Secretory phospholipases A_2 induce neurite outgrowth in PC12 cells

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$sPLA_2$s (secretory phospholipases A_2) belong to a broad and structurally diverse family of enzymes that hydrolyse the *sn*-2 ester bond of glycerophospholipids. We previously showed that a secreted fungal 15 kDa protein, named p15, as well as its orthologue from *Streptomyces coelicolor* (named Scp15) induce neurite outgrowth in PC12 cells at nanomolar concentrations. We report here that both p15 and Scp15 are members of a newly identified group of fungal/bacterial $sPLA_2$s. The phospholipid-hydrolysing activity of p15 is absolutely required for neurite outgrowth induction. Mutants with a reduced PLA_2 activity exhibited a comparable reduction in neurite-inducing activity, and the ability to induce neurites closely matched the capacity of various p15 forms to promote fatty acid release from live PC12 cells. A structurally divergent member of the $sPLA_2$ family, bee venom $sPLA_2$, also induced neurites in a phospholipase activity-dependent manner, and the same effect was elicited by mouse group V and X $sPLA_2$s, but not by group IB and IIA $sPLA_2$s. Lysophosphatidylcholine, but not other lysophospholipids, nor arachidonic acid, elicited neurite outgrowth in an L-type Ca^{2+} channel activity-dependent manner. In addition, p15-induced neuritogenesis was unaffected by various inhibitors that block arachidonic acid conversion into bioactive eicosanoids. Altogether, these results delineate a novel, Ca^{2+}- and lysophosphatidylcholine-dependent neurotrophin-like role of $sPLA_2$s in the nervous system.

Key words: L-type Ca^{2+} channel, lysophosphatidylcholine, neurite outgrowth, neuronal differentiation, PC12, secretory phospholipase A_2.

INTRODUCTION

Rat pheochromocytoma PC12 cells have been extensively utilized as a model system to study the intracellular signalling pathway(s) leading to neuronal differentiation. Stimulation by differentiation factors such as NGF (nerve growth factor) and subsequent activation of its receptor, TrkA, initiates a variety of signalling events that lead to cessation of growth, acquirement of electrical excitability, expression of neuron-specific marker proteins and neurite extension. Besides growth factor stimulation, Ca^{2+} influx through voltage-gated Ca^{2+} channels upon membrane depolarization also induces neuronal differentiation. Neurite extension induced by various kinds of stimuli, such as growth factors and depolarization, is a characteristic signature of neuronal differentiation. Ca^{2+} influx through voltage-gated channels, followed by an increase in intracellular Ca^{2+}, initiates a variety of cellular events leading to neurite outgrowth via the concerted action of various Ca^{2+}-binding proteins [1]. For example, calmodulin activation in growth cones leads to the formation of an active calcineurin form that is implicated in the dephosphorylation of the microtubule-associated protein tau and in promoting tubulin assembly. Elevation of intracellular Ca^{2+} levels also stimulates the expression of several Ca^{2+}-regulated genes, such as c-*fos* and brain-derived neurotrophic factor, through the activation of the Ras-MAPK (mitogen-activated protein kinase) cascade.

It has also been found that homophilic interaction of CAMs (cell adhesion molecules) stimulate a neurite outgrowth response that is dependent on the activity of L- and N-type Ca^{2+} channels. Co-culture of PC12 cells or cerebellar neurons on a monolayer of fibroblasts expressing neural CAM or N-cadherin leads to neurite extension through interaction with CAMs exposed on the surface of neighbouring support cells [2,3]. The second messenger pathway underlying CAM-induced neurite outgrowth has been analysed in detail. It has been shown, for example, that stimulation by CAMs leads to the phosphorylation and activation of the fibroblast growth factor receptor, which in turn transmits its signal to phospholipase Cγ [4]. Activated phospholipase Cγ hydrolyses phospholipids to produce diacylglycerol, which then serves as a substrate for the production of arachidonic acid through the action of diacylglycerol lipase. Arachidonic acid has thus been suggested to induce an influx of Ca^{2+} through plasma membrane-associated Ca^{2+} channels, ultimately leading to neurite outgrowth. The direct addition of arachidonic acid to the culture medium, however, did not cause any increase in steady-state Ca^{2+} levels in neuronal growth cones [5].

$sPLA_2$s (secretory phospholipases A_2) are enzymes of 13–20 kDa that hydrolyse the ester bond at the *sn*-2 position of glycerophospholipids in a Ca^{2+}-dependent manner [6,7]. The first members of this family of enzymes were identified in pancreatic juice and snake venoms. After decades of study, it has been recognized that $sPLA_2$s belong to a broad, structurally and functionally diverse family of proteins [6,7], that are present in various venoms as well as in animal organs (but also in filamentous bacteria and fungi [8,9]), and are endowed with a variety of pro-inflammatory, neurotoxic, myotoxic, cardiotoxic and other stress-response-inducing properties [10,11]. An increasing number of

Abbreviations used: PLA_2, phospholipase A_2; $bvPLA_2$, bee venom PLA_2; CAM, cell adhesion molecule; COX, cyclo-oxygenase; HA, haemagglutinin; LOX, lipoxygenase; lysoPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinase; NDGA, nordihydroguaiaretic acid; NGF, nerve growth factor; PA, palmitic acid; PC, phosphatidylcholine; $rbvPLA_2$, recombinant $bvPLA_2$; $sPLA_2$, secretory PLA_2; mcPAF, methylcarbamyl platelet-activating factor C-16; DMEM, Dulbecco's modified Eagle's medium.

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novel cellular $sPLA_2$s is being identified as the result of completed or ongoing whole genome sequencing projects. They are currently classified into eight different groups, I, II, III, V, IX, X, XI and XII, according to their primary structures and biochemical properties [6,7]. In mammals, $sPLA_2$s not only function as phospholipid-digesting enzymes, but are also involved in the production of signalling molecules, especially arachidonic acid, a key precursor of various eicosanoids including prostaglandins, thromboxanes and leukotrienes [7,12].

We reported previously the identification of a secreted 15 kDa fungal protein, named p15, with a potent neurite-inducing activity on PC12 cells [13,14]. Amino acid sequence analysis of p15 followed by a database search revealed the presence of a p15 homologue in *Streptomyces coelicolor* A3(2), that also displays neurite-inducing activity in PC12 cells [15]. We show here that these two proteins are novel members of a recently defined fungal/bacterial $sPLA_2$ group (XIII) [8,9] and that their *sn*-2 position-specific phospholipase activity is absolutely required for neurite outgrowth induction. $sPLA_2$-induced neuritogenesis was specifically blocked by the L-type Ca^{2+} channel blocker nicardipine, but not by inhibitors of eicosanoid-synthesizing enzymes. In the absence of exogenously supplied $sPLA_2$, neurite outgrowth was elicited by lysophosphatidylcholine (lysoPC), but not by other lysophospholipids, nor by arachidonic acid. Altogether, these results point to the involvement of $sPLA_2$s in a novel cellular pathway leading to neuronal differentiation.

EXPERIMENTAL

Materials

The high-fidelity, thermostable *Pfx* DNA polymerase used for DNA amplification, the expression vector pRSET-C and Lipofectamine 2000 reagent for the transfection of COS1 cells were from Invitrogen. Amplified DNA fragments were purified with GeneClean II (Bio101). Ni^{2+}-nitrilotriacetate agarose was purchased from Qiagen. [^{3}H]Arachidonic acid ([5,6,8,9,11,12,14,15-^{3}H]; 200 Ci/mmol) and [^{3}H]oleic acid ([9,10-^{3}H], 15 Ci/mmol) were from Moravek (MT-901) and New England Nuclear (NET-289), respectively. $bvPLA_2$ (bee venom PLA_2; Sigma catalogue no. P 9279), nicardipine (N 7510), arachidonic acid (A 9673), aspirin (A 5376), indomethacin (I 7378), nordihydroguaiaretic acid (NDGA; N 5023), AA-861 (N 3711), dipalmitoyl-*sn*-glycerophosphatidylcholine, 1-palmitoyl-2-lynoleyl phosphatidylcholine, 1-palmitoyl-2-lynoleyl phosphatidylethanolamine, 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysoPC; L 5245), sphingosylphosphorylcholine (S 4257), lysophosphatidylinositol (L 7635), lysophosphatidylethanolamine (L 4754), lysophosphatidylserine (L 3401) and 1-oleyl-*sn*-glycero-3-phosphate (L 7260) were from Sigma. Baicalein was purchased from Aldrich (46,511-9), and mcPAF (methylcarbamyl platelet-activating factor C-16) was from Cayman (catalogue no. 60908).

cDNA cloning and expression of $bvPLA_2$ and mouse $sPLA_2$s

A full-length cDNA coding for the N-terminally processed form of $bvPLA_2$ was PCR-amplified using reverse-transcribed total RNA from the venom gland of honey bee as a template (S. Nakashima, K. Kitamoto and M. Arioka, unpublished work; details can be obtained from the corresponding author on request), and the following oligonucleotide primers: 5′-cgagttggagctgcag-ATAATATATCCGGAACGTT-3′ (forward); 5′-cgtgaaagcttTCA-TACTTGCGAAGATCGA-3′ (reverse; uppercase letters indicate the cDNA sequences coding for the two ends of the mature $bvPLA_2$ polypeptide; the *Pst*I and *Hin*dIII sites are underlined). The cDNA obtained from such an amplification reaction was digested with *Pst*I and *Hin*dIII, and ligated to the corresponding site of the pRSET-C vector. The resulting construct, pRSET-$bvPLA_2$, was transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Invitrogen), which were then induced to express $rbvPLA_2$ (recombinant $bvPLA_2$) as described previously [14]. After cell lysis, the insoluble fraction was pelleted by centrifugation, solubilized with 6 M guanidine/HCl (pH 7.8), and applied to a Ni^{2+}-nitrilotriacetate agarose column, which was utilized for $rbvPLA_2$ purification following the manufacturer's instructions. Purified $rbvPLA_2$ was renatured according to previously reported procedures [16,17].

Mouse group IB (accession no. AF162712), IIA (AF162712), V (AF162713) and X (AF166097) cDNAs were amplified by PCR using *Pfx* DNA polymerase and mouse cDNA libraries from adult pancreas (IB and IIA) or fetal brain (V and X) as templates. The following oligonucleotides were utilized as amplification primers: 5′-agaattcgccaccATGAAACTCCTTCTGCTGGCTGCTC-3′ and 5′-gcacagatctccACAGAATTTCCCGGTGTCAAGGTTT-3′ for the group IB $sPLA_2$; 5′-agaattcgccaccATGAAGGTCCTCCTGC-TGCTAGCAG-3′ and 5′-gcatagatctccGCATTTGGGCTTCTTC-CCTTTGCAA-3′ for the group IIA $sPLA_2$; 5′-gaattcgccacc-ATGAAGGGTCTCCTCACA-3′ and 5′-atgcagatctgcGCAGAG-GAAGTTGGGGTAATAC-3′ for the group V $sPLA_2$; 5′-gaattc-gccaccATGCTGCTGCTACTGCTG-3′ and 5′-atgcagatctgcAT-TGCACTTGGGAGAGTCCTTC-3′ for the group X $sPLA_2$. Capital letters correspond to the coding regions, and lower-case underlined sequences identify the *Eco*RI and *Bgl*II sites used for cDNA cloning. Amplified cDNA fragments were digested with *Eco*RI and *Bgl*II, and ligated to the pMT vector for expression in COS1 cells. The pMT vector is a derivative of pME18S (kindly provided by Dr K. Maruyama, Department of Virology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan), that was modified in this laboratory so as to allow the expression of recombinant proteins tagged at the C-terminus with HA (haemagglutinin) and with a stretch of six histidines (His_6; M. Arioka, unpublished work; details can be obtained from the corresponding author on request). Recombinant proteins expressed from this vector contain the sequence G(or A)DLYPYDVPDYASHHHHHH (the HA epitope is underlined) as a C-terminal extension. Individual $sPLA_2$ constructs were transfected into COS1 cells by lipofection. Equal volumes of culture supernatants, recovered 3 days after transfection, were examined by immunoblot analysis before being assayed for neuritogenic activity in PC12 cells. Immunoblot analysis was performed with the SuperSignal Substrate Western blotting kit (Pierce), using the anti-HA antibody (Roche) and a peroxidase-labelled anti-mouse IgG secondary antibody (PI-2000; Vector).

Site-directed mutagenesis

A PCR-based mutagenesis protocol was used to generate the H34A mutant of $bvPLA_2$. Using the pRSET-$bvPLA_2$ plasmid as a template, mutated DNA fragments were first amplified by PCR using the following pairs of vector-annealing and mutagenic oligonucleotides as primers: T7 (5′-TAATACGACTCACTA-TAGGG-3′)/mutant antisense (5′-GCACATGTCGGCGGTTCG-ACA-3′) and pRSET RV (5′-TAGTTATTGCTCAGCGGTGG-3′)/mutant sense (5′-TGTCGAACCGCCGACATGTGC-3′; where the underlined sequence corresponds to the His-to-Ala mutation). The two resulting PCR fragments were mixed, followed by a second amplification reaction, carried out with the T7 and pRSET

RV primers, to generate the His_6-tagged H34A mutant version of rbvPLA$_2$. The amplicon thus obtained was then digested with *Pst*I and *Hin*dIII and ligated to the corresponding site of pRSET C.

A similar strategy was employed to generate the H47A (group V) and H46A (group X) active-site mutants of mouse sPLA$_2$s. The mutagenic primers were: 5′-TGTCAGATGgcCGAtCGTTGT-3′ and 5′-ACAACGaTCGgcCATCTGACA-3′ for the H47A mutant; and 5′-TGCTACCACgcCGACTGCTGC-3′ and 5′-GCAGCAGTCGgcGTGGTAGCA-3′ for H46A mutant. Mutated nucleotides are indicated by lower-case letters, with the replaced (His-to-Ala) codons underlined.

A distinct PCR-based mutagenesis protocol was used to generate the HDAA and D107A mutants of p15. The pRSETC-p15 plasmid, previously utilized to express His_6-p15 and to produce the C38/54S and C90/102S mutants [14], was employed as a template for mutagenic PCR in combination with the following sets of oligonucleotide primers: for the HDAA mutation, 5′-TGCCACCGCGCTGCCTTCGGCTACCGC-3′ (forward) and 5′-AGAGGATAAGAAGTCGAAGCCGAATGG-3′ (reverse) primers; for the D107A mutation, 5′-GCCGTTGCCGCTATCTACTACGAG-3′ (forward) and 5′-TTTGCAGGCAGCTCTTGTGAAGAT-3′ (reverse) primers [where the underlined sequences indicate the His-57-to-Ala/Asp-58-to-Ala (HDAA) and the Asp-107-to-Ala (D107A) mutations, respectively]. All His_6-p15 mutant proteins, as well as wild-type His_6-p15 and His_6-Scp15, were expressed in *E. coli* BL21 (DE3) pLysS cells and purified by Ni^{2+}-affinity chromatography under denaturing conditions as described previously [14].

Phospholipase activity assays

Three different assays were used to measure phospholipase activity. The first assay employed [^{3}H]oleic acid-labelled *E. coli* membranes as a substrate [18]. Approx. 4.8×10^5 d.p.m. equivalents of [^{3}H]oleic acid-labelled *E. coli* membranes were incubated with 10 nM His_6-p15 in 100 μl reaction mixtures containing 25 mM Tris/HCl (pH 7.4) and various concentrations (0–100 mM) of $CaCl_2$ for 2 h at 37 °C. An equal volume of 1 mg/ml BSA was then added, mixed by vortexing, followed by centrifugation at 18 500 ***g*** for 3 min. Aliquots of the resulting supernatants (100 μl each) were mixed with 1 ml of scintillation cocktail [0.55 % 2,5-diphenyloxazole, 0.01 % 1,4-bis(4-methyl-5-phenyl-2-oxazoly)benzene in 33.3 % (v/v) Triton X-100 and 66.7 % (v/v) toluene] and counted by liquid scintillation. A second type of assay, carried out with a commercially available sPLA$_2$ assay kit (Cayman Chemical, catalogue no. 765001), based on the synthetic substrate diheptanoyl thiophosphorylcholine, was used routinely for monitoring phospholipase activity. A third assay system, employing the synthetic radioactively labelled glycerophospholipids 1-palmitoyl-2-[1-^{14}C]palmitoyl-L-α-PC (phosphatidylcholine), 1,2-di[1-^{14}C] palmitoyl-L-α-PC, 1-palmitoyl-2-[1-^{14}C]linoleyl-L-α-PC or 1-palmitoyl-2-[1-^{14}C]linoleyl-L-α-phosphatidylethanolamine (Amersham Biosciences), were used to determine the ester bond position specificity and substrate preferences of His_6-p15. Enzyme reactions were conducted at 30 °C for various lengths of time (10–30 min) in 150 μl of reaction mixtures containing 140 mM NaCl, 0.1 % Triton X-100, 15 mM $CaCl_2$, 20 mM Hepes/KOH (pH 8.0), 0.1 μCi of each radioactive, *sn*-2-position-labelled phospholipid, plus the corresponding unlabelled phospholipid (40 μM), and various amounts (5–10 nM) of His_6-p15. Assays conducted in the absence of Ca^{2+} (plus 2 mM EDTA) or in the presence of increasing concentrations of $CaCl_2$ (0.1–100 mM) were used to determine the Ca^{2+} dependence of His_6-p15. The pH optimum for activity was determined using 20 mM sodium acetate buffer for pH values ranging from 4.3 to 6.5, and 20 mM Hepes/KOH from pH 7 to 9. The dual-labelled phospholipid 1,2-di[1-^{14}C]palmitoyl-L-α-PC (0.2 μCi/reaction), along with unlabelled di-palmitoyl-L-α-PC (40 μM), was used to assay for lysophospholipase activity. After blockage with 0.1 M EDTA/2 M NaCl (150 μl), organic solvent extraction [19] and TLC fractionation [20], reaction products were visualized and quantified by Phosphorimaging as described previously [8].

PC12 cell culture and neurite outgrowth assay

Rat pheochromocytoma PC12 cells were maintained in DMEM (Dulbecco's modified Eagle's medium; high-glucose type; Invitrogen) supplemented with 5 % horse serum and 5 % fetal calf serum. Cells were passaged every 3–4 days and maintained at 37 °C in 10 % CO_2 in humidified air. In a typical neurite-induction experiment, PC12 cells were seeded in the growth medium at 4.5×10^3 cells/cm^2 in collagen type I-coated 24-well culture plates (Becton Dickinson), allowed to grow for 24 h, and then supplemented with each of the various protein and/or non-protein additives specified in the text. When the culture supernatants of COS1 cells transfected with each sPLA$_2$ construct were examined for the neuritogenic activity, they were adjusted to 5 % fetal calf serum and 5 % horse serum by 2-fold dilution with fresh DMEM containing 10 % horse serum, and were directly applied to the PC12 cell cultures. After 48 h, neurite outgrowth was quantified by taking five random photographs/well; cells bearing processes longer than the cell diameter were judged as positive.

Assay of phospholipase-mediated fatty acid release from PC12 cells

PC12 cells grown at 1.0×10^5 cell/cm^2 in 24-well culture plates were incubated for 24 h in the presence of [^{3}H]arachidonic acid (0.5 μCi/ml) or [^{3}H]oleic acid (0.5 μCi/ml) in 500 μl of DMEM containing sera. After washing three times with DMEM without serum, cells were treated with p15, Scp15 or rbvPLA$_2$ for the indicated times at 37 °C. Culture media were then recovered, and cells were solubilized in 250 μl of 2 % Triton X-100. Both culture media and cell lysates (250 μl each) were mixed with 5 ml of scintillation cocktail, followed by radioactivity measurement in a liquid scintillation counter. The fraction of total radioactive fatty acid released into the culture medium by phospholipase action was calculated by dividing total counts present in the medium by the sum of the counts measured in the medium and in the corresponding cell lysate; background radioactivity measured in phospholipase-unsupplemented, control incubations was subtracted from each datapoint.

RESULTS

Identification of p15 as a sPLA$_2$

The closest homologue of p15 is a putative open reading frame from *S. coelicolor* A3(2) (CAB38593) named Scp15 [15]. They are similar in size (15 and 14 kDa for the N-terminally processed forms of p15 and Scp15, respectively) and both have four cysteine residues at conserved positions. Similar to p15, recombinant Scp15 also displays a dose-dependent neurite-inducing activity in PC12 cells, indicating that both proteins share structural as well as functional features [15]. Further search of the nucleotide and protein sequence databases revealed another p15/Scp15

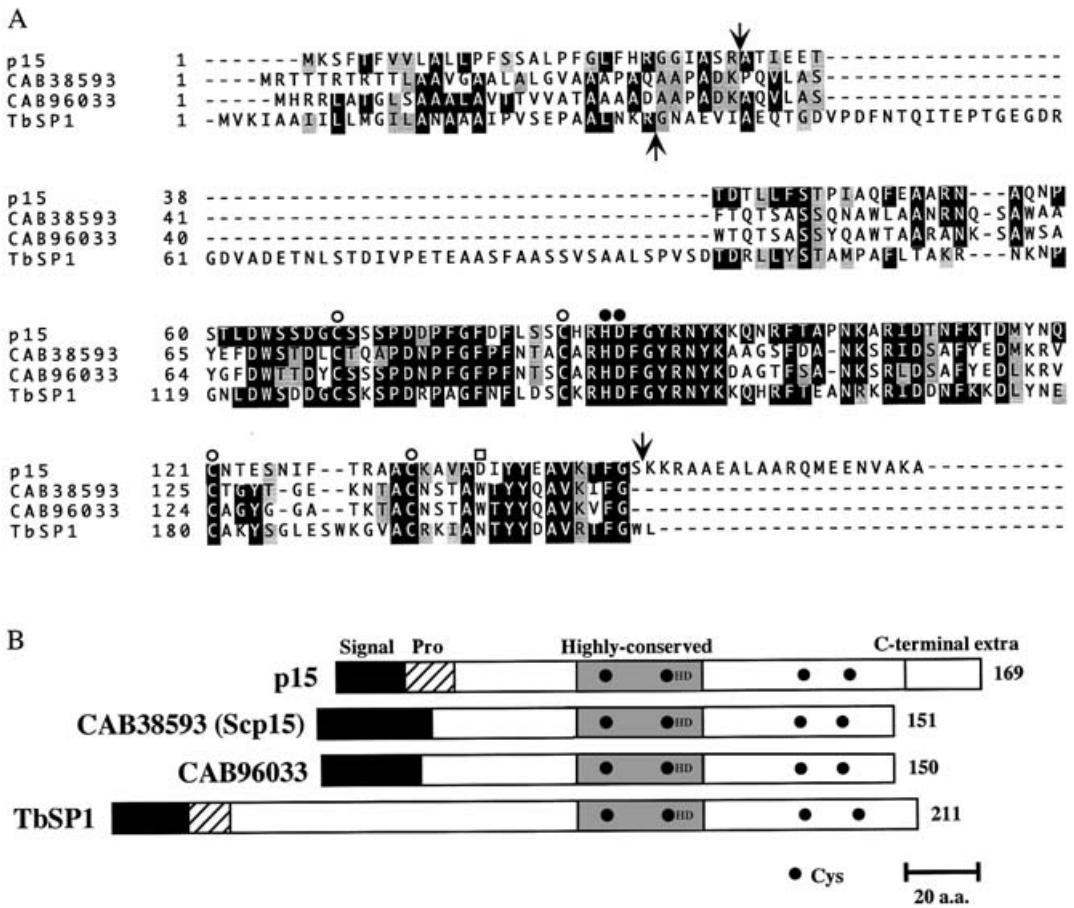

Figure 1 Comparative sequence analysis of p15 and of microbial sPLA$_2$s

(**A**) Alignment of p15 with its microbial homologues. Identical and similar amino acid residues are indicated by black and grey shading, respectively. The borders of experimentally determined (p15 and TbSP1) extra-sequences at the N- and C-termini are marked with arrows. The catalytic His-Asp dyad is indicated by closed circles. The four cysteine residues involved in the two linear disulphide bridges of p15 are indicated by open circles. D107A mutation is marked with an open square; see the text for details and for the species of origin of each sequence. As mentioned in the text, the amino acid sequences of CAB38593 and Scp15 from *S. coelicolor* A3(2), and E08479 from *S. violaceoruber* are identical. (**B**) Schematic representation of p15 and microbial sPLA$_2$s. Black and hatched boxes in the N-terminal region of p15 and TbSP1 indicate predicted secretion signal (pre-) and pro-peptide sequences, respectively. The central region containing the conserved His-Asp dyad (His-57 and Asp-58 in mature p15) that forms part of the catalytic centre of all PLA$_2$s known to date is enclosed in a grey box. The positions of cysteines (Cys-38, Cys-54, Cys-90 and Cys-102 in mature p15) are marked by dots. The total number of amino acid residues in each sequence is shown on the right.

homologue in *S. coelicolor* A3(2) (CAB96033) and one in *Streptomyces violaceoruber* (E08479), which has been identified recently as the first prokaryotic PLA$_2$ [11] (Figure 1A). Additional homologous sequences were found in other micro-organisms, such as the filamentous ascomycete *Tuber borchii*, where the TbSP1 polypeptide (AF162269; Figure 1A) has been recognized as the first Ca^{2+}-dependent sPLA$_2$ to be described in fungi [10]. As further shown in Figure 1(B), all these proteins contain either an experimentally documented (p15, TbSP1) or a putative (Scp15, CAB96033) N-terminal signal sequence. These observations prompted us to test whether p15 is a PLA$_2$, and whether neurite induction by p15 is correlated with its phospholipid-hydrolysing activity. As revealed by the results of the *in vitro* assays reported in Figure 2(A), we found that His$_6$-p15, the N-terminally His$_6$-tagged form of the recombinant p15 polypeptide produced in *E. coli*, indeed exhibits a Ca^{2+}-dependent lipid-hydrolysing activity toward [^{3}H]oleic acid-labelled *E. coli* membranes, with maximum activity in the presence of 1–2 mM $CaCl_2$. A Ca^{2+}-dependent phospholipase activity of both p15 and Scp15 was also revealed by independent *in vitro* assays employing arachidonoyl thio-phosphocholine as a substrate (results not shown). Similar results, albeit with an approx. 10-fold higher optimum Ca^{2+} concentration, were obtained in a third set of assays conducted on His$_6$-p15 at an optimum pH of 8.0, using synthetic di-palmitoyl-L-α-PC derivatives carrying 1-^{14}C-labelled palmitoyl groups at defined stereochemical positions. As shown in Figure 2(B), [^{14}C]PA (palmitic acid), rather than 2-[^{14}C]palmitoyl-lysoPC, was the only product released from 1-palmitoyl-2-[^{14}C]palmitoyl-L-α-PC, thus indicating that p15 is absolutely specific for the *sn*-2 ester bond position of glycerophospholipids and is devoid of any phospholipase A$_1$ activity. When 1,2-di[^{14}C]palmitoyl-L-α-PC was used as a substrate (Figure 2C), nearly equivalent amounts of [^{14}C]PA and 1-[^{14}C]palmitoyl-lysoPC were produced: in a 10 min reaction (Figure 2C, lane 2), 20 and 17.8 % of the original 1,2-di[^{14}C]palmitoyl-L-α-PC counts were converted into PA and 2-lysoPC, while the corresponding amounts generated in a 30 min reaction (Figure 2C, lane 3) were 33.4 and 30.4 %, respectively. These results clearly indicate that p15 has no lysophospholipase activity. Furthermore, additional assays (results not shown) revealed that the

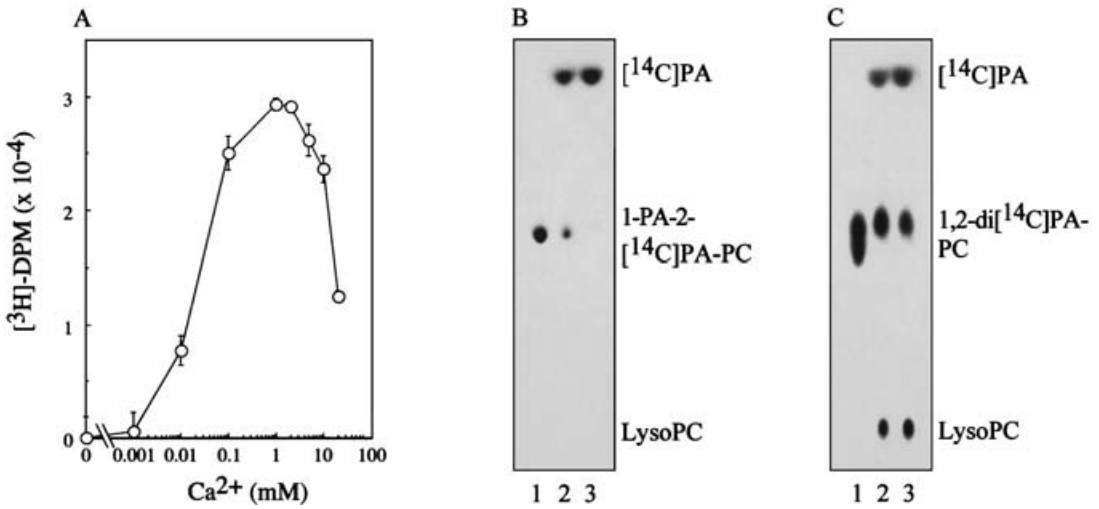

Figure 2 Identification of p15 as a $sPLA_2$

(**A**) [^{3}H]oleic acid release from labelled *E. coli* membranes, catalysed by His_6-p15 (10 nM) in the presence of the indicated concentrations of $CaCl_2$ (see the Experimental section for details). (**B**) *sn*-2 position-specific hydrolysis of a synthetic phospholipid substrate by p15. Hydrolysis of 1-palmitoyl-2-[1-^{14}C]palmitoyl-L-α-PC (20 min, 30 °C) in the presence of 5 nM (lane 2) or 10 nM (lane 3) His_6-p15, followed by TLC fractionation and Phosphorimager visualization of the residual radioactive substrate (1-PA-2-[^{14}C]PA-PC) and of radiolabelled hydrolysis products. The migration position of PA and the expected migration position of 1-palmitoyl-lysoPC are indicated, but note the absence of any radiolabelled lysoPC among the products of p15-catalysed 1-palmitoyl-2-[^{14}C]palmitoyl-L-α-PC hydrolysis. The results shown are from a representative lipolytic assay; a control reaction lacking His_6-p15 is shown in lane 1. (**C**) Hydrolysis of 1,2-di[1-^{14}C]palmitoyl-L-α-PC (1,2-di[^{14}C]PA-PC) upon incubation for 10 min (lane 2) or 30 min (lane 3) in the presence of 10 nM His_6-p15; a control reaction lacking His_6-p15 is shown in lane 1. Reaction conditions, TLC fractionation, Phosphorimager visualization and hydrolysis product abbreviations are the same as in (**B**). Note the production at both time points of equivalent amounts of [^{14}C]PA and [^{14}C]lysoPC, indicating the lack of lysophospholipase activity in p15 (see the text for details on these assays and on the quantification of radiolabelled hydrolysis products).

activity ratio measured in separate reactions containing either 1-palmitoyl 2-[^{14}C]linoleyl- or 1-palmitoyl 2-[^{14}C]palmitoyl-L-α-PC as substrate was 1.05 ± 0.25, and the corresponding ratio for the 1-palmitoyl-2-[^{14}C]linoleyl-PC/1-palmitoyl 2-[^{14}C]linoleyl-phosphatidylethanolamine pair was 0.69 ± 0.2. Altogether, these results demonstrate that p15 is an authentic PLA_2, lacking any strict specificity for the fatty acyl or polar head group moieties of its phospholipid substrates.

Fatty acid-release from PC12 cells by p15 and Scp15

The ability of exogenously added p15 or Scp15 to liberate fatty acids from live PC12 cells was next investigated by adding either of the two recombinant proteins to the culture medium of PC12 cells that had been pre-labelled with [^{3}H]arachidonic or [^{3}H]oleic acid, followed by the measurement of radioactivity in culture supernatants as well as in total cell lysates. As shown in Figure 3(A), p15 catalysed fatty acid release in a time-dependent manner. In keeping with the previously documented ability of p15 to act on variously substituted phospholipid substrates, arachidonic and oleic acids were released with nearly equal efficiencies, thus further supporting the notion that p15, like other $sPLA_2$s, has no strict fatty acyl selectivity (Figures 3B and 3C). A similar lack of fatty acid selectivity was observed for Scp15, even though the extent of fatty acid release by p15 was slightly higher than that by Scp15, which is consistent with our previous observation that p15 is a more potent neurite inducer than Scp15 [15]. In both cases, the release of fatty acids was not due to PLA_2-induced cell lysis, because the amount of total lactate dehydrogenase activity (a cytosolic marker) in the culture medium supernatants was usually very low and essentially the same in control and p15- or Scp15-treated PC12 cells (results not shown).

The PLA_2 activity of p15 is required for neuritogenesis

Next, we asked whether the catalytic activity of p15 is required for neurite formation. For this purpose, the mutant versions of His_6-p15, HDAA, D107A, C38/54S and C90/102S, were generated (Figure 4A), and tested for their catalytic and neurite-inducing activities. In the HDAA mutant, the catalytic His-57–Asp-58 dyad (amino acid residues are numbered from the N-terminus of the mature p15 protein) was replaced by two alanine residues. Similarly, in the D107A mutant, the Asp at position 107, which corresponds to the critical Asp-99 residue of mammalian $sPLA_2$s [23], was replaced by an alanine. In each of the C38/54S and C90/102S mutants two pairs of cysteines, previously shown to be involved in disulphide bond formation [13], were simultaneously replaced by serine residues. As revealed by PLA_2 activity assays conducted on each of these mutants with the synthetic substrate diheptanoyl thiophosphorylcholine (Figure 4B), HDAA and C38/54S exhibited essentially no enzymic activity, the D107A mutant retained approx. 60% of wild-type activity, while, unexpectedly, the C90/102S mutant exhibited a PLA_2 activity 1.4-fold higher than that of wild-type His_6-p15 (Figure 4B). When fatty acid-release from live PC12 cells by wild-type or mutant p15 proteins was examined (Figure 4C), a substantial reduction of both arachidonate and oleate release was observed in the case of the HDAA and C38/54S mutants. In contrast, only a modest decrease or nearly wild-type levels of fatty acid releasing activity were measured for the C90/102S and D107A mutants, respectively. Finally, as shown in Figure 4(D), we found that the neurite-outgrowth-inducing activity

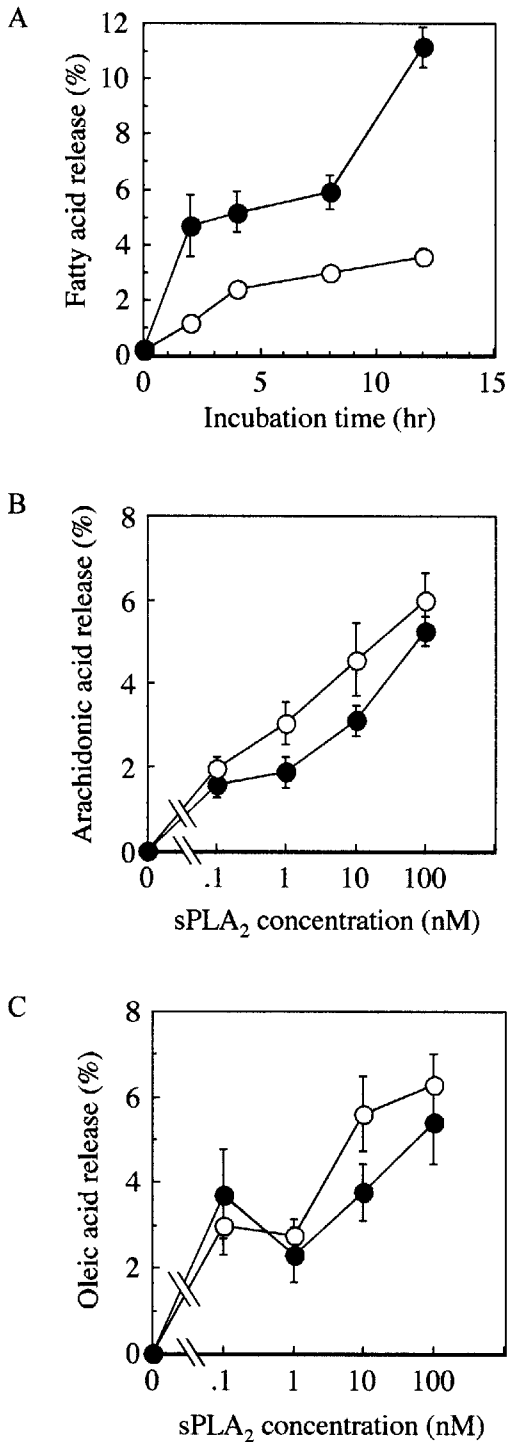

Figure 3 p15-mediated fatty acid release from PC12 cells

(**A**) PC12 cells, pre-labelled with [^{3}H]arachidonic acid (○) or [^{3}H]oleic acid (●), were incubated with 10 nM His_6-p15 (added directly to the culture medium) for the times indicated. The culture medium was then recovered and the cells lysed in 2% Triton X-100; for each data point, radioactivity was measured both in the culture medium and the corresponding cell lysate (see the Experimental section for details). Radiolabelled fatty acid release into the culture medium is expressed as the percentage of total (released and cell-associated) radioactivity, calculated by dividing the counts present in the medium by the sum of the counts measured in the medium and in the corresponding cell lysate; background radioactivity measured in His_6-p15-unsupplemented control incubations was subtracted from each data point. [^{3}H]Arachidonic acid or [^{3}H]oleic acid release from pre-labelled PC12 cells, incubated for 4 h at 37 °C in the presence of increasing concentrations of His_6-p15 (○) or Scp15 (●) are shown in (**B**) and (**C**), respectively; experimental conditions, data presentation and analysis are the same as in (**A**).

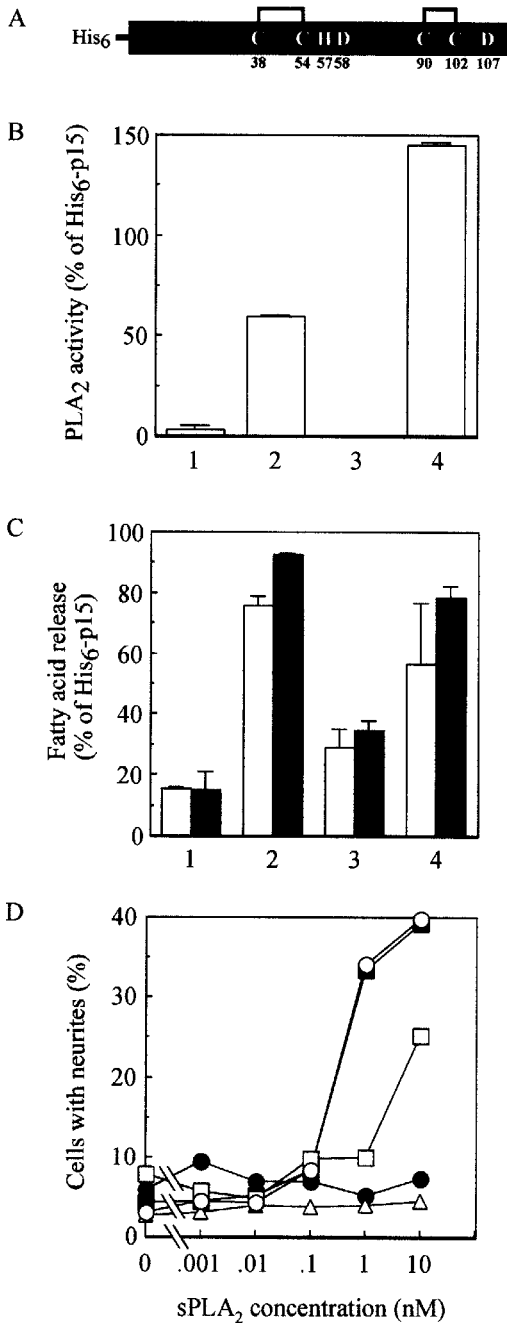

Figure 4 PLA_2 and neurite-inducing activities of p15 mutant forms

(**A**) Schematic representation of the arrangement of p15 amino acid residues targeted by site-directed mutagenesis: the catalytic His-Asp dyad (positions 57 and 58) was changed into an Ala pair in the HDAA mutant; the Asp residue at position 107 was turned into an Ala to generate the D107A mutant. The two linear disulphide bridges (Cys-38–Cys-54, Cys-90–Cys-102) destroyed in the C38/54S and C90/102S mutants are indicated. (**B**) The PLA_2 activity of the HDAA (1), D107A (2), C38/54S (3) and C90/102S (4) p15 mutants (each at 10 nM) was measured with the diheptanoyl thiophosphorylcholine-based assay (see the Experimental section for details). The enzyme activities of the various mutants are given as percentages of wild-type His_6-p15 activity. (**C**) [^{3}H]Arachidonic acid (open bars) or [^{3}H]oleic acid (closed bars) release from pre-labelled PC12 cells by the HDAA (1), D107A (2), C38/54S (3) and C90/102S (4) p15 mutants (each at a 10 nM). Incubation conditions (4 h at 37 °C) and data analysis are the same as those specified in the Figure 3 legend; data for the various mutants are given as the percentage of fatty acid release promoted by wild-type His_6-p15. (**D**) Neurite outgrowth induced by a 48 h treatment of PC12 cells with the indicated concentrations of wild-type His_6-p15 (○), or the HDAA (△), D107A (■), C38/54S (●) and C90/102S (□) mutants. Cells with neurite extensions longer than the cell diameter were judged as positive; the number of such cells is given as the percentage of the total cell number.

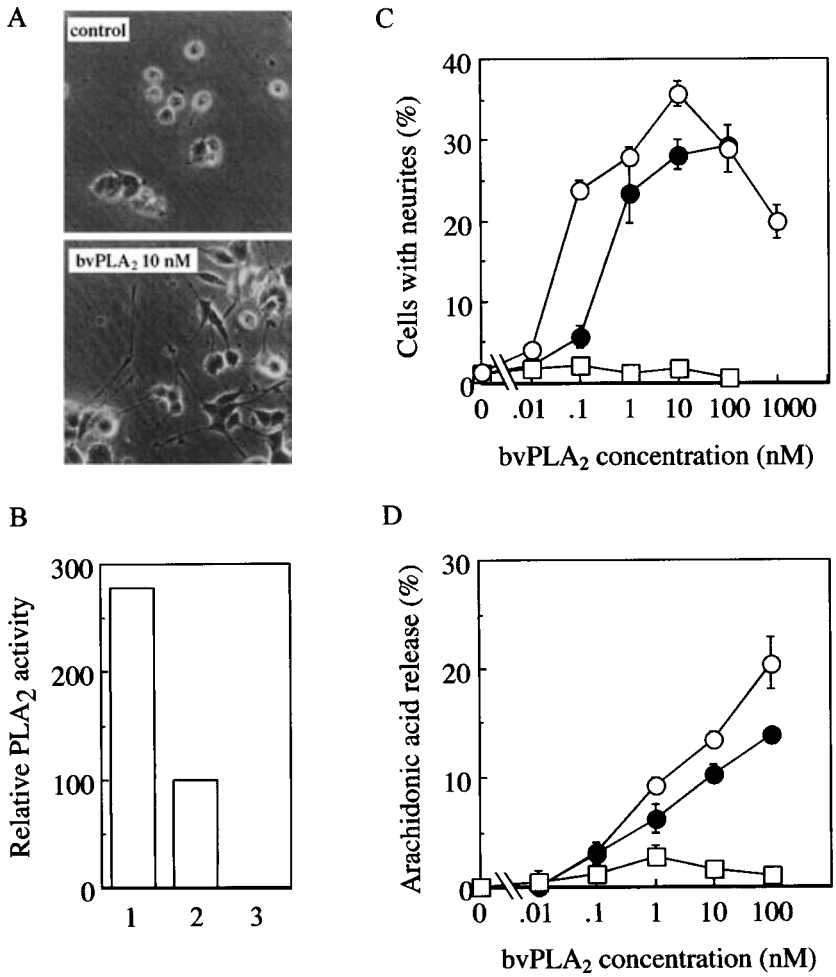

Figure 5 PLA_2 activity-dependent neuritogenesis induced by natural and recombinant $bvPLA_2$

(**A**) Untreated (control) or natural $bvPLA_2$-treated (10 nM, 48 h) PC12 cells. (**B**) The PLA_2 activities of natural $bvPLA_2$ (1), $rbvPLA_2$ (2) and the H34A $bvPLA_2$ mutant (3) were measured by diheptanoyl thiophosphorylcholine-based assay (see the Experimental section for details); activities are given as relative values, normalized with respect to the activity of $rbvPLA_2$. (**C**) Neurite outgrowth induced by a 48 h treatment of PC12 cells with the indicated concentrations of natural $bvPLA_2$ (○), $rbvPLA_2$ (●) or the H34A $bvPLA_2$ mutant (□); neurite-positive cell assessment and data presentation are the same as those specified for Figure 4(D). (**D**) [^{3}H]Arachidonic acid release from pre-labelled PC12 cells incubated with the indicated concentrations of natural $bvPLA_2$ (○), $rbvPLA_2$ (●) or the H34A $bvPLA_2$ mutant (□). Incubation conditions (4 h at 37 °C) and data analysis are the same as those specified for Figure 3; arachidonic acid released by the various $bvPLA_2$s is given as the percentage of the total amount of [^{3}H]arachidonic acid (released plus cell-associated) incorporated by the cells.

of wild-type and mutant p15 proteins matches quite closely their ability to release fatty acids from live PC12 cells. The HDAA and C38/54S mutations completely abolished neuritogenesis, while no loss of neurite inducing activity, or an approx. 40 % reduction (at 10 nM) of neuritogenic activity compared with wild-type p15 were observed in the case of the D107A and C90/102S mutants, respectively. Therefore, with the sole exception of the C90/102S mutant which displayed a higher PLA_2 than neurite-inducing activity, there is a fairly good correlation between PLA_2, and fatty acid releasing- and neurite-inducing activities, implying that the catalytic activity of p15 is essential for neuritogenesis.

Phospholipase activity-dependent neuritogenesis induced by $bvPLA_2$

If p15 induces neurite outgrowth through its PLA_2 activity, one would expect that other $sPLA_2$s should also be capable of inducing neurites. This possibility was examined by testing the neuritogenic activity of a group III $sPLA_2$ from honey bee venom ($bvPLA_2$). As shown in Figure 5(A), a commercially available preparation of natural $bvPLA_2$ potently induced neurites in PC12 cells; at a concentration as low as 0.1 nM, which was almost completely ineffective for either p15 or Scp15, $bvPLA_2$ potently induced neurites (Figure 5B). Moreover, unlike p15 or Scp15, $bvPLA_2$ exhibited cytotoxicity at concentrations higher than 10 nM, which is probably the reason for the reduction in neurite-inducing activity observed at these $bvPLA_2$ concentrations.

To find out whether the PLA_2 activity of $bvPLA_2$ is required for neurite induction, we isolated the $bvPLA_2$ cDNA from the venom gland of honey bee, and produced histidine-tagged, wild-type and mutant (H34A) versions of the honey bee enzyme in *E. coli*. In the H34A mutant, the catalytic His at position 34 [21] was replaced by an Ala residue. Both forms of the recombinant $bvPLA_2$ enzyme were almost completely insoluble in *E. coli* and were purified by inclusion-body solubilization,

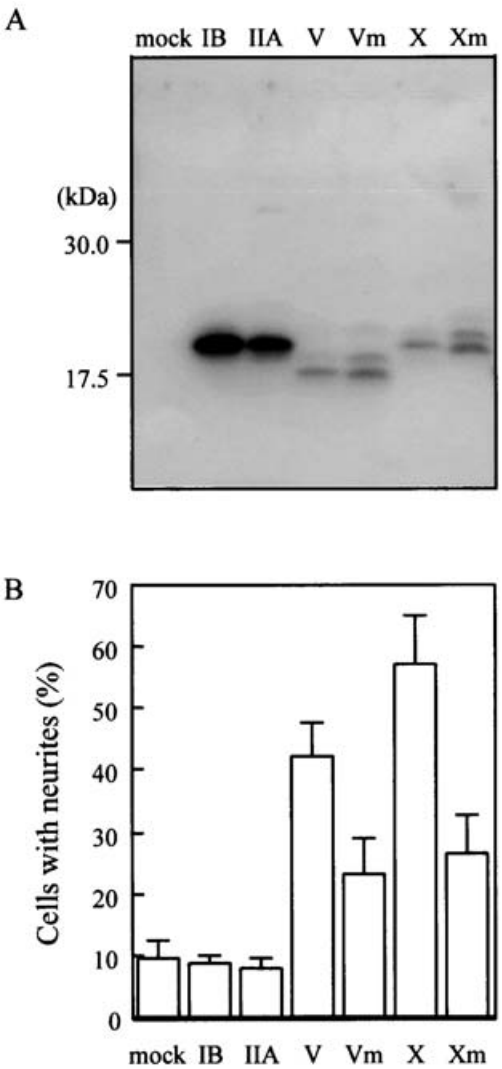

Figure 6 Neurite induction activity of mammalian sPLA$_2$s

Immunoblot analysis (**A**) and neuritogenic activity assay (**B**) of the supernatants of COS1 cells expressing recombinant (HA-tagged) group IB, IIA, V and X mouse sPLA$_2$s, and the active-site mutants of group V (Vm) and X (Xm) enzymes (see the Experimental section for details). Equal amounts of each culture supernatant were subjected to the standard PC12 neurite-outgrowth assay, as specified for Figure 4(D).

followed by Ni^{2+}-agarose chromatography under denaturing conditions and subsequent renaturation. Even though the enzymic activity of recombinant wild-type bvPLA$_2$ (rbvPLA$_2$) towards the synthetic substrate diheptanoyl thiophosphorylcholine was significantly lower than that of the natural honey bee venom PLA$_2$ (Figure 5B), both enzymes induced neurite outgrowth in PC12 cells in a dose-dependent manner (Figure 5C). Again, PLA$_2$ activity correlated quite well with both neurite-inducing and arachidonic acid-releasing activities (Figure 5D). Instead, the catalytically inactive rbvPLA$_2$ H34A mutant completely lacked neurite-inducing and fatty acid-releasing activities, thus further supporting the existence of a strong correlation between neurite-induction and PLA$_2$ activity.

Induction of neurite outgrowth by mammalian sPLA$_2$s

We next wished to find out whether mammalian sPLA$_2$s also display neuritogenic activity. To address this question, we cloned mouse group IB, IIA, V and X sPLA$_2$s and expressed them as C-terminally HA epitope-tagged proteins in COS1 cells, whose culture supernatants were assayed for recombinant sPLA$_2$ expression and neurite-induction activity. As shown in Figure 6(A), group IB and IIA sPLA$_2$s were both expressed at fairly high levels, while expression of the group V and X enzymes was barely detectable. The PLA$_2$ activities, examined with the [^{3}H]oleic acid-labelled *E. coli* membrane assay, of culture supernatants containing IB and IIA sPLA$_2$s were higher than those containing group V or X, which paralleled the amount of each recombinant sPLA$_2$ produced (results not shown). However, when the same culture supernatants were assayed for neurite-induction activity, a prominent neuritogenic effect was observed with culture supernatants containing group V and X, but not group IB and IIA, sPLA$_2$s (Figure 6B). As further shown in Figure 6(B), culture supernatants containing the catalytic mutant forms of group V and X sPLA$_2$s (respectively, H47A and H46A, in which the active-site His was replaced by an Ala residue) elicited a substantially reduced neuritogenic effect. Although the lack of a neuritogenic effect by group IB and IIA sPLA$_2$s was somewhat unexpected (see the Discussion), the impairment of neuritogenic activity observed with group V and X mutants indicates once again that PLA$_2$ activity is essential for the neurite outgrowth response of PC12 cells.

Calcium-dependent neuritogenic activity of exogenously supplied PLA$_2$ hydrolysis products

It has been reported that arachidonic acid formation is involved in CAM-dependent neurite outgrowth both in PC12 cells and in cerebellar neurons [4]. In fact, homophilic binding of CAMs initiates a signalling cascade that, through the activation and tyrosine phosphorylation of fibroblast growth factor receptor, leads to the production of arachidonic acid via diacylglycerol lipase-catalysed diacylglycerol hydrolysis. Arachidonic acid thus produced is thought to promote the opening of L- and/or N-type Ca^{2+} channels and to induce neurite outgrowth through as yet unknown mechanisms. Furthermore, the direct addition of arachidonic acid to the culture medium of cerebellar neurons or PC12 cells grown on a fibroblast monolayer also stimulated neurite outgrowth. Since neurite outgrowth induction by either microbial (p15, Scp15) or animal (bvPLA$_2$) sPLA$_2$s, but not NGF-induced neuritogenesis, has been found to be sensitive to the L-type Ca^{2+} channel inhibitor nicardipine (Figure 7A) as well as to nifedipine and nimodipine (results not shown), it was conceivable to imagine that sPLA$_2$-induced neuritogenesis might be mediated by an arachidonic acid-dependent mechanism. This possibility was tested by examining the possible neuritogenic effect of exogenously supplied arachidonic acid, directly added to the culture medium of PC12 cells, in the presence or absence of p15. As shown in Figure 7(B), arachidonic acid, even though it was readily incorporated by PC12 cells (see Figure 3), did not induce neurites or enhance p15-dependent neuritogenesis in our assay system. Also without effect was the diacylglycerol lipase inhibitor RHC-80267, which blocks CAM-induced neuritogenesis by interfering with arachidonic acid release [4] (results not shown).

Having found that arachidonic acid release itself is not a determining factor in PC12 cell neuritogenesis, we asked whether bioactive derivatives of arachidonic acid, such as prostaglandins or leukotrienes, might be involved in sPLA$_2$-induced neurite outgrowth. The effect on PC12 neuritogenesis of inhibitors of COX (cyclo-oxygenase) and LOX (lipoxygenase), two key enzymes of arachidonic acid metabolism, was thus examined. As shown in Figure 7(C), neither aspirin nor indomethacin, inhibitors

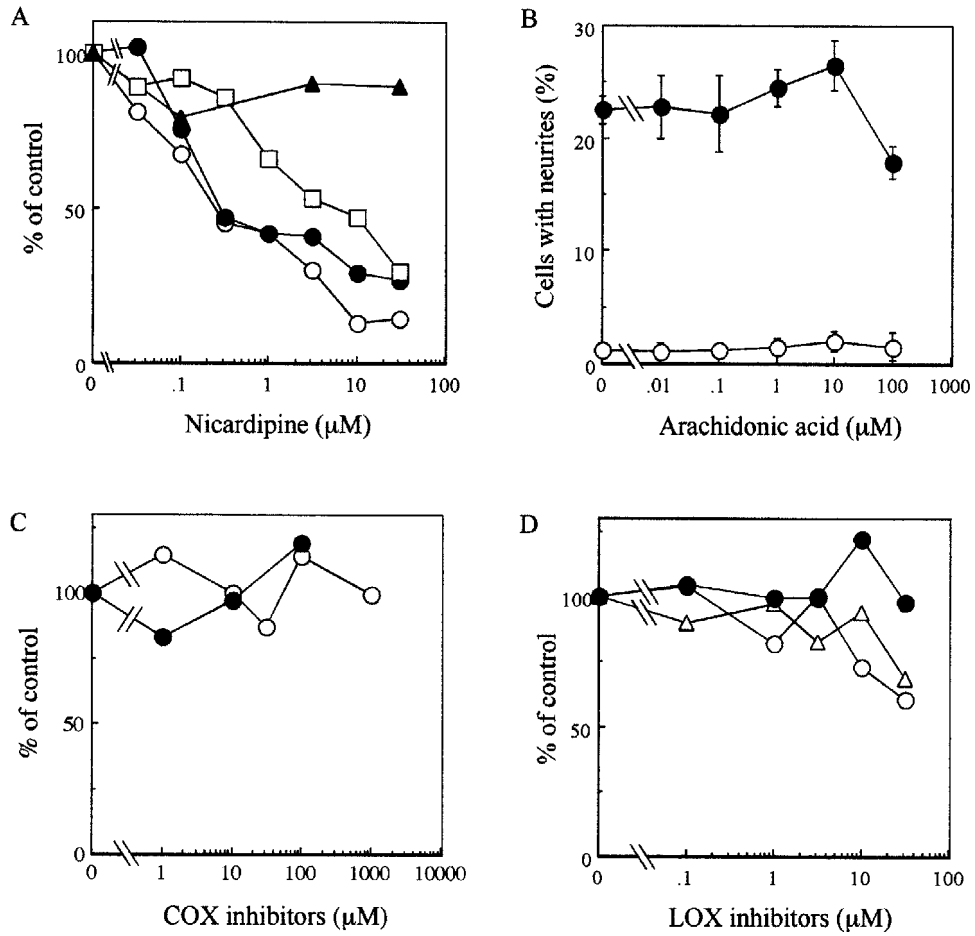

Figure 7 Effect of L-type Ca^{2+} channel and eicosanoid biosynthesis inhibitors and of exogenously supplied arachidonic acid on PC12 cells neuritogenesis

(**A**) PC12 cells were treated for 48 h with His_6-p15 (10 nM; ○), Scp15 (100 nM; ●), natural bvPLA$_2$ (10 nM; □) or NGF (50 ng/ml; ▲) in the presence of the indicated concentrations of the L-type Ca^{2+} channel inhibitor, nicardipine. Data are expressed as the percentage of neurite-positive cells at various concentrations of nicardipine, relative to the number of such cells measured in the absence of nicardipine. (**B**) Effect of the indicated concentrations of arachidonic acid on neurite outgrowth from PC12 cells incubated for 48 h in the absence (○) or presence (●) of 1 nM p15. Data are the percentage of neurite-positive cells at various concentrations of arachidonic acid. The effects on p15 (10 nM)-induced neuritogenesis of the COX inhibitors aspirin (○) and indomethacin (●), and of the LOX inhibitors NDGA (○), AA-861 (△) and baicalein (●), are shown in (**C**) and (**D**), respectively. Data are the percentage of neurite-positive cells at the indicated concentrations of the various drugs, relative to the number of such cells measured in the absence of drug supplementation. Neurite-positive cell assessment was done as described for Figure 4(D).

of COX-1 and COX-1/2 respectively, had any detectable effect on p15-induced neuritogenesis, even though both drugs (at equivalent or even lower concentrations) were previously shown to inhibit kainic acid-induced prostaglandin E_2 generation in mouse cortical neurons [22]. Also without any effect were NDGA, an inhibitor of both 5- and 12-LOX, the 5-LOX inhibitor AA-861, and the 12-LOX inhibitor baicalein, which was previously shown to block NGF-induced neurite outgrowth in PC12 cells [23] (Figure 7D). Altogether, these results indicate that arachidonic acid and its bioactive metabolites neither mimic nor affect sPLA$_2$-induced neuritogenesis.

We then focused on a possible pro-neuritogenic role of lysophospholipids, the other product of PLA$_2$-catalysed phospholipid hydrolysis. We found that 2-lysoPC, but no other 2-lysophospholipids, such as lysophosphatidylinositol, lysophosphatidylethanolamine, lysophosphatidylserine and lysophosphatidic acid, nor sphingosylphosphorylcholine (also known as lysosphingomyelin), induced neurite outgrowth when added to the culture medium of PC12 cells at 100 μM (Figure 8A). The platelet-activating factor receptor agonist mcPAF also did not exert any neuritogenic effect (Figure 8A). As shown in Figure 8(B), the neuritogenic response induced by lysoPC was dose-dependently blocked by nicardipine to the same extent as nicardipine inhibition of sPLA$_2$-induced neuritogenesis (cf. 7A and 8B). This suggests that 2-lysoPC (either exogenously added or generated *in situ* by sPLA$_2$-catalysed PC hydrolysis) acts through an L-type Ca^{2+} channel-dependent mechanism. As in the case of sPLA$_2$-promoted neurite outgrowth, no synergistic enhancement of lysoPC-induced neuritogenesis was produced by the co-addition of 100 μM arachidonic acid (results not shown).

DISCUSSION

We have shown here that a secreted fungal protein named p15, a potent inducer of neurite outgrowth in PC12 cells, is an sPLA$_2$ capable of releasing fatty acids from membrane phospholipids of live PC12 cells. Neurite induction by p15 requires its PLA$_2$ activity. In fact, the two mutants with the most strongly reduced phospholipase activity (HDAA and C38/54S) also displayed the strongest reduction in neurite-induction activity. The possibility

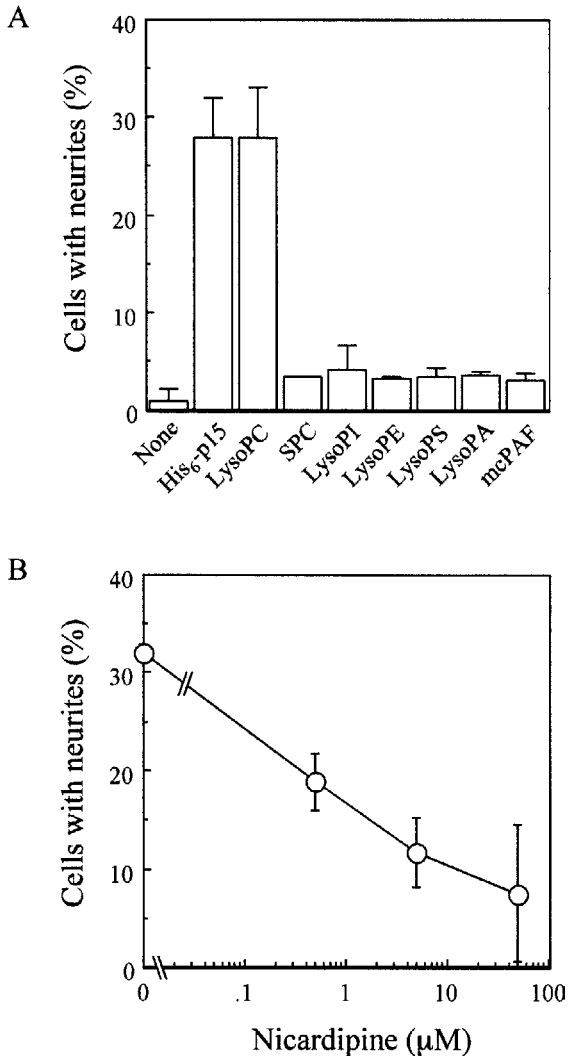

Figure 8 Neurite-induction activity of 2-lysoglycerophospholipids and other phospholipid derivatives

(**A**) PC12 cells assay of the neuritogenic activity of the indicated 2-lysophospholipids, sphingosylphosphorylcholine (SPC; each at 100 μM) and mcPAF (10 μM). Mock-treated PC12 cells (None) and cells incubated with 100 nM recombinant p15 (His_6-p15) were used as negative and positive controls, respectively. Neurite-positive cell assessment was done as described for Figure 4(D). LysoPI, lysophosphatidylinositol; LysoPE, lysophosphatidylethanolamine; LysoPS, lysophosphatidylserine; LysoPA, 1-oleyl-*sn*-glycero-3-phosphate. (**B**) Effect of the indicated concentrations of the L-type Ca^{2+} channel inhibitor nicardipine on lysoPC (100 μM)-induced neurite outgrowth.

that the introduced mutations may have altered other properties of p15, besides catalytic activity, cannot be ruled out completely. However, the fairly close matching between *in vitro* phospholipid hydrolysis, fatty acid release from PC12 cells and neuritogenic activities of wild-type and mutant forms of p15 strongly suggests that PLA_2 activity (especially towards choline polar-head-group phospholipids; see below) is the primary if not the sole determinant of p15-induced neuritogenesis. Further support to the notion that PLA_2 activity is absolutely required for neurite induction was provided by the observation that $sPLA_2$s from either closely or distantly related organisms, for example Scp15 from *S. coelicolor* A3(2), TbSP1 from *T. borchii* ([8], and M. Arioka and S. Ottonello, unpublished work), wild-type $bvPLA_2$ and mouse group V and X $sPLA_2$s, but not their active site mutants, also elicited neuritogenesis.

We have found that $sPLA_2$-, but not NGF-induced neuritogenesis is blocked by L-type Ca^{2+}-channel inhibitors. This finding is reminiscent of the results of previous studies showing that CAM- but not NGF-mediated neurite outgrowth depends on the activity of L- and N-type Ca_2^+ channels and is accompanied by arachidonic acid release [4]. At variance with such studies, however, we find that exogenously supplied arachidonic acid (which was effectively incorporated by PC12 cells and readily released from them upon p15 action) neither caused neurite outgrowth nor stimulated p15-induced neuritogenesis. Also, neither COX nor LOX inhibitors were capable of preventing neurite outgrowth induction by p15. In contrast, 2-lysoPC, but not other lysophospholipids (nor platelet-activating factor), potently induced neurites in a nicardipine-sensitive manner, strongly suggesting that lysoPC triggers neurite outgrowth through the same mechanism underlying $sPLA_2$-induced neuritogenesis. The striking lysophospholipid specificity of this effect might explain the inability of group IB and IIA $sPLA_2$s, but not of group V and X enzymes, to promote neurite outgrowth. The latter two enzymes, in fact, have been shown to display a much higher specific activity (up to 60-fold) toward phosphatidylcholine compared with group IB and IIA $sPLA_2$s [24]. Indeed, at variance with their lack of neurite induction activity on intact PC12 cells, recombinant group IB and IIA enzymes were both catalytically active when assayed on heat-killed [^{3}H]oleic acid-labelled bacterial cells, which are known to contain mainly phosphatidylethanolamine and anionic phospholipids, but not PC, among their surface lipid constituents (results not shown). So, even though other features of this set of mammalian $sPLA_2$s (e.g. their ability to interact with PC12 cells, either non-specifically or through specific receptors) may contribute to their differential ability to induce neurite outgrowth, it appears that a high hydrolytic activity towards PC and the consequent production of adequate amounts of lysoPC represents a key requirement for their neuritogenic effect. These data also suggest that different groups of mammalian $sPLA_2$s may be involved in profoundly different responses. For example, a type II $sPLA_2$ (presumably belonging to the same IIA group we find to be inactive in neurite induction) was reported to be involved in degranulation of, and catecholamine secretion by, NGF-treated neuronally differentiated PC12 cells [25].

How does lysoPC induce neurite outgrowth? This PC derivative has been shown to be specifically involved in a variety of cellular responses such as activation of phospholipase C and protein kinase C, increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and activation and inhibition of MAPK [26]. Interestingly, we found previously that an activation of the Src-Ras-MAPK cascade, which is known to occur through membrane depolarization, is induced upon p15 treatment, whereas the expression of a dominant-negative form of Ras or the addition of the MAPK kinase inhibitor PD98059 blocks neuritogenesis elicited by p15 [13,14]. At least some of the effects of lysoPC are likely to be mediated by the recently identified G protein-coupled lysoPC receptors G2A and GPR4 [27,28], which in response to lysoPC induce the release of calcium ions from intracellular stores through a G_i-dependent mechanism. We do not know, at present, whether the neuritogenic effect of lysoPC is mediated by G2A or GPR4 receptors, or by another type of receptor. It should be noted, however, that such an effect takes place at lysoPC concentrations (10–100 μM) that are well above the apparent K_d values of these receptors for lysoPC (65–160 nM), and that lysoPC, also at micromolar concentrations, exerts neurotrophic effects on cerebellar granule neurons *in vitro* and on hippocampal neurons *in vivo* [29]. Various explanations can be imagined for

this concentration discrepancy; including, for example, the sequestration by albumin or other serum proteins (present in the culture medium) of lysoPC, which would then become unavailable for receptor interaction and response activation. Indeed, the addition of an excess of BSA to immortalized MCF10A cells blocked the $[Ca^{2+}]_i$ increase induced by lysoPC upon interaction with the GPR4 receptor [28].

At variance with the neurite-inducing activity of $sPLA_2$s demonstrated in this study, which is reminiscent of the action(s) of neurotrophic factors (such as NGF and basic fibroblast growth factor) on PC12 cells, it has been reported that mammalian group IB and IIA $sPLA_2$s can promote apoptotic cell death in primary cultures of rat cortical neurons [30,31]. The same effect, however, was also produced by an anti-$sPLA_2$ receptor antibody, as if receptor binding itself, rather than phospholipase activity, were chiefly responsible for cell-death induction in this system [32]. On a similar note, the OS2 but not the OS1 $sPLA_2$ from taipan snake venom caused extensive histological damage when co-injected with glutamate into rat brain, and the same enzyme was found to increase glutamate-induced cell death and intracellular Ca^{2+} concentration, along with an enhanced arachidonic acid mobilization, in primary neuronal cell cultures [33,34]. A glutamate-independent cell-death effect, involving the activation of a cytosolic PLA_2, arachidonic acid release, platelet-activating factor production and induction of COX-2, was also reported to occur in neuronal cell cultures exposed to neurotoxic concentrations of the OS2 phospholipase [35]. Whether or not these neurotoxic and pro-apoptotic effects of $sPLA_2$s are relevant to our results is presently unclear. It is likely, however, that different $sPLA_2$s can perform distinct functions depending on their biochemical properties, dosage and mode of delivery, and on the particular cellular context (e.g. neuronal cell cultures versus undifferentiated PC12 cells) in which they are examined. Following up to this initial demonstration of a Ca^{2+}- and lysoPC-dependent differentiation role of $sPLA_2$s, further studies will be needed to gain a more detailed understanding of the mode of action and of the full complement of physiological roles played by these enzymes in mammalian cells, especially those in the nervous system.

This work was supported by a Grant-in-Aid for Scientific Research (no. 12460041 to K. K. and no. 14560058 to M. A.) from the Ministry of Education, Science, Sports and Culture of Japan, and by grants from the Ministry of Education, University and Research of Italy (FIRB Program to A. B.) and from the Regione Emilia Romagna (to S. O.).

REFERENCES

1 Kater, S. B. and Mills, L. R. (1991) Regulation of growth cone behavior by calcium. J. Neurosci. **11**, 891–899

2 Doherty, P., Ashton, S. V., Moore, S. E. and Walsh, F. S. (1991) Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca^{2+} channels. Cell **67**, 21–33

3 Williams, E. J., Doherty, P., Turner, G., Reid, R. A., Hemperly, J. J. and Walsh, F. S. (1992) Calcium influx into neurons can solely account for cell contact-dependent neurite outgrowth stimulated by transfected L1. J. Cell Biol. **119**, 883–892

4 Walsh, F. S. and Doherty, P. (1997) Neural cell adhesion molecules of the immunoglobulin superfamily: role in axon growth and guidance. Annu. Rev. Cell Dev. Biol. **13**, 425–456

5 Harper, S. J., Bolsover, S. R., Walsh, F. S. and Doherty, P. (1994) Neurite outgrowth stimulated by L1 requires calcium influx into neurons but is not associated with changes in steady state levels of calcium in growth cones. Cell Adhes. Commun. **2**, 441–453

6 Six, D. A. and Dennis, E. A. (2000) The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim. Biophys. Acta **1488**, 1–19

7 Murakami, M. and Kudo, I. (2002) Phospholipase A_2. J. Biochem. (Tokyo) **131**, 285–292

8 Soragni, E., Bolchi, A., Balestrini, R., Gambaretto, C., Percudani, R., Bonfante, P. and Ottonello, S. (2001) A nutrient-regulated, dual localization phospholipase A_2 in the symbiotic fungus *Tuber borchii*. EMBO J. **20**, 5079–5090

9 Sugiyama, M., Ohtani, K., Izuhara, M., Koike, T., Suzuki, K., Imamura, S. and Misaki, H. (2002) A novel prokaryotic phospholipase A_2. J. Biol. Chem. **277**, 20051–20058

10 Dennis, E. A. (1997) The growing phospholipase A_2 superfamily of signal transduction enzymes. Trends Biol. Sci. **22**, 1–2

11 Lambeau, G. and Lazdunski, M. (1999) Receptors for a growing family of secreted phospholipase A_2. Trends Pharmacol. Sci. **20**, 162–170

12 Balsinde, J., Winstead, M. V. and Dennis, E. A. (2002) Phospholipase A_2 regulation of arachidonic acid mobilization. FEBS Lett. **531**, 2–6

13 Wakatsuki, S., Arioka, M., Dohmae, N., Takio, K., Yamasaki, M. and Kitamoto, K. (1999) Characterization of a novel fungal protein, p15, which induces neuronal differentiation of PC12 cells. J. Biochem. (Tokyo) **126**, 1151–1160

14 Wakatsuki, S., Yokoyama, T., Nakashima, S., Nishimura, A., Arioka, M. and Kitamoto, K. (2001) Molecular cloning, functional expression and characterization of p15, a novel fungal protein with potent neurite-inducing activity in PC12 cells. Biochim. Biophys. Acta **1522**, 74–81

15 Nakashima, S., Wakatsuki, S., Yokoyama, T., Arioka, M. and Kitamoto, K. (2003) Identification and characterization of Scp15, a novel protein from *Streptomyces coelicolor* A3(2) with neurite-inducing activity in PC12 cells. Biosci. Biotechnol. Biochem. **67**, 77–82

16 Han, S. K., Yoon, E. T. and Cho, W. (1998) Bacterial expression and characterization of human secretory class V phospholipase A_2. Biochem. J. **331**, 353–357

17 Valentin, E., Koduri, R. S., Scimeca, J. C., Carle, G., Gelb, M. H., Lazdunski, M. and Lambeau, G. (1999) Cloning and recombinant expression of a novel mouse-secreted phospholipase A_2. J. Biol. Chem. **274**, 19152–19160

18 Elsbach, P. and Weiss, J. (1991) Utilization of labeled *Escherichia coli* as phospholipase substrate. Methods Enzymol. **197**, 24–31

19 McIntosh, J. M., Ghomashchi, F., Gelb, M. H., Dooley, D. J., Stoehr, S. J., Giordani, A. B., Naisbitt, S. R. and Olivera, B. M. (1995) Conodipine-M, a nove phospholipase A_2 isolated from the venom of the marine snail *Conus magus*. J. Biol. Chem. **270**, 3518–3526

20 Merkel, O., Fido, M., Mayr, J. A., Pruger, H., Raab, F., Zandonella, G., Kohlwein, S. D. and Paltauf, F. (1999) Characterization and function *in vivo* of two novel phospholipases B/lysophospholipases from *Saccharomyces cerevisiae*. J. Biol. Chem. **274**, 28121–28127

21 Scott, D. L., Otwinowski, Z., Gelb, M. H. and Sigler, P. B. (1990) Crystal structure of bee-venom phospholipase A_2 in a complex with a transition-state analogue. Science **250**, 1563–1566

22 Kim, E. J., Lee, J. E., Kwon, K. J., Lee, S. H., Moon, C. H. and Baik, E. J. (2001) Differential roles of cyclooxygenase isoforms after kainic acid-induced prostaglandin E_2 production and neurodegeneration in cortical and hippocampal cell cultures. Brain Res. **908**, 1–9

23 DeGeorge, J. J., Walenga, R. and Carbonetto, S. (1988) Nerve growth factor rapidly stimulates arachidonate metabolism in PC12 cells: potential involvement in nerve fiber growth. J. Neurosci. Res. **21**, 323–332

24 Singer, A. G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G. and Gelb, M. H. (2002) Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A_2. J. Biol. Chem. **277**, 48535–48549

25 Matsuzawa, A., Murakami, M., Atsumi, G., Imai, K., Prados, P., Inoue, K. and Kudo, I. (1996) Release of secretory phospholipase A_2 from rat neuronal cells and its possible function in the regulation of catecholamine secretion. Biochem. J. **318**, 701–709

26 Xu, Y. (2002) Sphingosylphosphorylcholine and lysophosphatidylcholine: G protein-coupled receptors and receptor-mediated signal transduction. Biochim. Biophys. Acta **1582**, 81–88

27 Kabarowski, J. H., Zhu, K., Le, L. Q., Witte, O. N. and Xu, Y. (2001) Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A. Science **293**, 702–705

28 Zhu, K., Baudhuin, L. M., Hong, G., Williams, F. S., Cristina, K. L., Kabarowski, J. H., Witte, O. N. and Xu, Y. (2001) Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. J. Biol. Chem. **276**, 41325–41335

29 Blondeau, N., Lauritzen, I., Widmann, C., Lazdunski, M. and Heurteaux, C. (2002) A potent protective role of lysophospholipids against global cerebral ischemia and glutamate excitotoxicity in neuronal cultures. J. Cereb. Blood Flow Metab. **22**, 821–834

30 Yagami, T., Ueda, K., Asakura, K., Hata, S., Kuroda, T., Sakaeda, T., Takasu, N., Tanaka, K., Gemba, T. and Hori, Y. (2002) Human group IIA secretory phospholipase A_2 induces neuronal cell death via apoptosis. Mol. Pharmacol. **61**, 114–126

31 Yagami, T., Ueda, K., Asakura, K., Hayasaki-Kajiwara, Y., Nakazato, H., Sakaeda, T., Hata, S., Kuroda, T., Takasu, N. and Hori, Y. (2002) Group IB secretory phospholipase A_2 induces neuronal cell death via apoptosis. J. Neurochem. **81**, 449–461

32 Yagami, T., Ueda, K., Asakura, K., Hata, S., Kuroda, T., Sakaeda, T., Kishino, J., Sakaguchi, G., Itoh, N. and Hori, Y. (2002) Group IB secretory phospholipase A_2 induces cell death in the cultured cortical neurons: a possible involvement of its binding sites. Brain Res. **949**, 197–201

33 Kolko, M., Bruhn, T., Christensen, T., Lazdunski, M., Lambeau, G., Bazan, N. G. and Diemer, N. H. (1999) Secretory phospholipase A_2 potentiates glutamate-induced rat striatal neuronal cell death *in vivo*. Neurosci. Lett. **274**, 167–170

34 Rodriguez De Turco, E. B., Jackson, F. R., DeCoster, M. A., Kolko, M. and Bazan, N. G. (2002) Glutamate signalling and secretory phospholipase A_2 modulate the release of arachidonic acid from neuronal membranes. J. Neurosci. Res. **68**, 558–567

35 Kolko, M., Rodriguez de Turco, E. B., Diemer, N. H. and Bazan, N. G. (2003) Neuronal damage by secretory phospholipase A_2: modulation by cytosolic phospholipase A_2, platelet-activating factor, and cyclooxygenase-2 in neuronal cells in culture. Neurosci. Lett. **338**, 164–168

Received 5 June 2003/27 August 2003; accepted 11 September 2003
Published as BJ Immediate Publication 11 September 2003, DOI 10.1042/BJ20030830