Release of secretory phospholipase A_2 from rat neuronal cells and its possible function in the regulation of catecholamine secretion

Atsushi MATSUZAWA*, Makoto MURAKAMI*†, Gen-ichi ATSUMI*†, Kazuhiro IMAI*, Pablo PRADOS*, Keizo INOUE* and Ichiro KUDO*†‡

*Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 and †School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

Here we show that secretory phospholipase A_2 ($sPLA_2$) that is immunochemically indistinguishable from type II $sPLA_2$ is (i) stored in neuroendocrine cells, (ii) released in response to neurotransmitters or depolarization, and (iii) involved in the regulation of catecholamine secretion by these cells. Rat brain synaptic vesicle fractions contained PLA_2 activity, which was neutralized completely by an antibody raised against rat type II $sPLA_2$. $sPLA_2$ immunoreactive with anti-(type II $sPLA_2$) antibody was released from synaptosomes in response to depolarization evoked by a high concentration of potassium in the presence of Ca^{2+}. Rat pheochromocytoma PC12 cells, which differentiated into adherent cells similar to sympathetic neurons in response to nerve growth factor, were used for the detailed analysis of the dynamics and function of $sPLA_2$ in neuronal cells. Antibody against rat type II $sPLA_2$ precipitated $\sim 80\,\%$ of the PLA_2 activity in PC12 cell lysates. Transcript for type II $sPLA_2$ was detected in PC12 cells by reverse transcriptase-PCR. When neuronally differentiated PC12 cells were stimulated with carbamylcholine or potassium, $sPLA_2$ was released into the medium and reached a maximal $\sim 40\,\%$ release by 15 min. Inhibitors specific to type II $sPLA_2$ suppressed catecholamine secretion by PC12 cells which had been activated by carbamylcholine. Furthermore, treatment of PC12 cells with exogenous type II $sPLA_2$ alone elicited catecholamine secretion. These observations indicate that $sPLA_2$ released from neuronal cells may regulate the degranulation process leading to release of neurotransmitters and are compatible with our earlier finding that this enzyme is involved in the degranulation of rat mast cells.

INTRODUCTION

Phospholipase A_2 (PLA_2; EC 3.1.1.4) is a lipolytic enzyme which hydrolyses the *sn*-2 position of glycerophospholipid to generate free fatty acid and lysophospholipid. Recent studies have revealed the presence of at least two families of PLA_2 in mammalian cells [1]. Secretory PLA_2 ($sPLA_2$) enzymes, with a molecular mass of ~ 14 kDa, are subdivided into several groups based upon the position of cysteine residues. Type I $sPLA_2$ (pancreatic type) and type II $sPLA_2$ (inflammatory type) are the best-characterized forms of $sPLA_2$ among a still expanding family. $sPLA_2$ enzymes require submillimolar concentrations of Ca^{2+} for effective hydrolysis of substrates without fatty acid selectivity [1]. Cytosolic PLA_2 ($cPLA_2$), with a molecular mass of ~ 85 kDa, shows strict substrate specificity for arachidonate-containing phospholipids and is activated by submicromolar Ca^{2+} through translocation from the cytosol to membrane compartments [2–5]. Phosphorylation of $cPLA_2$ by mitogen-activated protein kinase increases its intrinsic enzyme activity severalfold [6]. Based on these characteristics, $cPLA_2$ is considered to have a crucial role in the liberation of arachidonic acid in response to various agonists that mobilize intracellular Ca^{2+}. Evidence has been accumulating that type II $sPLA_2$ may also participate in the arachidonate metabolism elicited by cytokine stimulation of several cell types [7–10].

Several lines of evidence have suggested the participation of certain PLA_2s in exocytosis in, for example, mast cells, chromaffin cells and neuroendocrine cells [11–15]. Classical PLA_2 inhibitors, such as *p*-bromophenacyl bromide and mepacrine, are reported to inhibit histamine release from mast cells or neurotransmitter release from neuroendocrine cells [11–13]. Some snake venom PLA_2s or a PLA_2-activator, mellitin, trigger mast cell degranulation [14,15]. We have reported that mast cells express type II $sPLA_2$ and that inhibitors specific for type II $sPLA_2$ suppress histamine release [16]. Furthermore, exogenously added type II $sPLA_2$ directly elicits degranulation by mast cells [17]. These observations have provided the first demonstration that type II $sPLA_2$ may regulate exocytosis by mast cells.

Neuronal endocrine cells secrete their granule contents in response to several kinds of stimuli, such as neurotransmitters or depolarization. Several investigators have reported that exocytosis from neuronal cells is prevented by classical PLA_2 inhibitors [12,13]. We now report that synaptic vesicle fractions isolated from rat brain contain $sPLA_2$ immunochemically identical to type II $sPLA_2$, which is released from synaptosomes by depolarization. By use of the rat pheochromocytoma PC12 cell line, which differentiates into neuron-like cells morphologically and functionally following treatment with nerve growth factor (NGF) [18], we found that $sPLA_2$ is secreted upon activation by neurotransmitter or by depolarization. The possible involvement of endogenous and exogenous type II $sPLA_2$ in catecholamine secretion by PC12 cells is discussed.

EXPERIMENTAL

Purification of synaptic vesicles from rat brain

Subcellular fractions of rat brain were separated from cerebra of Wistar rats (Nippon Bio-Supply Center) as described previously

Abbreviations used: PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; $cPLA_2$, cytosolic PLA_2; NGF, nerve growth factor; TBS, Tris-buffered saline; RT-PCR, reverse transcriptase-PCR; SSV, small synaptic vesicle; LDCV, large dense-core vesicle; CSV, crude synaptic vesicle; CSM, crude synaptic membrane.

‡ To whom correspondence should be addressed at: Department of Health Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan.

[19]. Briefly, cerebral tissue of 20 rats (male, 7-week-old) was homogenized in 200 ml of 0.32 M sucrose containing 1 mM (*p*-amidinophenyl)methanesulphonyl fluoride hydrochloride, 1 mM $NaHCO_3$, 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ by 12 strokes at 500 rev./min in a Potter–Elvehjem homogenizer (B. BRAUN). The homogenate was spun for 10 min at 1400 ***g*** to yield a pellet (designated P1) and a supernatant (designated S1). S1 was further centrifuged for 10 min at 13800 ***g***. The resulting pellet was washed once with 180 ml of 0.32 M sucrose, yielding the crude synaptosomal pellet P2. P2 was resuspended in 40 ml of 0.32 M sucrose containing 1 mM $NaHCO_3$ and layered on top of a discontinuous sucrose gradient (0.85 M, 1.0 M and 1.2 M sucrose). After centrifugation for 2 h at 82500 ***g***, the gradient was collected in four fractions: the synaptosomal fraction between 1.0 and 1.2 M sucrose (P2C), the fractions on top (P2A) and underneath (P2B), and the pellet fraction (P2D). Then 160 ml of 0.32 M sucrose was added to P2C followed by centrifugation for 20 min at 32800 ***g***. The pellet was resuspended in 200 ml of 6 mM Tris/HCl (pH 7.4), stirred for 45 min at 4 °C, and then centrifuged for 20 min at 32800 ***g*** to separate crude synaptic vesicles (CSVs) in the supernatant from crude synaptic membranes (CSMs) in the pellet. The supernatant, containing CSVs, was centrifuged for 2 h at 78000 ***g*** to remove the synaptosomal supernatant (SS). The resulting CSV pellet was suspended in 5 ml of 1 mM $NaHCO_3$ solution. The CSM pellet was resuspended in 8 ml of 0.32 M sucrose. The efficiency of separation of synaptic vesicles was estimated by following the distribution of synaptophysin, which occurred specifically in synaptic vesicles [20], by immunoblotting using an anti-synaptophysin monoclonal antibody (PROGEN).

PLA_2 assay

PLA_2 activity was measured by Dole's method [21] using 1-acyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphoethanolamine (57 mCi/mmol; DuPont–NEN) as the substrate. The standard incubation mixture for assaying PLA_2 comprised 250 μl of 100 mM Tris/HCl (pH 9.0), 4 mM $CaCl_2$, substrate (2 μM), and an enzyme preparation. The incubation was carried out at 37 °C for 30 min. The reaction was stopped by adding 1.25 ml of isopropanol/n-heptane/0.5 M H_2SO_4 (78:20:2, by vol.). Free arachidonic acid released was extracted into an organic phase and counted in a β-scintillation counter (Beckman).

Affinity purification of type II $sPLA_2$

A monoclonal antibody raised against rat type II $sPLA_2$, MD7.1 [22], was conjugated with CNBr-activated Sepharose CL-4B (Pharmacia) as described previously [23]. Samples were applied to the MD7.1-conjugated column that had been equilibrated with Tris-buffered saline (TBS; 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl), with a flow rate of 10 ml/h. After washing extensively with TBS, the bound PLA_2 was eluted with 50 mM glycine/HCl, pH 2.2.

Absorption of PLA_2 activity by antibodies

Samples (20 μl) were incubated with a monoclonal antibody against rabbit $cPLA_2$, RHY-4 [24], for 90 min at 4 °C. Then, 20 μl of anti-(mouse IgG) immunobeads (Bio-Rad) were added and incubated for 30 min at room temperature. After centrifugation for 3 min at 10000 ***g***, the supernatant was collected for measuring PLA_2 activity. Alternatively, 20 μl samples were incubated with a polyclonal anti-(rat type II $sPLA_2$) antibody, R377 [25], which neutralized the activity of rat type II $sPLA_2$, for 90 min at 4 °C. Residual PLA_2 activity was then measured.

SDS/PAGE/immunoblot analysis

Samples were applied to SDS/15 %-polyacrylamide gels and electrophoresed under non-reducing conditions. The separated proteins were electroblotted on to nitrocellulose membranes (Bio-Rad) using a semi-dry MilliBlot-SDE system (Millipore) at 25 mA/cm for 0.5 h. The membranes were then sequentially treated with the following: 3 % BSA in 10 mM phosphate buffer, pH 7.2, containing 140 mM NaCl and 2 mM KCl (PBS) overnight at 4 °C; a polyclonal antibody against type II $sPLA_2$ (R377) at a dilution of 1:1000 in PBS containing 0.05 % Tween (PBS-Tween) and 0.1 % BSA for 1 h at room temperature; PBS-Tween for six washes; biotinylated goat anti-(rabbit IgG) (Zymed) in PBS-Tween (1:1000 dilution) for 1 h at room temperature; PBS-Tween for six washes; and horseradish peroxidase-conjugated streptavidin (Zymed) (1:1000 dilution) in PBS-Tween for 1 h at room temperature. After six washes, the protein bands were visualized with a chemiluminescence technique using an enhanced chemiluminesence Western blot analysis system (Amersham).

Activation of synaptosomes

P2C fractions obtained from the brains of five rats were resuspended in TBS at 5 mg of protein/ml. After incubation for 10 min at 4 °C, 500 μl portions of the synaptosome suspension were treated with 50 mM KCl in the presence or absence of 2 mM $CaCl_2$ for 10 min at 37 °C. After centrifugation for 5 min at 13800 ***g***, the PLA_2 activity in the supernatant and in the pellet was measured. The percentage release of PLA_2 was calculated using the formula $[S/(S+P)] \times 100$, where S and P are PLA_2 activities in equal portions of supernatant and pellet, respectively.

Cell culture

Rat pheochromocytoma PC12 cells (RIKEN Cell Bank) were maintained in RPMI 1640 medium supplemented with 10 % (v/v) horse serum and 5 % (v/v) fetal-calf serum. For neuronal differentiation, 2×10^4 PC12 cells/cm^2 were cultured with 50 ng/ml NGF (Chemicon) in collagen-coated 24-well plates (Corning) for 7 days.

Heparin–Sepharose column chromatography

PC 12 cells (1×10^7 cells equivalent) were suspended in 10 ml of 10 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA and then sonicated on ice for 1-min pulses (60 % work cycle, setting 6) with a Branson Sonifier (Branson Sonic Power Co.). The cell lysate was centrifuged for 1 h at 100000 ***g*** at 4 °C. The supernatant was then applied to 3 ml of heparin–Sepharose CL-6B (Pharmacia) that had been equilibrated with TBS, pH 7.4, at a flow rate of 10 ml/h. After washing with TBS, the bound proteins were eluted with 10 mM Tris/HCl, pH 7.4, containing 1 M NaCl.

Release of catecholamines from neuron-like differentiated PC12 cells

NGF-treated PC12 cells were incubated with [^{3}H]noradrenaline (1 μCi/ml) (DuPont–NEN) in the culture medium for 4 h at 37 °C. The cells were washed three times with culture medium to remove unincorporated [^{3}H]noradrenaline and then treated with carbamylcholine, KCl, or type II $sPLA_2$ that had been purified to near homogeneity from rat platelets [26] at 37 °C in the culture medium. The radioactivity released into the supernatant, as well as that remaining in the cells (which was extracted by 1 % SDS),

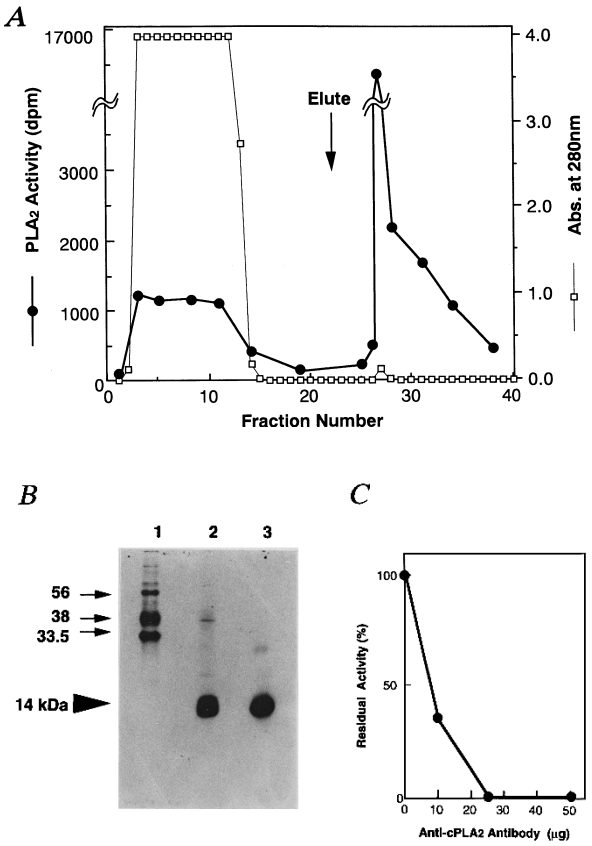

Figure 1 Immunochemical detection of type II $sPLA_2$ in rat brain

(**A**) Elution profile of PLA_2 activity (●) and proteins (□) in the 100 000 ***g*** supernatant of rat brain homogenate from the anti-(type II $sPLA_2$) monoclonal antibody (MD7.1)-conjugated Sepharose column. (**B**) Aliquots of fraction 27 in (**A**) (10 μg of protein; lane 2) and 100 ng of type II $sPLA_2$ purified from rat platelets (lane 3) were applied to an SDS/15%-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with the anti-(type II $sPLA_2$) polyclonal antibody R377. Lane 1 contained molecular-mass markers (Bio-Rad). (**C**) Absorption of PLA_2 activity in the flow-through fractions in (**A**) by the anti-$cPLA_2$ antibody RHY-4. The sample was incubated with RHY-4, followed by incubation with anti-(mouse IgG)-conjugated beads. Residual activity remaining in the supernatant is shown, with the PLA_2 activity in the absence of RHY-4 regarded as 100%.

was counted in a β-scintillation counter. In another series of experiments, the cells were stimulated with type II $sPLA_2$, and the amounts of endogenous dopamine released into the supernatants or remaining in the cells were measured after purification by HPLC, as described previously [27]. Briefly, samples were loaded on to a fully automated HPLC system, including a direct in-line extraction of catecholamines from samples on a weak cation-exchange precolumn, separation of catecholamines on a reversed-phase HPLC column, fluorogenic derivatization of catecholic compounds with ethylenediamine, and sensitive detection of fluorophores with a peroxylate chemiluminescence reaction. The percentage release of catecholamines was calculated by using the formula $[S/(S+P)] \times 100$, where S and P are the catecholamine contents of equal portions of supernatant and pellet, respectively.

Immunohistochemical staining

PC12 cells were seeded on poly(L-lysine)-coated coverglasses (Iwaki) at 2×10^4 cells/cm^2 and cultured for 24 h in the presence of 50 ng/ml NGF in 500 μl of culture medium. The cells were washed with PBS and fixed with 3.6% paraformaldehyde in PBS for 20 min at room temperature. For permeabilization, the cells were treated for 30 min with 0.1% saponin in PBS. The cells on the coverslips were then sequentially treated with 3% BSA in PBS for 2 h at 4 °C, 1 μg/ml anti-(rat type II $sPLA_2$) antibody R377 overnight at 4 °C, biotinylated anti-(rabbit IgG) (Zymed; 1:1000 dilution) for 2 h at 4 °C, and fluorescein isothiocyanate-conjugated streptavidin (Zymed; 1:1000 dilution) for 2 h at 4 °C. Alternatively, the fixed cells were treated sequentially with 1 μg/ml anti-synaptophysin monoclonal antibody overnight at 4 °C, and fluorescein isothiocyanate-conjugated anti-(mouse IgG) (Zymed; 1:1000 dilution) for 2 h at 4 °C. The coverslips were mounted in 90% (w/v) glycerol and examined by fluorescence microscopy.

Reverse transcriptase-PCR (RT-PCR)

Total RNA was purified from PC12 cells using TRIZOL reagent (GIBCO BRL). A 5 μg portion of total RNA was incubated with avian myeloblastosis virus (AMV) reverse transcriptase (Takara) for 30 min at 50 °C in the presence of a 19-mer primer 5′-TCAGCAACTGGGCGTCTTC-3′, corresponding to an antisense oligonucleotide for the C-terminus of rat type II $sPLA_2$ [28]. After a 5 min incubation at 94 °C, a 2 μl portion of the RT product was mixed with a 22-mer rat type II $sPLA_2$ sense primer 5′-ATGAAGGTCCTCCTGCTAGCAG-3′ and the antisense 19-mer primer shown above. PCR was performed by adding *ex Taq* polymerase (Takara) with 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The size of the PCR product was analysed by electrophoresis on a 2% agarose gel and staining with ethidium bromide.

The PCR product separated on the gel was then transferred to Immobilon-N (Millipore) and was hybridized with a cDNA probe for rat type II $sPLA_2$ [28] that was labelled by random priming (Takara) with [α-^{32}P]dCTP (3000 Ci/mmol; DuPont–NEN). Hybridization was performed at 42 °C overnight in 50% formamide (Merck)/0.75 M NaCl/75 mM sodium citrate/0.2% SDS/5 mM EDTA/10 mM sodium phosphate (pH 6.8)/2 × Denhardt's solution/10% dextran sulphate/100 μg/ml salmon sperm DNA (Sigma). The DNA blot was washed twice with 150 mM NaCl/15 mM sodium citrate/1 mM EDTA/0.2% SDS/10 mM sodium phosphate, pH 6.8, for 5 min each, followed by two washes at 65 °C in 30 mM NaCl/3 mM sodium citrate/1 mM EDTA/0.2% SDS/10 mM sodium phosphate, pH 6.8, for 15 min each. The blot was visualized by autoradiography with Kodak XAR-5 film.

The PCR product was subcloned into the eukaryotic TA cloning vector pCR™3 (Invitrogen) and sequenced using a Sequenase DNA sequence kit (Amersham) according to the manufacturer's instructions. COS-7 cells (American Type Culture Collection), maintained in RPMI 1640 containing 10% (v/v) fetal-calf serum, were cultured in a 35-mm-diam. dish (Corning) with ~60% confluency. Approximately 2 μg of pCR™3, with or without an insert of the aforementioned PCR product, was mixed with 10 μg/ml CellFECTIN™ reagent (GIBCO BRL) and transfected into COS-7 cells in Opti-MEM medium (GIBCO BRL) according to the manufacturer's instructions. After a 6 h incubation at 37 °C, medium was replaced with RPMI 1640 containing 10% (v/v) fetal-calf serum. After 72 h of culture, the supernatant was collected and assayed for $sPLA_2$ activity.

Other methods

Protein was quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce) with BSA as a standard. Thielocin A1, a type

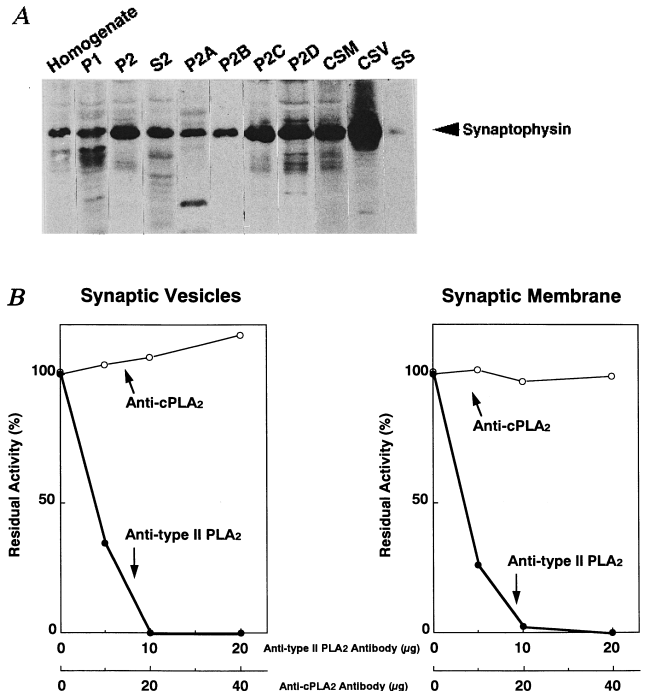

Figure 2 Effect of anti-PLA_2 antibodies on PLA_2 activity in rat synaptic vesicles

(**A**) SDS/PAGE/immunoblotting of each subfraction obtained from rat brain homogenate with an anti-synaptophysin monoclonal antibody. A representative blot of four independent experiments is shown. (**B**) A lysate of the synaptic vesicle fraction (approx. 1 unit of PLA_2 activity) was incubated with the indicated amounts of anti-(type II $sPLA_2$) antibody R377 or anti-$cPLA_2$ antibody RHY-4 as described in the Experimental section. Residual PLA_2 activity was measured, with PLA_2 activity in the absence of each antibody regarded as 100%.

Table 1 PLA_2 activity in subcellular fractions of rat brains

Abbreviations: P1, nucleus and cell debris; P2, mitochondria, myelin and synaptosome; S2, microsome and cytosol; P2A and P2B, myelin; P2C, synaptosome; P2D, mitochondria; and SS, synaptosomal supernatant. Values are means ± S.D. of three independent experiments.

Fraction	Specific activity (pmol/min per mg of protein)	Total activity (pmol/min)	Distribution (%)
Starting material (homogenate)	1.6 ± 0.4	6518 ± 38	
P1	0.9 ± 0.3	438 ± 242	9.5 ± 5.2
P2	1.5 ± 0.5	2106 ± 134	45.6 ± 2.9
S2	1.7 ± 0.2	2077 ± 117	44.9 ± 2.5
Total (P1 + P2 + S2)		4621 ± 493	100
Starting material (P2)	1.5 ± 0.5	2106 ± 134	
P2A	0.8 ± 0.3	252 ± 65	11.8 ± 3.0
P2B	0.7 ± 0.0	199 ± 9	9.3 ± 0.4
P2C	1.5 ± 0.3	1400 ± 70	65.6 ± 3.3
P2D	0.9 ± 0.2	283 ± 97	13.3 ± 4.5
Total (P2A + P2B + P2C + P2D)		2134 ± 241	100
Starting material (P2C)	1.5 ± 0.3	1400 ± 70	
CSM	3.5 ± 0.4	1001 ± 171	72.6 ± 12.4
CSV	27.6 ± 5.4	292 ± 48	21.2 ± 3.5
SS	0.9 ± 0.1	86 ± 24	6.2 ± 1.7
Total (CSM + CSV + SS)		1397 ± 243	100

II $sPLA_2$-selective inhibitor, was described previously [29–32]. Data were analysed by Student's *t*-test and the results expressed as means ± S.D.

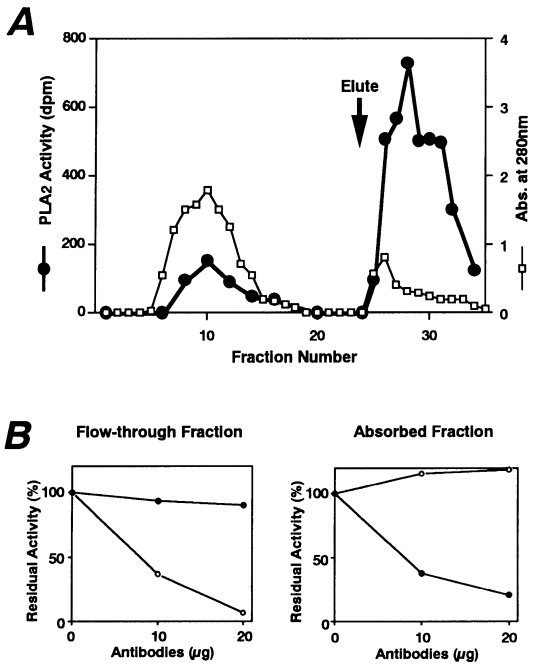

Figure 3 Detection of type II $sPLA_2$ in PC12 cells

(**A**) Soluble fractions of lysates of 1×10^7 PC12 cells were applied to a heparin–Sepharose column as described in the Experimental section, and PLA_2 activity (●) and proteins (□) were monitored. Each fraction contains 1 ml. (**B**) The flow-through and absorbed fractions from heparin–Sepharose columns shown in (**A**) were each incubated with the indicated amounts of either the anti-(type II $sPLA_2$) antibody R377 (●) or the anti-$cPLA_2$ antibody RHY-4 (○) as described in the Experimental section. Residual PLA_2 activity was measured, with PLA_2 activity in the absence of each antibody regarded as 100%.

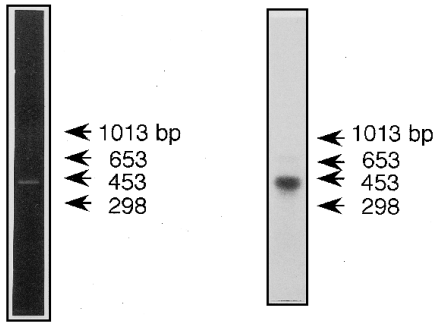

Figure 4 Detection of type II PLA_2 transcript in PC12 cells by RT-PCR

Total RNA obtained from undifferentiated PC12 cells was subjected to RT-PCR analysis with rat type II $sPLA_2$ primers as described in the Experimental section. After staining the gel with ethidium bromide (left), Southern hybridization using rat type II $sPLA_2$ cDNA as a probe was carried out (right).

RESULTS

Detection of immunoreactive type II $sPLA_2$ in rat brain

Rat brains were homogenized with a Potter–Elvehjem homogenizer, sonicated, and centrifuged for 1 h at 100000 ***g***. The supernatant was applied to the anti-(rat type II $sPLA_2$) monoclonal antibody (MD7.1)-conjugated Sepharose column. Approximately 80% of PLA_2 activity in the 100000 ***g*** supernatant was bound to the antibody column and eluted with

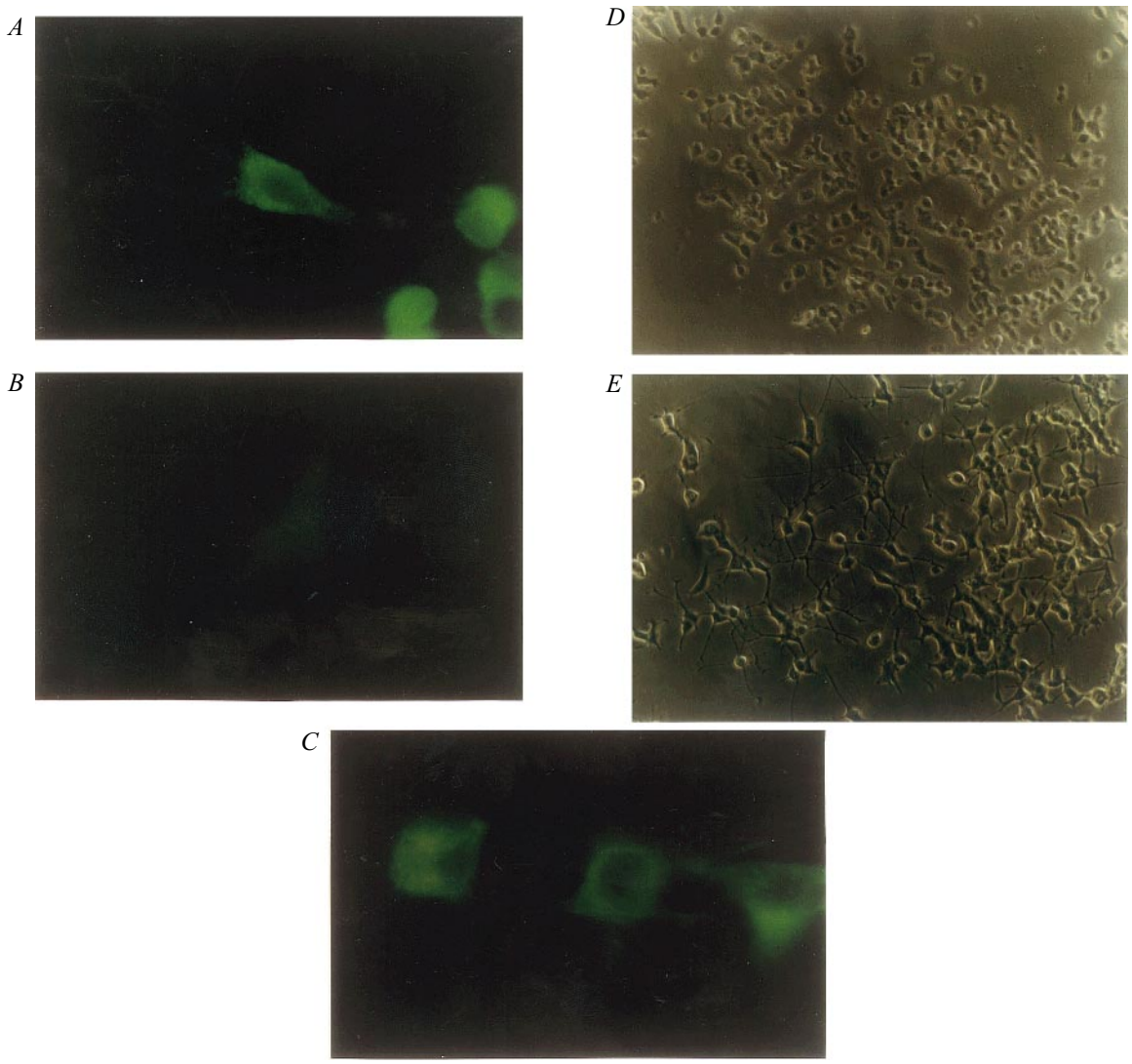

Figure 5 Immunohistochemical staining of PC12 cells

NGF-treated PC12 cells, which had been treated with (**A** and **C**) or without (**B**) saponin, were stained with either the anti-(type II $sPLA_2$) antibody R377 (**A** and **B**) or the anti-synaptophysin antibody (**C**) as described in the Experimental section. Morphological change in PC12 cells before (**D**) and after (**E**) culture for 1 week with NGF is also shown. (**A**) to (**C**), $\times 400$ magnification; (**D**) and (**E**), $\times 100$ magnification.

50 mM glycine/HCl, pH 2.2 (Figure 1A). When an aliquot (10 μg of protein) of the heparin-binding fraction was applied to an SDS/15 %-PAGE gel, transferred to nitrocellulose, and immunblotted with an anti-(rat type II $sPLA_2$) polyclonal antibody, R377, a single protein band with a molecular mass of $\sim$14 kDa, which co-migrated with 14 kDa type II $sPLA_2$ purified from rat platelets, was visualized (Figure 1B). PLA_2 activity recovered in the flow-through fractions of the anti-(type II $sPLA_2$) antibody-conjugated column was completely absorbed by the anti-(rabbit $cPLA_2$) monoclonal antibody, RHY-4 (Figure 1C). Thus, the soluble fraction of rat brain homogenates contained both $sPLA_2$ and $cPLA_2$ with a ratio of 4:1 as assessed in terms of enzyme activity.

To determine the subcellular localization of $sPLA_2$, rat brain was gently homogenized so as not to disrupt intracellular organelles. The particulate fraction of rat brain homogenate was then separated into subcellular fractions by a discontinuous sucrose density gradient to obtain synaptosomes and synaptic vesicles as described in the Experimental section. The efficiency of subcellular fractionation was confirmed by SDS/PAGE/immunoblotting with an antibody against synaptophysin, a synaptic vesicle-specific protein, in that 38 kDa immunoreactive synaptophysin was concentrated into synaptosomes (P2C–P2D), and further into synaptic vesicle (CSV) and synaptic membrane (CSM) fractions (Figure 2A). PLA_2 activity was concentrated in P2C, which contained 65 % of the total PLA_2 activity in the P2 fraction (Table 1). When P2C was further fractionated, more than 90 % of the PLA_2 activity was recovered in the CSV and CSM fractions. PLA_2 activities in CSVs and CSMs represented approximately 20 % and 70 % of the total PLA_2 activity in synaptosome fractions, respectively. Of particular note is that the specific PLA_2 activity in CSVs (27.6 pmol/min per mg) was $\sim$17-fold higher than that of total brain homogenate (1.6 pmol/min per mg). The anti-(type II

sPLA$_2$) antibody R377 absorbed the PLA$_2$ activity almost completely in CSVs and in CSMs, whereas the anti-cPLA$_2$ antibody RHY-4 did not (Figure 2B).

When the synaptosome fraction was treated with 50 mM KCl in the presence of Ca^{2+}, $10.9 \pm 1.0\,\%$ of PLA$_2$ activity was released into the medium relative to $4.1 \pm 0.3\,\%$ or $4.4 \pm 0.9\,\%$ by Ca^{2+} alone or by K^+ alone, respectively ($n = 3$, $P < 0.05$). There was no appreciable release of lactate dehydrogenase ($< 1\,\%$) under any of these conditions. These results indicate that sPLA$_2$ is stored in synaptic vesicles and released in response to depolarization in the presence of extracellular Ca^{2+}.

Presence of immunoreactive type II sPLA$_2$ in the rat neuronal cell line PC12 cells

Lysate of rat pheochromocytoma PC12 cells was applied to a heparin–Sepharose column. Proteins bound to the heparin–Sepharose column were eluted stepwise with 1 M NaCl. PLA$_2$ activities were detected in both the flow-through and absorbed fractions (Figure 3A). PLA$_2$ activity recovered in the absorbed fractions was neutralized by the anti-(type II sPLA$_2$) antibody R377, but was not absorbed by the anti-cPLA$_2$ antibody RHY-4 (Figure 3B). Conversely, PLA$_2$ activity in the flow-through fractions was completely absorbed by the anti-cPLA$_2$ antibody but not by the anti-(type II sPLA$_2$) antibody (Figure 3B). Thus, as in the case of rat brain, PC12 cells contained sPLA$_2$ and cPLA$_2$ as assessed by reactivity with each antibody. SDS/PAGE/immunoblotting with the anti-(type II sPLA$_2$) polyclonal antibody revealed a single protein band with a molecular mass of about 14 kDa, which co-migrated with rat platelet type II sPLA$_2$, in PC12 cells (results not shown).

To confirm that PC12 cells express type II sPLA$_2$, RT-PCR analysis was carried out. Using a set of rat type II sPLA$_2$-specific primers that could amplify full-length sPLA$_2$, an approx. 440 bp fragment (which was hybridized with cDNA probe for rat type II sPLA$_2$ under high stringency conditions), was specifically amplified from RNA obtained from PC12 cells (Figure 4). DNA sequencing revealed that this PCR fragment indeed encoded rat type II sPLA$_2$ (results not shown). When this PCR product was subcloned into the mammalian expression vector pCR™3 and then transfected into COS-7 cells, appreciable PLA$_2$ activity was released in the supernatant relative to cells transfected with vector alone (results not shown).

When PC12 cells were cultured for 7 days with NGF, they were tightly bound to collagen-coated dishes and exhibited neurite outgrowth (Figures 5D and 5E). These NGF-treated, neuronally differentiated PC12 cells were then detached, seeded on poly(L-lysine)-coated coverglasses, and incubated for an additional 24 h in the presence of NGF. The adherent cells were fixed with paraformaldehyde and stained immunochemically with antibodies against either type II sPLA$_2$ (Figures 5A and 5B) or synaptophysin (Figure 5C). The antibody against type II sPLA$_2$ visualized granule-like organelles in PC12 cells that had been permeabilized by treatment with saponin (Figure 5A). A similar staining pattern was seen when permeabilized PC12 cells were treated with the anti-synaptophysin antibody (Figure 5C). In contrast, PC12 cells that had not been permeabilized by saponin were not reactive with the anti-(type II sPLA$_2$) antibody (Figure 5B).

Release of type II sPLA$_2$ from neuronally differentiated PC12 cells upon stimulation

PC12 cells, which have differentiated into neuron-like cells by treatment with NGF, are reported to be activated by stimulation through their acetylcholine receptors or voltage-gated Ca^{2+} channels [18]. NGF-treated PC12 cells were prelabelled for 4 h with [^{3}H]noradrenaline, stimulated with carbamylcholine or high concentrations of potassium ions, and the release of incorporated [^{3}H]noradrenaline into the supernatant was examined. Release of

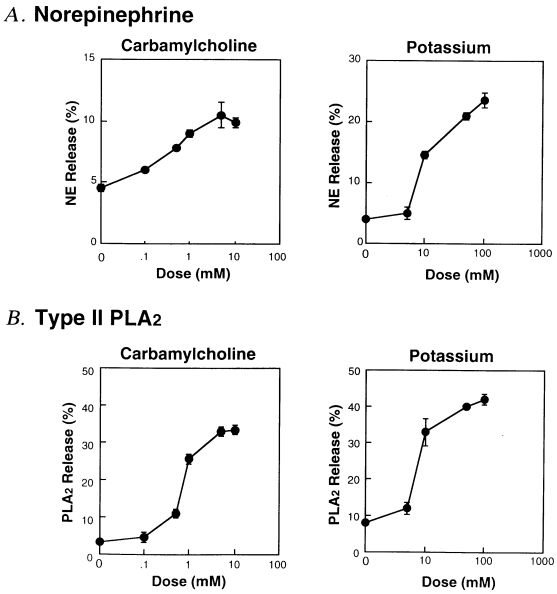

Figure 6 Dose-dependent release of [^{3}H]noradrenaline (norepinephrine, NE) and sPLA$_2$ from PC12 cells

NGF-treated PC12 cells were activated for 30 min at 37 °C in the presence of the indicated concentrations of carbamylcholine or potassium. Release of [^{3}H]noradrenaline (**A**) and sPLA$_2$ (**B**) were assessed as described in the Experimental section. There was no appreciable release when potassium ion was replaced with the same osmolarity of sodium ion.

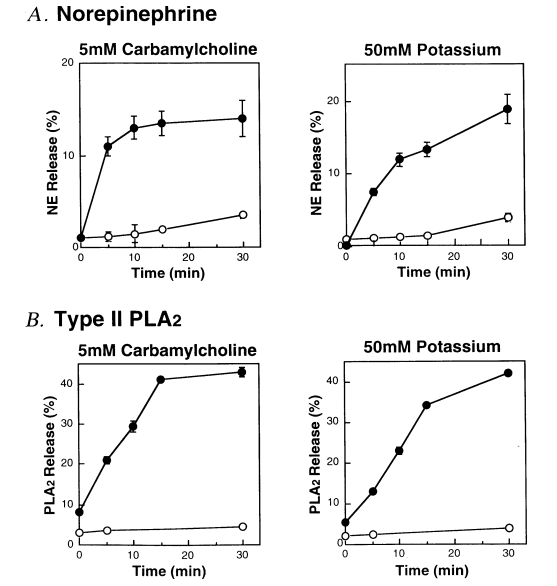

Figure 7 Kinetics of release of [^{3}H]noradrenaline (norepinephrine, NE) and sPLA$_2$ from PC12 cells

NGF-treated PC12 cells were activated with (●) or without (○) 5 mM carbamylcholine or 50 mM KCl for the indicated periods at 37 °C. Release of [^{3}H]noradrenaline (**A**) and sPLA$_2$ (**B**) were assessed as described in the Experimental section.

Table 2 Effect of type II $sPLA_2$ inhibitors on [^{3}H]noradrenaline release from PC12 cells stimulated with carbamylcholine

PC12 cells, cultured with 50 ng/ml NGF for 7 days, were incubated for 4 h with [^{3}H]noradrenaline, washed, and incubated for 5 min with the indicated concentrations of thielocin A1 or anti-(type II $sPLA_2$) antibody. The cells were then activated with 5 mM carbamylcholine for 30 min in the continued presence of thielocin A1 or antibody. Net [^{3}H]noradrenaline release after each treatment is shown. Relative [^{3}H]noradrenaline release (%) = (net [^{3}H]noradrenaline released from cells treated with inhibitors)/(that released from cells without inhibitors) × 100. Values are means ± S.D. of three to four independent experiments. *$P < 0.001$ and †$P < 0.05$ versus absence of inhibitors.

Inhibitors (concentration)	Net release (%)	Relative release (%)
None	11.9 ± 0.75	100
Thielocin A1		
(1 ng/ml)	7.43 ± 0.61*	62.4 ± 3.6*
(10 ng/ml)	3.75 ± 0.90*	31.5 ± 5.4*
(100 ng/ml)	3.48 ± 1.10*	29.2 ± 6.3*
(1000 ng/ml)	3.18 ± 0.87*	26.7 ± 4.8*
Anti-type II $sPLA_2$ antibody		
(1 μg/ml)	11.1 ± 0.44†	93.3 ± 2.9†
(10 μg/ml)	8.65 ± 2.00†	72.7 ± 13.0†
(100 μg/ml)	4.41 ± 0.95†	37.1 ± 6.2†

[^{3}H]noradrenaline was dependent on the concentrations of each stimulus, reaching maximum values of 10% and 25% release with 10 mM carbamylcholine and 100 mM potassium, respectively (Figure 6A). When the cells were stimulated with carbamylcholine, [^{3}H]noradrenaline release reached a plateau 10 min after stimulation, whereas activation by depolarization resulted in a progressive release of [^{3}H]noradrenaline over 30 min (Figure 7A). There was no appreciable leakage of lactate dehydrogenase from the cytosol as a result of these stimuli (results not shown).

After 7 days culture of PC12 cells with NGF, the PLA_2 activity in the cell lysate increased by approximately 1.5-fold. When the cells were stimulated with carbamylcholine or KCl, PLA_2 activity was released into the medium in a dose-dependent manner and reached a maximum of approx. 40% release following these stimuli at concentrations similar to those which elicited the release of [^{3}H]noradrenaline (Figure 6B). ED_{50} values for [^{3}H]noradrenaline release by carbamylcholine and KCl were 0.5 mM and 10 mM, respectively, and those for $sPLA_2$ release were 0.8 mM and 8 mM, respectively. $sPLA_2$ release reached a maximum plateau level 15 min after activation by these stimuli (Figure 7B). $sPLA_2$ activity released into the supernatant was completely neutralized by the anti-(type II $sPLA_2$) antibody R377 (results not shown). As assessed by PLA_2 activity, approx. 0.7 ng of $sPLA_2/10^6$ cells was released upon stimulation.

Regulation of catecholamine secretion by type II $sPLA_2$ in PC12 cells

When [^{3}H]noradrenaline-labelled PC12 cells were stimulated with carbamylcholine in the presence of thielocin A1, a type II $sPLA_2$-specific inhibitor [29–32], the release of [^{3}H]noradrenaline was suppressed in a dose-dependent manner with an IC_{50} of < 10 ng/ml (Table 2). The neutralizing antibody against type II $sPLA_2$, R377 [25], also suppressed [^{3}H]noradrenaline release in a dose-dependent manner (Table 2). Nearly 70% of release was suppressed by either 10 ng/ml thielocin A1 or by 100 μg/ml antibody. These results indicate that endogenous $sPLA_2$ may be involved in catecholamine secretion from PC12 cells.

In order to assess whether extracellular type II $sPLA_2$ could directly elicit exocytosis or not, the effect of exogenously added type II $sPLA_2$ on secretion of catecholamine was examined.

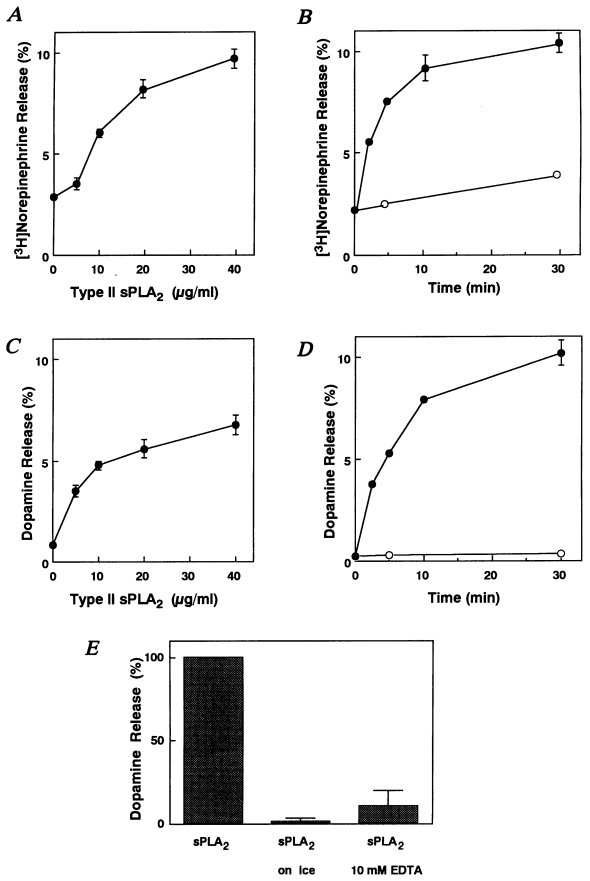

Figure 8 Effect of exogenous type II $sPLA_2$ on catecholamine release from PC12 cells

(**A** and **C**) NGF-treated PC12 cells were incubated with the indicated concentrations of rat platelet-derived type II $sPLA_2$ for 10 min at 37 °C. (**B** and **D**) NGF-treated PC12 cells were incubated for the indicated periods with (●) or without (○) 40 μg/ml $sPLA_2$ at 37 °C. (**E**) NGF-treated PC12 cells were incubated with 40 μg/ml $sPLA_2$ for 10 min under various conditions. Release of [^{3}H]noradrenaline (norepinephrine) (**A** and **B**) and dopamine (**C** to **E**) were assessed as described in the Experimental section. In (**E**), relative dopamine release (%) is shown, with the release by $sPLA_2$ at 37 °C in the absence of EDTA regarded as 100%.

When type II $sPLA_2$ purified from rat platelets was added to NGF-treated PC12 cells, there was a dose-dependent release of [^{3}H]noradrenaline, reaching a maximum release of ~10% at 20–40 μg/ml $sPLA_2$ (Figure 8A) after 10–30 min of incubation (Figure 8B). We also quantified the release of endogenous dopamine, which was stored ~10-fold more abundantly in PC12 granules than endogenous noradrenaline [33]. Dopamine release elicited by type II PLA_2 almost paralleled [^{3}H]noradrenaline release, reaching a maximum release of 5–10% at 20–40 μg/ml $sPLA_2$ (Figure 8C) and reaching a plateau 10–30 min after stimulation (Figure 8D). There was no detectable release of lactate dehydrogenase after treatment with exogenous $sPLA_2$ (results not shown). Release of dopamine by exogenous $sPLA_2$ was not observed when the cells were treated at 4 °C or when the cells were treated in the absence of extracellular Ca^{2+} (Figure 8E).

DISCUSSION

Several investigators have reported the presence of $cPLA_2$ in brain [4,34,35] and of type II PLA_2 in glial cells in brains of rats

with endotoxin shock [36]. We have now shown that rat brain synaptic vesicle fractions contain $sPLA_2$ (Figures 1 and 2, Table 1). Immunoreactive type II $sPLA_2$ was detected in neuronally differentiated PC12 cells (Figures 3 and 5), indicating that neuronal cells express $sPLA_2$. Of particular note is that $sPLA_2$ was released from rat brain synaptosomes or from neuronally differentiated PC12 cells (Figures 6 and 7) upon stimulation via acetylcholine receptors or via voltage-dependent Ca^{2+} channels through depolarization, thereby provoking the possibility that $sPLA_2$ may play a role in the function of neuronal cells.

Several studies have demonstrated the involvement of certain PLA_2 types in the degranulation process in various cells. The possible involvement of type II $sPLA_2$ in the secretory process has been shown in mast cells [16,17,37]. Classical PLA_2 inhibitors, such as *p*-bromophenacyl bromide or mepacrine, are reported to suppress exocytosis not only by mast cells but also by neuronal cells or by chromaffin cells [11–13]. Moskowitz et al. [12] have reported that stimulation of Ca^{2+}-dependent PLA_2 activity in synaptic vesicles correlates with the induction of vesicle–vesicle aggregation and they suggested that the synaptic vesicle PLA_2 may play a central role in presynaptic neurotransmission. Based upon these observations, we hypothesized that type II $sPLA_2$ might have a general function in the regulation of degranulation by these cells. Indeed, the release of catecholamine from activated PC12 cells was markedly suppressed by thielocin A1, an inhibitor specific to type II $sPLA_2$, or by anti-(type II PLA_2) antibody (Table 2). Because the antibody could not pass through the plasma membrane without permeabilization (Figure 5), it is likely that $sPLA_2$ may act from outside the cells and so become accessible to the antibody after cell activation. This is supported by the fact that exogenously added $sPLA_2$ directly elicited dopamine release from PC12 cells in an extracellular Ca^{2+}-dependent manner (Figure 8).

The concentrations of each stimulant required for release of $sPLA_2$ and [^{3}H]noradrenaline from PC12 cells were comparable (Figure 6). However, maximal release of $sPLA_2$ reached approx. 40 % in response to carbamylcholine or potassium, whereas that of [^{3}H]noradrenaline was dependent upon the nature of the stimulus, reaching only 10 % and 25 % in response to carbamylcholine and potassium, respectively (Figure 6). Furthermore, the time courses of $sPLA_2$ release and of [^{3}H]noradrenaline release were not parallel in that the release of $sPLA_2$ and [^{3}H]noradrenaline reached a plateau 15 min and 10 min, respectively, after activation with carbamylcholine (Figure 7). These results suggest that $sPLA_2$ and catecholamine may be stored in different kinds of granules in PC12 cells. Studies have established that each neuron can secrete a cocktail of peptide and non-peptide neurotransmitter molecules via at least two types of secretory granules [38]. Small synaptic vesicles (SSVs) are involved in the release of non-peptide neurotransmitters only, while peptides are secreted via larger vesicles, the so-called large dense-core vesicles (LDCVs). The latter are similar in their biochemical nature and in their mechanism of packaging to secretory granules in the well-established secretory pathway of endocrines. Both LDCVs and secretory granules are known to contain peptides, including regulatory peptides, some enzymes and proteins. Because $sPLA_2$ is often detected in secretory granules in various cells and tissues [1], it might be stored in LDCVs rather than SSVs in PC12 cells. It is not clear, however, where $sPLA_2$ is stored in rat brain synaptosomes because vesicles in synaptosomes are mostly SSVs.

The molecular mechanism whereby $sPLA_2$ functions is presently unknown. One of the possible explanations is the generation of lipid products which directly regulate fusion between the plasma membrane and secretory granules. Some lipid components generated by the enzymic action of PLA_2, such as lysophospholipids, free fatty acids and their metabolites, may be critical for the fusion step. Lysophospholipids have been suggested as potential 'fusogenic' substances and several reports have demonstrated the potential importance of lysophospholipids in the secretory process [39]. Karli et al. [40] described a model for membrane fusion, in which Ca^{2+}-dependent PLA_2 triggered fusion between granules and plasma membranes isolated from bovine adrenal chromaffin cells, and stated that the fatty acids liberated were metabolized to fusogenic substances, thus rendering the membranes ready for fusion. $sPLA_2$ derived from PC12 cells may act on plasma membranes in an autocrine or paracrine manner to generate such fusogenic compound(s), which then enhance the efficiency of fusion between the plasma membrane and chromaffin granules.

The concentration of exogenous type II $sPLA_2$ required for catecholamine secretion from PC12 cells (IC_{50} approx. 5 μg/ml) is higher than that released from activated PC12 cells (0.7 ng/10^6 cells), as in the case of mast cells [16,17]. These results indicate, as we have currently suggested, that some additional signals, which occur at the early stage of cell activation following exposure to agonists, might be necessary for the secretory process to work effectively. In this regard, Fourcade et al. [41] reported that $sPLA_2$ is stimulated by certain degradation products of sphingomyelin and generates lysophosphatidic acid in membrane microvesicles shed from activated cells. Kim et al. [42] reported that stimulation of cortical neuronal cultures from rat brain with glutamate enhances the activity of 14 kDa PLA_2 with heparin affinity, the Ca^{2+} requirement of which was shifted from millimolar to micromolar as a result of stable modification of the enzyme induced by cell activation.

Alternatively, the effect of type II $sPLA_2$ may be mediated through a receptor-dependent process [43–46]. However, the cloned 180 kDa receptor binds only to the mature form of type I isoenzyme but not to type II isoenzyme except for the receptor cloned from rabbit [44]. Furthermore, transcript for this receptor was undetectable in rat neuronal tissues [46]. Therefore it seems unlikely that this receptor mediates neuronal cell activation by type II $sPLA_2$ isoenzyme. It is of interest to note that some neurotoxic snake venom $sPLA_2$s, such as crotoxin that belongs to the group II $sPLA_2$ family, bind to a 45 kDa protein in the neuronal membrane and affect neurotransmitter release [47,48].

Arachidonic acid metabolism elicited by neurotransmitters or by depolarization, which mobilizes intracellular Ca^{2+}, is more likely to be regulated by $cPLA_2$. However, it is also possible that $sPLA_2$ might hydrolyse membrane phospholipids to liberate arachidonic acid, which participates in the acceleration of cell activation, leading to catecholamine secretion. The effect of type II $sPLA_2$ on arachidonic acid metabolism in neuronal cells is now under investigation.

Recently, two laboratories have independently reported the natural disruption of the type II $sPLA_2$ gene in some inbred mouse strains, such as C57BL/6J and 129/Sv [49,50]. It is not clear whether the intrinsic lack of type II $sPLA_2$ affects neuronal functions in these strains. There are at least two new classes of low-molecular-mass $sPLA_2$s identified in mammals. These are $sPLA_2$ with 12 cysteine residues that is abundant in heart [51], and $sPLA_2$ with 16 cysteine residues that is abundant in testis [52]. Whether or not these other $sPLA_2$s compensate for the function of type II $sPLA_2$ in these mice strains remains to be determined.

We thank Dr. T. Yoshida (Shionogi Co. Ltd.) for kindly providing thielocin A1, and Drs. S. Araki (Eisai Co. Ltd.) and T. Iwatsubo (The University of Tokyo) for technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Kudo, I., Murakami, M., Hara, S. and Inoue, K. (1993) Biochim. Biophys. Acta **1170**, 217–231
2 Kim, D. K., Kudo, I. and Inoue, K. (1991) Biochim. Biophys. Acta **1083**, 80–88
3 Channon, J. Y. and Leslie, C. C. (1990) J. Biol. Chem. **265**, 5409–5413
4 Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) Cell **65**, 1043–1051
5 Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R., Becker, P. L., Kang, L. H., Roberts, E. F. and Kramer, R. M. (1991) J. Biol. Chem. **266**, 14850–14853
6 Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) Cell **72**, 269–278
7 Barbour, S. E. and Dennis, E. A. (1993) J. Biol. Chem. **268**, 21875–21882
8 Murakami, M., Kudo, I. and Inoue, K. (1993) J. Biol. Chem. **268**, 839–844
9 Murakami, M., Kudo, I. and Inoue, K. (1991) FEBS Lett. **294**, 247–251
10 Suga, H., Murakami, M., Kudo, I. and Inoue, K. (1993) Eur. J. Biochem. **218**, 807–813
11 McGivney, A., Morita, V., Crews, F. T., Hirata, F., Axelrod, J. and Siraganian, R. P. (1981) Arch. Biochem. Biophys. **212**, 572–580
12 Moskowitz, N., Schook, W. and Puszkin, S. (1982) Science **216**, 305–307
13 Bradford, P. G., Marinetti, G. V. and Abood, L. G. (1983) J. Neurochem. **41**, 1684–1693
14 Wang, J.-P. and Teng. C.-M. (1990) Eur. J. Pharmacol. **190**, 347–354
15 Knepel, W. and Meyen, G. (1986) Neuroendocrinology **43**, 44–48
16 Murakami, M., Kudo, I., Suwa, Y. and Inoue, K. (1992) Eur. J. Biochem. **209**, 257–265
17 Murakami, M., Hara, N., Kudo, I. and Inoue, K. (1993) J. Immunol. **151**, 5675–5684
18 Shafer, T. J. and Atchison, W. D. (1991) Neurotoxicology **12**, 473–492
19 Ueda, T., Greengard, P., Berzins, K., Cohen, R. S., Blomberg, F., Grab, D. J. and Siekevitz, P. (1979) J. Cell Biol. **83**, 308–319
20 Jahn, R., Schiebler, W., Ouimet, C. and Greengard, P. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4137–4141
21 Dole, V. P. and Menertz, H. (1960) J. Biol. Chem. **235**, 2595–2599
22 Murakami, M., Kobayashi, T., Umeda, M., Kudo, I. and Inoue, K. (1988) J. Biochem. (Tokyo) **104**, 884–888
23 Mizushima, H., Kudo, I., Horigome, K., Murakami, M., Hayakawa, M., Kim, D. K., Kondo, E., Tomita, M. and Inoue, K. (1989) J. Biochem. (Tokyo) **105**, 520–525
24 Fujimori, Y., Kudo, I., Fujita, K. and Inoue, K. (1993) Eur. J. Biochem. **218**, 629–635
25 Murakami, M., Kudo, I., Natori, Y. and Inoue, K. (1990) Biochim. Biophys. Acta **1043**, 34–42
26 Horigome, K., Hayakawa, M., Inoue, K. and Nojima, S. (1987) J. Biochem. (Tokyo) **101**, 625–631
27 Prados, P., Higashidate, S. and Imai, K. (1994) Biomed. Chromatogr. **8**, 1–8
28 Komada, M., Kudo, I., Mizushima, H., Kitamura, N. and Inoue, K. (1989) J. Biochem. (Tokyo) **106**, 545–547
29 Yoshida, T., Nakamoto, S., Sakazaki, R., Matsumoto, K., Terui, Y., Sato, T., Arita, H., Matsutani, S., Inoue, K. and Kudo, I. (1991) J. Antibiotics **44**, 1467–1470
30 Tanaka, K., Matsutani, S., Matsumoto, K. and Yoshida, T. (1992) J. Antibiotics **45**, 1071–1078
31 Tanaka, K., Kato, T., Matsumoto, K. and Yoshida, T. (1993) Inflammation **17**, 107–119
32 Tanaka, K., Matsutani, S., Matsumoto, K. and Yoshida, T. (1995) Eur. J. Pharmacol. **279**, 143–148
33 Kudo, I., Matsuzawa, A., Imai, K., Murakami, M. and Inoue, K. (1996) J. Lipid. Med. Cell. Sig. in the press
34 Yoshihara, Y. and Watanabe, Y. (1990) Biochem. Biophys. Res. Commun. **170**, 484–490
35 Oka, S. and Arita, H. (1991) J. Biol. Chem. **266**, 9956–9960
36 Fujimori, Y., Murakami, M., Kim, D. K., Hara, S., Takayama, Y., Kudo, I. and Inoue, K. (1992) J. Biochem. (Tokyo) **111**, 54–60
37 Murakami, M., Kudo, I., Fujimori, Y., Suga, H. and Inoue, K. (1991) Biochem. Biophys. Res. Commun. **181**, 714–721
38 Camilli, P. D. and Jahn, R. (1990) Annu. Rev. Physiol. **52**, 625–645
39 Poole, A. R., Howell, J. I. and Lucy, J. A. (1970) Nature (London) **227**, 810–814
40 Karli, U. O., Schafer, T. and Burger, M. M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5912–5915
41 Fourcade, O., Simon, M.-F., Viade, C., Rugan, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L. and Chop, H. (1995) Cell **80**, 919–927
42 Kim, D. K., Rordorf, G., Nemenoff, A., Koroshetz, W. J. and Bonventre, J. V. (1995) Biochem. J. **310**, 83–90
43 Ishizaki, J., Hanasaki, K., Higashino, K., Kishino, J., Kikuchi, N., Ohara, O. and Arita, H. (1994) J. Biol. Chem. **269**, 5897–5904
44 Lambeau, G., Ancian, P., Barhanin, J. and Lazdunski, M. (1994) J. Biol. Chem. **269**, 1575–1578
45 Ancian, P., Lambeau, G., Mattei, M.-G. and Lazdunski, M. (1995) J. Biol. Chem. **270**, 8963–8970
46 Higashino, K., Ishizaki, J., Kishino, J., Ohara, O. and Arita, H. (1994) Eur. J. Biochem. **225**, 375–382
47 Yen, C.-H. and Tzeng, M.-C. (1991) Biochemistry **30**, 11473–11477
48 Tzeng, M.-C., Ten, C.-H., Hseu, M.-J., Dupureur, C. M. and Tsai, M.-D. (1995) J. Biol. Chem. **270**, 2120–2123
49 MacPhee, M., Chepenik, K. P., Liddell, R. A., Nelson, K. K., Siracusa, L. D. and Buchberg, A. M. (1995) Cell **81**, 957–966
50 Kennedy, B. P., Payette, P., Mudgett, J., Vadas, P., Pruzanski, W., Kwan, M., Tang, C., Rancourt, D. E. and Cromlich, W. A. (1995) J. Biol. Chem. **270**, 22378–22385
51 Chen, J., Engle, S. J., Seilhamer, J. J. and Tischfield, J. A. (1994) J. Biol. Chem. **269**, 2365–2368
52 Chen, J., Engle, S. J., Seilhamer, J. J. and Tischfield, J. A. (1994) J. Biol. Chem. **269**, 23018–23024

Received 12 January 1996/25 April 1996; accepted 14 May 1996