Data are expressed as means ± SEM ($n = 4$). Comparisons were made by ANOVA followed by Dunnett's test. $^{**}p < 0.01$, compared with control. $^{\#\#}p < 0.01$, compared with sPLA$_2$-IIA alone.

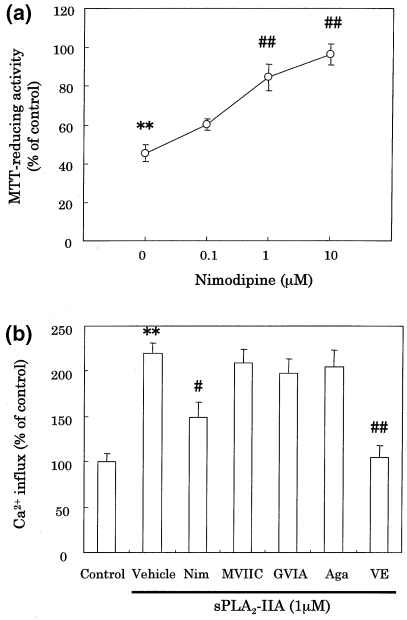


**Fig. 4** Effects of VSCC blockers and a radical scavenger on sPLA$_2$-IIA-induced neuronal cell death and influx of $Ca^{2+}$. (a) Cortical neurons were treated with nimodipine at the indicated concentrations in the presence of 1 μM sPLA$_2$-IIA. MTT reducing activity was determined 48 h later. Control value of MTT-reducing activity was 0.37 ± 0.01. Data are expressed as means ± SEM ($n = 4$). $^{**}p < 0.01$, compared with control (no addition of sPLA$_2$-IIA) by Student's *t*-test. $^{\#\#}p < 0.01$, compared with vehicle by ANOVA followed by Dunnett's test. (b) $Ca^{2+}$ influx: cortical neurons were treated with 1 μM sPLA$_2$-IIA in the absence or presence of 10 μM nimodipine (Nim), ω-conotoxin MVIIC (MVIIC), ω-conotoxin GVIA (GVIA), ω-agatoxin GVIA (Aga), or 130 μM vitamin E (VE). Influx of $Ca^{2+}$ was measured 18 h later. Control value of $Ca^{2+}$ influx was 5176 ± 624 cpm. Data are expressed as means ± SEM ($n = 4$). $^{**}p < 0.01$, compared with control (no addition of sPLA$_2$-IIA) by Student's *t*-test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with vehicle by ANOVA followed by Dunnett's test.

VSCC blocker (ω-conotoxin-GVIA) nor P/Q-type VSCC blockers (ω-agatoxin-IVA and ω-conotoxin-MVIIC) affected sPLA$_2$-IIA-induced neuronal cell death (Fig. 3b). Nimodipine significantly prevented neurons from undergoing sPLA$_2$-IIA-induced cell death (Fig. 3b). The neuroprotective effect of nimodipine was dependent on its concentration (Fig. 4a).

## Effect of L-VSCC blockers on sPLA$_2$-IIA-induced $Ca^{2+}$ influx

The potentiation of $Ca^{2+}$ uptake by sPLA$_2$-IIA was significantly reduced by L-VSCC blockers (Fig. 4b). On the other hand, neither an N-type VSCC blocker (ω-conotoxin-GVIA) nor P/Q-type VSCC blockers (ω-agatoxin-IVA and ω-conotoxin-MVIIC) affected sPLA$_2$-increased $Ca^{2+}$ influx (Fig. 4b). These results indicated that sPLA$_2$ induced the influx of $Ca^{2+}$ into neurons through L-VSCC.

## Effects of L-VSCC blockers on sPLA$_2$-IIA-induced apoptotic features

We evaluated the condensation of chromatin, a characteristic feature of apoptosis, in neurons (Figs 5a–c and 6a). sPLA$_2$-IIA-treated neurons were stained with Hoechst 33258 fluorescent dye. In untreated cultures, cells showed little fluorescence in the nucleus (Fig. 5a). On the other hand, condensed and fragmented chromatin was clearly observed in cultures treated with sPLA$_2$-IIA for 48 h (Fig. 5b). The amount of condensed chromatin in sPLA$_2$-IIA-treated neurons was decreased significantly by nimodipine (Figs 5c and 6a).

We also studied another apoptotic feature, fragmentation of DNA (Figs 5d–f and 6b). With the TUNEL technique, it is possible morphologically to discriminate between the apoptotic nuclei by observing the presence of strand breaks in the DNA when the nicked ends of DNA are labeled. After

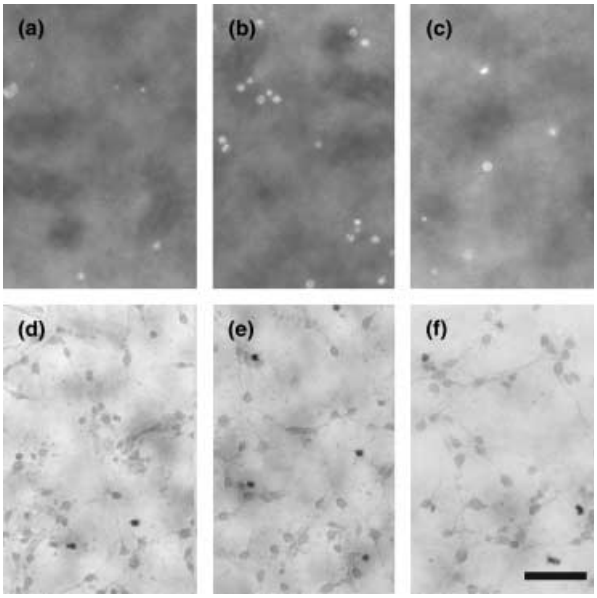


**Fig. 5** Effect of an L-VSCC blocker on $sPLA_2$-IIA-induced apoptotic features. Cortical neurons were treated with vehicle (a and d), 1 μM $sPLA_2$-IIA (b and e), or 1 μM $sPLA_2$-IIA + 10 μM nimodipine (c and f). Neurons were stained with 10 μM Hoechst 33258 for 10 min 48 h later (a, b and c). Neurons were fixed with 4% paraformaldehyde, washed twice with phosphate-buffered saline, and stained by the TUNEL technique 48 h later (d, e and f). Bar = 100 μm.

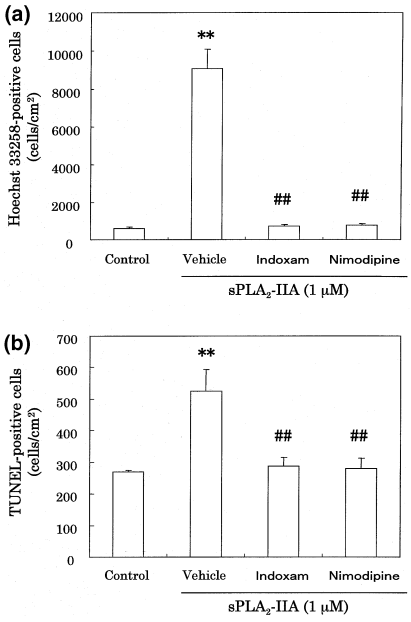


**Fig. 6** Effect of an L-VSCC blocker on $sPLA_2$-IIA-induced apoptosis. Cortical neurons were treated with 1 μM $sPLA_2$-IIA in the absence or presence of 10 μM indoxam or 10 μM nimodipine. (a) Hoechst 33258-positive and (b) TUNEL-positive neurons were detected 48 h later. Data are expressed as means ± SEM ($n = 4$). Comparisons were made by ANOVA followed by Dunnett's test. $^{**}p < 0.01$, compared with control. $\#\#p < 0.01$, compared with $sPLA_2$-IIA alone.

neurons were incubated with or without $sPLA_2$-IIA for 48 h, the number of TUNEL-positive nuclei was increased in $sPLA_2$-IIA-treated neurons (Fig. 5e) as compared to untreated controls (Fig. 5d). The number of TUNEL-positive nuclei in $sPLA_2$-IIA-treated neurons (Fig. 5f) was decreased significantly by nimodipine (Fig. 6b).

### Effects of radical scavengers on $sPLA_2$-IIA-induced ROS

Free radicals can activate L-VSCC (Ueda *et al.* 1997a). To ascertain whether radical scavengers affect the neurotoxicity of $sPLA_2$-IIA, we evaluated their effect on $sPLA_2$-IIA-induced neuronal cell death. ROS were generated 15 h after $sPLA_2$-IIA treatment (Fig. 7a). ROS were reduced significantly by radical scavengers, including vitamin E (VE), probucol (PRB), butylated hydroxytoluene (BHT), and chlorpromazine (CPZ; Fig. 7a). On the other hand, nimodipine (NIM) did not affect the production of ROS after

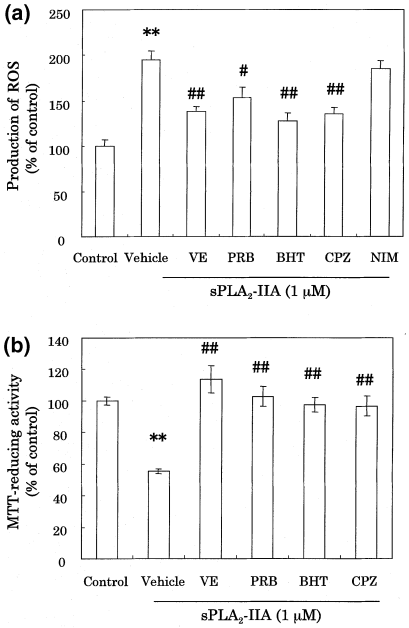


**Fig. 7** Effects of radical scavengers and an L-VSCC blocker on the neurotoxicity of $sPLA_2$-IIA. (a) Free radicals: cortical neurons were treated with vehicle, 130 μM vitamin E (VE), 10 μM probucol (PRB), 10 μM butylated hydroxytoluene (BHT), 10 μM chlorpromazine (CPZ), or 10 μM nimodipine (NIM) in the presence of 1 μM $sPLA_2$-IIA. Production of ROS was measured 15 h later. Control value of ROS was 0.32 ± 0.01. Data are expressed as means ± SEM ($n = 4$). $^{**}p < 0.01$, compared with control (no addition of $sPLA_2$-IIA) by Student's *t*-test. $\#p < 0.05$, $\#\#p < 0.01$, compared with vehicle by ANOVA followed by Dunnett's test. (b) Cortical neurons were treated with vehicle, 130 μM VE, 10 μM PRB, 10 μM BHT, or 10 μM CPZ in the presence of 1 μM $sPLA_2$-IIA. MTT-reducing activity was determined 48 h later. Control value of MTT-reducing activity was 0.38 ± 0.03. Data are expressed as means ± SEM ($n = 4$). $^{**}p < 0.01$, compared with control (no addition of $sPLA_2$-IIA) by Student's *t*-test. $\#\#p < 0.01$, compared with vehicle by ANOVA followed by Dunnett's test.

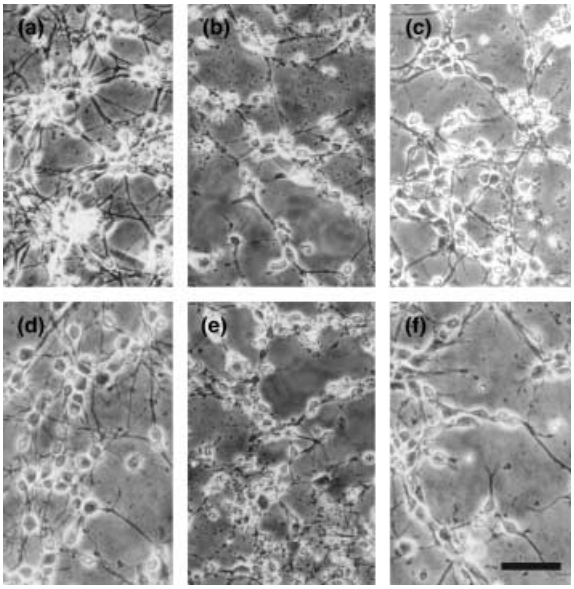


**Fig. 8** Effect of an sPLA$_2$-IIA inhibitor, $Ca^{2+}$ channel blockers and a radical scavenger on sPLA$_2$-IIA-induced morphologic changes in cortical neurons. Cortical neurons were treated with vehicle (a), 1 μM sPLA$_2$-IIA (b), 1 μM sPLA$_2$-IIA + 10 μM indoxam (c), 1 μM sPLA$_2$-IIA + 10 μM nimodipine (d), 1 μM sPLA$_2$-IIA + 30 μM MK-801 (e), or 1 μM sPLA$_2$-IIA + 130 μM vitamin E (f). Neurons were examined by phase-contrast microscopy 48 h later. Bar = 100 μm.

sPLA$_2$-IIA treatment. Vitamin E also suppressed $Ca^{2+}$ influx as well as the L-VSCC blocker (Fig. 4b). In addition, radical scavengers including vitamin E, probucol, butylated hydroxytoluene, and chlorpromazine significantly prevented neurons from undergoing sPLA$_2$-IIA-induced neuronal cell death (Fig. 7b).

### Effects of L-VSCC blockers on sPLA$_2$-IIA-induced light-microscopic changes in neurons

We examined light microscopic changes in neurons at 48 h after sPLA$_2$-IIA treatment (Fig. 8). In control cultures, neurons had extended neurites and smooth, round cell bodies (Fig. 8a). On the other hand, most cell bodies shrank and lost their neurites in cultures treated with sPLA$_2$-IIA (Fig. 8b). Indoxam (Fig. 8c), nimodipine (Fig. 8d), and vitamin E (Fig. 8f) significantly ameliorated the morphologic disruption in neurons treated with sPLA$_2$-IIA, whereas MK-801 (Fig. 8e) had no effect on them.

## Discussion

In the present study, we found that the influx of $Ca^{2+}$ into neurons was involved in sPLA$_2$-IIA-induced neuronal cell death. Furthermore, sPLA$_2$-IIA potentiated $Ca^{2+}$ influx through L-VSCC. To our knowledge, this is the first report that sPLA$_2$-IIA exhibits a biological response via the influx of $Ca^{2+}$ through L-VSCC.

How does sPLA$_2$-IIA trigger the elevation of the intracellular concentration of calcium ([$Ca^{2+}$]i) prior to neuronal cell death? In the human astrocytoma cell line 1321 N1, sPLA$_2$-IIA elicits a rapid and transient peak of [$Ca^{2+}$]i (Hernández *et al*. 1999). Hernández *et al*. (1999) proposed the hypothesis that sPLA$_2$-IIA interacts with a plasma membrane binding structure(s), activates herbimycin-sensitive tyrosine kinase and pertussis toxin-sensitive GTP binding protein, stimulates PLCγ, produces inositol triphosphate, and mobilizes [$Ca^{2+}$]i. The possibility was not supported in our system for the following reasons. First, herbimycin had no effect on sPLA$_2$-IIA-induced neuronal cell death and apoptotic features (data not shown). Second, no tyrosine phosphorylation specific for herbimycin was detected (data not shown). Third, a [$Ca^{2+}$]i peak is elicited several minutes after the addition of sPLA$_2$-IIA, whereas the time course of neuronal cell death is of the order of days (Yagami *et al*. 2002a). Collectively, these results suggest that the influx of $Ca^{2+}$ from extracellular fluid, but not the efflux of $Ca^{2+}$ from intracellular store, might contribute to sPLA$_2$-IIA-induced neuronal cell death.

The NMDA receptor antagonist MK-801 partially blocks the neurotoxicity of sPLA$_2$-IA from Taipan snake venom (Kolko *et al*. 1996). NMDA receptors possess an intrinsic $Ca^{2+}$-conducting pore that is activated by the combination of ligand binding and membrane depolarization. Non-mammalian sPLA$_2$s causes cell death in primary cultures of rat cortical neurons (Clap *et al*. 1995; Kolko *et al*. 1996). In the present study, NMDA receptor blockers did not prevent neurons from cell death induced by the mammalian sPLA$_2$-IIA. Other ionotropic glutamate receptors are AMPA/kinate receptors. AMPA/kinate receptors are highly permeable to $Na^+$ and play a major role in fast synaptic transmission. Membrane depolarization resulting from glutamate binding to AMPA/kinate receptors activates voltage-dependent calcium channels. However, the AMPA/kinate receptor blocker did not affect the neurotoxicity of sPLA$_2$-IIA. Neither NMDA receptors nor AMPA/kinate receptors appeared to play a role in sPLA$_2$-IIA-induced neuronal cell death.

In the present study, L-VSCC blockers significantly suppressed sPLA$_2$-IIA-induced $Ca^{2+}$ influx. Furthermore, the blocker rescued neurons from sPLA$_2$-IIA-induced cell death, and it ameliorated the apoptotic features. This L-VSCC-dependent mechanism is supported by others (Ueda *et al*. 1997a; Ekinci *et al*. 1999). Aggregated deposits of Aβ, a neurotoxin, are generally assumed to have a causative role in neurodegeneration and development of AD. Aβ is a 39- to 43-amino-acid hydrophobic peptide that causes neuronal cell death in primary cultures of rat cortical neurons, accompanying several characteristic features of apoptosis, such as formation of cell surface blebs, chromatin condensation, and DNA fragmentation (Pike *et al*. 1991; Forloni *et al*. 1993; Ueda *et al*. 1994, 1996). Aβ causes elevation of [$Ca^{2+}$]i and collapse of $Ca^{2+}$ homeostasis (Mattson *et al*. 1992; Ueda

*et al*. 1997b). Moreover, Aβ potentiates $Ca^{2+}$ influx through L-VSCC (Weiss *et al*. 1994; Ueda *et al*. 1997a). These findings suggest that there are common L-VSCC-dependent mechanisms by which $sPLA_2$-IIA and Aβ lead to neuronal cell death on rat cortical neurons.

A transient increase of $[Ca^{2+}]i$ resulting from electrochemical stimulation and opening of voltage-sensitive $Ca^{2+}$ channels mediates information-coding processes in neuronal circuits (Malenka 1991) and regulates growth cone behaviors in developing neurons (Kater *et al*. 1988). However, uncontrolled prolonged elevation of $[Ca^{2+}]i$ can result in neuronal degeneration and death (Mattson *et al*. 1992; Ueda *et al*. 1997b). Calcium appears to damage cellular proteins and membranes by activating several enzymes such as proteases (Siman and Noszeck 1988), endonuclease (Arends *et al*. 1990), and protein kinases (Ueda *et al*. 1996).

How does $sPLA_2$-IIA activate L-VSCC? ROS is produced during arachidonic acid oxidative metabolism. Indeed, ROS was generated before $sPLA_2$-IIA-induced neuronal cell death. Radical scavengers not only suppressed the elevated level of ROS, but also prevented neurons from $sPLA_2$-IIA-induced $Ca^{2+}$ influx and cell death. Furthermore, L-VSCC can be opened by ROS (Ueda *et al*. 1997a). Thus, $sPLA_2$-IIA appears to stimulate L-VSCC via ROS.

Following the production of ROS, intracellular oxidants have the potential to trigger apoptosis in several ways. First, ROS damage membranes of cells by the peroxidation of lipids. The unsaturated bonds of fatty acids and cholesterol are particularly vulnerable to free-radical attack, yielding lipid peroxides that are also ROS. There are many functional proteins such as receptors, channels, and transporters. The functional impairment of these membrane proteins can destroy the intracellular homeostasis and trigger apoptosis. Second, oxidation of intracellular proteins may modify either their function or their ability to be recognized by other proteins. Oxidative damage can increase the susceptibility of some proteins to degradation by non-lysosomal proteinase complex (Davies and Goldberg 1987). Third, ROS can change nuclear gene transcription, which activates the apoptotic pathway. Several transcription factors such as Fos and Jun have critical cysteine residues involved in DNA binding, their thiol oxidation causing a large decrease in their efficiency of DNA-binding (Abate *et al*. 1990). In contrast, DNA-binding activity of the nuclear factor kappa B (NF-κB) is indirectly activated by oxidative events (via enhanced proteolysis of its inhibitory factor I-κB), thereby initiating transcription of NF-κB-responsive genes (Staal *et al*. 1990). Finally, direct oxidative damage to DNA can initiate an apoptotic response. In lymphocytes, radiation produces ROS and causes oxidation of DNA, leading to apoptosis (Yamada and Ohyama 1988).

$sPLA_2$-IIA exhibited neurotoxicity at concentrations of about 1 μM *in vitro*. Does the concentration of endogenous $sPLA_2$-IIA reach such a high level *in vivo*? Concentrations of $sPLA_2$-IIA *in vivo* were calculated under the assumption that $sPLA_2$-IIA contributed to most of the $sPLA_2$ activity. Its concentration was about 3.5 nM in the area of neurodegeneration induced by middle cerebral artery occlusion, if spread uniformly in that area. This gave rise to the question why the concentration of $sPLA_2$-IIA required *in vitro* was approximately 300 times as high as that expressed *in vivo*. This discrepancy could be explained in several reports. First, $sPLA_2$-IIA generated from cytokine-stimulated astrocytes might reach such a high concentration microenvironmentally at surfaces of neurons via attachment to heparan sulfate proteoglycan (Koduri *et al*. 1998). Second, $sPLA_2$-IIA might cause neuronal cell death at lower concentrations in the presence of co-factors (Fourcade *et al*. 1995; Murakami *et al*. 1999). This was not found in the present study. Third, the sensitivity of the cells to endogenously produced $sPLA_2$-IIA is higher than to exogenously added $sPLA_2$-IIA (Murakami *et al*. 1999). The striking difference between the amounts of $sPLA_2$-IIA required by different systems, e.g. exogenously added (μM) versus endogenously produced (nM), implies that the continued supply of $sPLA_2$-IIA, which occurs in the latter situation, may be an important factor for its adequate action during cellular (particularly prolonged) responses. Although $IC_{50}$ of indoxam is 1.5 nM (Yokota *et al*. 1999), 10 μM indoxam was used in the present study. Yokota *et al*. (1999) have reported that indoxam exhibited inhibitory effect against 0.4 ng/250 μL (0.11 nM) $sPLA_2$-IIA with $IC_{50}$ of 1.5 nM. In their condition, the ratio of $sPLA_2$-IIA : indoxam was 1 : 13. On the other hand, the ratio of $sPLA_2$-IIA : indoxam was 1 : 10 in the present study. Thus, the concentrations of indoxam and $sPLA_2$-IIA in the present study was different from those in the report of Yokota *et al*. (1999), whereas the ratio of indoxam to $sPLA_2$-IIA in the former was very similar to those in the latter.

The application of $sPLA_2$-IIA for 48 h decreased MTT-reducing activity by about 12 500 cells/$cm^2$, whereas it increased the Hoechst staining cells by about 8000 cells/$cm^2$ and the TUNEL-positive cells by about 250 cells/$cm^2$. The first of these assays was performed for evaluation of neuronal survival, and the latter two methods were used for quantifying apoptosis. The values obtained by these methods were not the same. Did $sPLA_2$-IIA induce neuronal cell death via necrosis? Previously, we have reported that $sPLA_2$-IIA induced neuronal cell death via apoptosis by the ultrastructural analysis as well as the biochemical analysis (Yagami *et al*. 2002a). In the analysis by transmission electron microscopy, the process of neuronal cell death induced by $sPLA_2$-IIA showed ultrastructural changes such as progressive cell shrinkage, blebbing of the plasma membrane, loss of cytosolic organelles, clumping of chromatin, and fragmentation of DNA. However, necrotic features such as mitochondrial abnormalities and nuclear swelling were not detected

during neuronal cell death. The MTT-reduction assay, Hoechst 33258-staining, and TUNEL reflect mitochondrial succinate dehydrogenase activity, clumping of chromatin, and fragmentation of DNA, respectively. On the analysis by transmission electron microscopy and light microscopy, sPLA$_2$-IIA triggered impairment of mitochondrial function, chromatin condensation, and DNA fragmentation sequentially, but not entirely in parallel. Thus, the different values obtained by the three methods appeared to be attributable to the different sensitivity of sPLA$_2$-IIA-treated neurons to these methods.

Apoptotic cell membranes are potent targets for the enzyme (Atsumi *et al*. 1997). Neuronally differentiated PC12 cells deprived of nerve growth factor and serum, mast cells deprived of hematopoietic cytokines, and anti-Fas antibody-treated U937 monocytic leukemia cells, all of which display the classical changes of apoptosis, become sensitive to sPLA$_2$-II-mediated liberation of arachidonic acid. Under our culture conditions, sPLA$_2$-IIA liberated arachidonic acid significantly from neurons prior to apoptosis, but not from myocytes, which are resistant to the enzyme (Yagami *et al*. 2002a). A specific sPLA$_2$ inhibitor, indoxam, significantly suppressed the sPLA$_2$-IIA-induced liberation of arachidonic acid from neurons (Yagami *et al*. 2002a). Indoxam and p-BPB prevented neurons from sPLA$_2$-IIA-induced apoptosis (Yagami *et al*. 2002a), suggesting that the neurotoxicity of sPLA$_2$-IIA depends on the susceptibility of cells to sPLA$_2$-IIA-mediated liberation of arachidonic acid. Arachidonic acid metabolites, especially PGD$_2$, might be involved in the neurotoxicity of sPLA$_2$-IIA (Yagami *et al*. 2002a). How are the influx of $Ca^{2+}$ and the generation of ROS associated with the neurotoxic PGD$_2$? We propose a mechanism showing how these mediators are involved in sPLA$_2$-IIA-induced apoptosis. First, sPLA$_2$-IIA acts on phospholipids to release free fatty acids, including arachidonic acid. Arachidonic acid oxidative metabolism produces eicosanoids, e.g. PGD$_2$, and ROS. Second, ROS activate L-VSCC in the plasma membranes of neurons, and potentate $Ca^{2+}$ influx into neurons. Third, the increase in intracellular calcium stimulates cytosolic PLA$_2$, producing a second cycle of arachidonic acid release. Finally, calcium, ROS, and PGD$_2$ cooperatively damage cellular membranes and proteins, leading to apoptosis.

In conclusion, we demonstrated that sPLA$_2$-IIA potentiates the influx of $Ca^{2+}$ into neurons through L-VSCC. We suggested that ROS generated during arachidonic acid oxidative metabolism stimulate L-VSCC. Furthermore, the present study sheds light on the cooperative involvement of eicosanoids, ROS, and $Ca^{2+}$ in sPLA$_2$-IIA-induced neuronal apoptosis.

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