Kainate Receptors Mediate Regulated Exocytosis of Secretory Phospholipase A₂ in SH-SY5Y Neuroblastoma Cells

Aung Than^a Yan Tan^b Wei-Yi Ong^{b, c} Akhlaq A. Farooqui^d Peng Chen^a

^aDivision of Bioengineering, Nanyang Technological University, and ^bDepartment of Anatomy and

^cNeurobiology and Ageing Research Program, National University of Singapore, Singapore;

^dDepartment of Molecular and Cellular Biochemistry, Ohio State University, Columbus, Ohio, USA

Key Words

Secretory phospholipase A₂ • Group IIA secretory phospholipase A₂ • Kainate receptors • Protein kinase C • GluR5 • Exocytosis • Neurotransmission • Synaptic plasticity • Nociception • Pain

Abstract

Secretory phospholipase A₂ (sPLA₂) isoforms are widely expressed in the brain and spinal cord. Group IIA sPLA₂ (sPLA₂-IIA) has been shown to stimulate exocytosis and release of neurotransmitters in neuroendocrine PC12 cells and neurons, suggesting a role of the enzyme in neuronal signaling and synaptic transmission. However, the mechanisms by which sPLA₂ is itself released, and a possible relation between glutamate receptors and sPLA₂ exocytosis, are unknown. This study was carried out to elucidate the effects of glutamate receptor agonists on exocytosis of sPLA₂-IIA in transfected SH-SY5Y neuroblastoma cells. sPLA₂-IIA enzyme was packaged in fusion-competent vesicles and released constitutively or upon stimulation, suggesting regulated secretion. The signal peptide of sPLA₂-IIA is required

for its vesicular localization and exocytosis. External application of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (KA) induced vesicular exocytosis and release of sPLA₂-IIA. UBP 302, a GluR5-specific KA receptor antagonist, abolished the effect of KA, confirming the role of KA receptors in mediating sPLA₂-IIA secretion. Moreover, KA-induced sPLA₂-IIA secretion is dependent on Ca²⁺ and protein kinase C. Together, these findings provide evidence of a link between glutamate receptors and regulated sPLA₂ secretion in neurons that may play an important role in synaptic plasticity, pain transmission and neurodegenerative diseases.

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Introduction

Phospholipases A₂ (PLA₂) are lipolytic enzymes that cleave the *sn*-2-acyl ester linkage of glycerophospholipid to liberate a free fatty acid and lysophospholipid [1]. The secretory type of mammalian PLA₂ (sPLA₂) has a low molecular weight of 13–19 kDa and is classified into sev-

W.-Y.O and P.C. are both corresponding authors.
A.T. and Y.T. contributed equally to this work.

Dr. Peng Chen
Division of Bioengineering
Nanyang Technological University
Singapore 637456 (Singapore)
Tel. +65 6514 1086, E-Mail chenpeng@ntu.edu.sg

Dr. Wei-Yi Ong
Department of Anatomy
National University of Singapore
Singapore 119260 (Singapore)
Tel. +65 6516 3662, E-Mail wei_yi_ong@nuhs.edu.sg

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eral subtypes, sPLA₂-IB, -IIA, -IIC, -IID, -IIE, -IIF, -III, -V, -X, -XIIA and -XIIB based mainly on the position of cysteine residues [2, 3]. Many sPLA₂ isoforms contain a signal peptide essential for their secretion. sPLA₂-IIA, -IIC, -IID, -IIE, -IIF and -V are clustered on the same chromosome locus (1p34-p36) [4]. sPLA₂-IB, -IIA, -IIC, -IIE, -III, -V and -X are found in the central nervous system (CNS) [5–7]. Relatively high levels of sPLA₂ activity are detected in the hippocampus, medulla oblongata and pons [8]. PLA₂ and its enzymatic products are involved in neurotransmission and synaptic plasticity [9, 10]. Stimulation of glutamate receptors not only results in Ca²⁺ influx essential for long-term potentiation (LTP) [11] but also activates PLA₂. This leads to formation of arachidonic acid and lysophospholipids [12, 13] which increase vesicle exocytosis [14, 15]. PLA₂ facilitates neurotransmission in hippocampal neurons [16] whereas inhibition of PLA₂ decreases LTP [17, 18].

sPLA₂-IIA is localized in the CNS [6, 19]. Dense immunolabeling of sPLA₂-IIA is observed in the rat brainstem and spinal cord, where the enzyme is present in a postsynaptic location in neurons [6]. sPLA₂-IIA plays a role in nociception from the paw and orofacial region [20, 21] and may contribute to neuronal injury in acute and chronic neurodegenerative diseases. sPLA₂-IIA activity and expression are upregulated after cerebral ischemia [22, 23] and spinal cord injury [24]. Enzyme expression is increased in Alzheimer's disease, especially in regions containing amyloid- β plaques [25]. The latter induces the expression of proinflammatory cytokines such as TNF- α and IL-1 β , which in turn upregulate sPLA₂-IIA [26, 27]. Treatment of cortical neurons with sPLA₂-IIA induces release of arachidonic acid and apoptosis [28]. sPLA₂ could mediate neuronal death through ionotropic glutamate receptors [29]. The enzyme also increases Ca²⁺ influx through L-type voltage-gated calcium channels [30].

α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptors are classified as ionotropic glutamate receptors [31]. AMPA receptors are composed of GluR1-4 subunits whilst KA receptors consist of GluR5-7 or KA1-2 subunits [32–34]. Many studies show that glutamate receptors mediate synaptic transmission and plasticity in the brain [33, 35, 36]. In addition, glutamate receptors regulate secretion of neuromodulators such as substance P from synaptosomes [37, 38]. There is also evidence that KA receptors can have metabotropic actions via G-protein signaling cascades linked to protein kinase C (PKC) [33, 36, 39]. sPLA₂-IIA stimulates exocytosis and release of neurotransmitters from neurons [40] and could itself

be released from synaptosomes upon potassium-induced depolarization [41]. However, to date, a possible relation between glutamate receptor activation and sPLA₂-IIA exocytosis is unknown. In this study, we elucidated the differential effects of glutamate receptor agonists on vesicular exocytosis and release of sPLA₂-IIA in human neuroblastoma SH-SY5Y cells.

Materials and Methods

Construction of the Rat Spinal sPLA₂-IIA cDNAs and Expression Vectors

Total RNAs from the rat spinal cord was extracted and isolated using Trizol reagent (Invitrogen, USA), and purified using the RNeasy Mini Kit (Qiagen Inc., USA). The spinal cord was chosen as the tissue source for sPLA₂-IIA mRNA since our recent study showed high relative expression of the enzyme in this organ [6]. The RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with the following conditions: 25°C for 10 min, 37°C for 120 min and 85°C for 5 s. The sPLA₂-IIA enzyme includes a signal peptide [6, 42]. By using the SignalP 3.0 Server, the sequence of sPLA₂-IIA without N-terminal signal peptide was determined to be from the 121st to 441st base pairs of sPLA₂-IIA (NM_031598.3).

Two sets of primers were designed based on the sequence of rat sPLA₂-IIA gene: (1) F1 5'-TGGATGAAGTCCTCCTGTTGCT-3' and B1 5'-TCAGCAACTGGGCGTCTTCCCTT-3' for insertion into the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen) and (2) F2 5'-CAGTCTCGAGCGGATGAAGTCCTCCTGTTGCTA-3' and B2 5'-GTACGAATTCCTATCAGCAACTGGGCGT-3' for insertion into the pIRES2-EGFP vector (BD Biosciences Clontech, Mountain View, Calif., USA). The primers for sPLA₂-IIA without signal peptide sequence for insertion into the pcDNA3.1/CT-GFP-TOPO vector were: F1 5'-TGGATGAGCC-TTCTGGAGTTTGG-3' and B1 5'-TCAGCAACTGGGCGTCTTCCCTT-3'. The PCR temperature profile was 95°C for 30 s followed by 33 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. PCR products were separated on agarose gel and the fragments containing the cDNA of either full-length sPLA₂-IIA or sPLA₂-IIA without signal peptide were extracted using the QIAquick Gel Extraction protocol (Qiagen). To obtain sPLA₂-IIA-GFP fusion protein, cDNA inserts of sPLA₂-IIA (sPLA₂-IIA with or without signal peptide) were cloned into pcDNA3.1/CT-GFP-TOPO using the CT-GFP Fusion TOPO Expression kit (Invitrogen). Recombinant plasmid sPLA₂-IIA-IRES-EGFP (full length sPLA₂-IIA) was produced by cloning the cDNA insert for sPLA₂-IIA with signal peptide, into pIRES2-EGFP at the sites of *Xho*I and *Eco*RI. Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen) and verified by DNA sequencing to confirm integrity and orientation.

Cell Culture and Generation of Stable Cell Lines

Human neuroblastoma SH-SY5Y cells (American Type Culture Collection, Rockville, Md., USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, Md., USA) supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (Gibco). Cells

were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂. In order to establish stable cell lines, FuGENE Transfection Reagent (Roche, Indianapolis, Ind., USA) was used. Specifically, after 70–80% confluence, cells were transfected with 1–2 µg of recombinant expression vectors, sPLA₂-IIA-GFP (sPLA₂-IIA with or without signal peptide), or sPLA₂-IIA-IRES-EGFP (sPLA₂-IIA with signal peptide), or empty vector, followed by incubation of cells at 37°C for 2 days in culture medium. The transient cell lines were subcultured and grown in culture medium containing selection reagent (G418 sulfate, 500 µg/ml; Invitrogen) for 10 days, with a change of medium every 2 days. Subsequently, transfected cells were transferred into a 96-well plate ensuring that only a single colony is present in each well, grown for another 2 weeks and subcultured into larger wells to obtain stable cell lines.

Total Internal Reflection Fluorescence Microscopy Analysis

Total Internal Reflection Fluorescence Microscopy (TIRFM) experiments were performed at 37°C using a Zeiss Axiovert-200M inverted microscope system (Carl Zeiss, Göttingen, Germany) equipped with a 100× 1.45 NA TIRF objective lens. Glass coverslips (refractive index = 1.52) with SH-SY5Y cells were mounted in an imaging chamber containing bath solution (150 mM NaCl, 5.4 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 10 mM HEPES, pH 7.4). The laser beam of 488 nm was incident on the coverslip at 68–70° from normal. Penetration depth of the evanescent field was calculated to be around 100 nm. The emission of green fluorescence protein (GFP) was collected at 520 nm. Using MetaMorph 6.3 software (Molecular Devices, Downingtown, Pa., USA), real-time motion of GFP-labeled sPLA₂-IIA vesicles was acquired as time-lapse digital images from a single cell with a CCD camera for 2 min with exposure times of 25 ms and 2-Hz sampling frequency, and with time intervals of 0.3 or 0.5 s. Fusion events of sPLA₂-IIA vesicles in SH-SY5Y cells stably expressed with sPLA₂-IIA-GFP were analyzed by ImageJ software (National Institute of Health, <http://rsb.info.nih.gov/ij/>). These are characterized by rapid increase of fluorescence signal followed by diffusion to the peripheral region. The number of events was counted in randomly chosen cells during a 2-min time interval, and the mean and standard error calculated.

Immunocytochemical Analyses

SH-SY5Y cells stably expressing sPLA₂-IIA-GFP (full-length sPLA₂-IIA) were immunolabeled with synaptotagmin-1 or neuropeptide Y (NPY; Santa Cruz Biotech, Santa Cruz, Calif., USA) to determine possible colocalization of the enzyme with other vesicles markers. The cells were plated on glass cover slips and fixed with 4% formaldehyde for 10 min. They were then permeabilized with 0.1% Triton X-100 and blocked with 1.5% bovine albumin before overnight incubation with a mouse monoclonal antibody to synaptotagmin-1 (Synaptic Systems GmbH, Göttingen, Germany; diluted 1:100) or a rabbit polyclonal antibody to NPY (diluted 1:100). The cells were washed with PBS and incubated with donkey anti-mouse secondary antibody conjugated with Alexa Fluor 555 (Invitrogen) or goat anti-rabbit secondary antibody conjugated with Atto 647 NHS (Sigma-Aldrich, St. Louis, Mo., USA) for 1 h at room temperature. Cells were then washed with PBS and viewed using a LSM 510 Meta confocal laser scanning microscope (Carl Zeiss).

Western Blot Analysis

SH-SY5Y cells stably expressing sPLA₂-IIA were seeded in 75-cm² flasks (4 × 10⁵ cells/flask) and grown to confluence. Cells (5 × 10⁶ cells/flask) were then washed 3 times with PBS and incubated in serum-free DMEM medium with or without test chemicals for 120 min at 37°C. The medium was then harvested and stored at –20°C. Cells were washed 3 times with ice-cold PBS and lysed using RIPA lysis buffer containing protease inhibitors (Santa Cruz Biotech). Protein concentrations in the collected medium or the cell lysates were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, San Diego, Calif., USA). Protein samples were separated on 12% SDS polyacrylamide gels under reducing conditions and blotted onto nitrocellulose membranes using a Mini-Transblot apparatus (Bio-Rad). The membranes were blocked for 2 h with 5% nonfat milk in Tris-buffered saline-Tween (TBST) buffer (10 mM Tris, 150 mM NaCl and 0.1% Tween-20, pH 7.4) and incubated overnight with rabbit primary antibodies to GFP, GAPDH (1:200–400; Biovision, Mountain View, Calif., USA), or sPLA₂-IIA (1:5,000) in TBST, followed by washing 3 times with TBST buffer. The anti-sPLA₂-IIA antibody was prepared by immunizing a rabbit with purified recombinant rat sPLA₂-IIA as described earlier [43]. Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; Thermo Scientific, Rockford, Ill., USA) for 6 h at room temperature. The enzyme protein was visualized with optiCN detection kit and quantified with QualityOne software (Bio-Rad). Immunoblots of secreted proteins and GAPDH were obtained using the same sample sets. GAPDH served as an internal control. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich, Saint Louis, Mo., USA. UBP302 and AMPA were purchased from Tocris (Tocris Bioscience, Ellisville, Mo., USA).

Statistical Analysis

Results are reported as mean ± SEM, and statistical significance was analyzed by one-way ANOVA with Bonferroni's post hoc test. *p* < 0.05 was considered significant.

Results

sPLA₂-IIA Proteins Are Packed into Fusion-Competent Vesicles

Individual secretory vesicles in the subplasmalemmal region and their fusion with the plasma membrane were visualized using the technique of TIRFM, which evanescently illuminates the thin region (<200 nm) immediately adjacent to the cell membrane [44]. sPLA₂-IIA protein (GFP-tagged full-length sPLA₂-IIA) was found in vesicle-like structures, in human neuroblastoma SH-SY5Y cells stably expressing sPLA₂-IIA-GFP (fig. 1a). The vesicles undergo constant lateral (parallel to the plasma membrane) and vertical trafficking (vesicle transit between the inner cytosol to the subplasmalemmal region) (fig. 1c). Spontaneous fusion events of sPLA₂-IIA vesicles were frequently observed by TIRFM similar to those observed for secretory vesicles in neuroendocrine

Fig. 1. sPLA₂-IIA enzymes are packed into fusion-competent vesicles. **a** Typical TIRFM image. **b** Bright-field image of an SH-SY5Y cell stably expressed with GFP-tagged sPLA₂-IIA fusion protein (full-length sPLA₂-IIA). Individual sPLA₂-IIA vesicles can be resolved as bright dots (**a**). Scale bar = 5 μ m. **c** Typical motion trajectory of a subplasmalemmal sPLA₂-IIA vesicle. Scale bar = 200 nm. **d** Sequential images of fusion event (top) of a sPLA₂-IIA vesicle. The curves in the bottom panel represent the change in mean fluorescence intensity over time within the region <0.5 μ m away from the vesicle center (solid line) and the change in fluorescence intensity within the annular region >0.5 and <0.65 μ m away from the vesicle center (dotted line). A full-fusion event of a vesicle is characterized by a simultaneous transient increase in these two curves due to rapid release of the fluorescent molecules and their diffusion to the peripheral region. The vertical dashed line indicates the onset of the fusion event. a.u. = Arbitrary unit.

Fig. 2. sPLA₂-IIA enzymes are colocalized with synaptotagmin-1. Distribution of GFP-tagged sPLA₂-IIA molecules (full-length sPLA₂-IIA) (left lane) in SH-SY5Y cells, with immunostained synaptotagmin-1 (AlexaFluor 555 secondary antibody) (**a**, middle) or immunostained NPY (Atto 647 secondary antibody) (**b**, middle) were analyzed using confocal microscopy. Respective colocalizations (merge of left and middle) are shown in the right lane. Scale bar = 5 μ m.

Color version available online

cells and synaptic vesicles in neurons [44–46]. They appeared as an abrupt increase in fluorescence intensity due to rapid release of fluorescent molecules from a vesicle, followed by broadening and disappearance of fluorescence as the molecules diffuse away [47] (fig. 1d). These observations suggest that sPLA₂-IIA molecules are contained in fusion-competent vesicles which are released constitutively or upon stimulation. Confocal microscopy of SH-SY5Y cells expressing GFP-tagged full-length sPLA₂-IIA revealed a punctuate fluorescence pattern characteristic of secretory vesicles (fig. 2). We also examined possible colocalization of sPLA₂-IIA with synaptotagmin-1, a Ca²⁺ sensor localized in secretory vesicles [48] or NPY, a marker of large dense core vesicles [49]. sPLA₂-IIA containing vesicles were found to be colocalized with synaptotagmin-1 (fig. 2a), but not NPY (fig. 2b).

KA Induces Vesicular Release of sPLA₂-IIA

The effects of glutamate and its analogs on the exocytosis of sPLA₂-IIA from SH-SY5Y cells stably transfected with sPLA₂-IIA-GFP were examined by TIRFM. Increased number of fusion events of sPLA₂-IIA vesicles was observed 30 min after application of 10 μM KA (fig. 3a). This is corroborated by the detection of increased sPLA₂-IIA release (sPLA₂-IIA-GFP, about 42 kDa) ($p < 0.05$) after 2 h of incubation with 10 μM of KA (fig. 3b). This effect was dose dependent and observed after treatment with 100 nM to 10 μM KA (fig. 3c). Addition of AMPA (10 μM, 2 h of incubation) also induced sPLA₂-IIA secretion. However, no effect on sPLA₂-IIA secretion or fusion frequency was detected after addition of 10 μM of NMDA or glutamate (fig. 3a, b).

Effect of CNQX on sPLA₂-IIA Secretion

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) is an inhibitor of AMPA receptors. As shown in figure 4a, 20 μM CNQX did not significantly suppress KA-induced vesicular exocytosis of sPLA₂-IIA. CNQX, however, significantly inhibited AMPA-induced sPLA₂ secretion ($p < 0.05$; fig. 4b).

KA-Induced sPLA₂-IIA Secretion Occurs through the GluR5-Containing KA Receptor

KA receptors include GluR5 or GluR6 subunits which have been localized pre- or postsynaptically and are involved in synaptic transmission and plasticity [36]. A GluR5-specific KA receptor antagonist UBP302 [S-1-(2-amino-2-carboxyethyl)-3-(2-carboxybenzyl) pyrimidine-2,4-dione] [50] or a GluR6-specific antagonist NS

102 (6,7,8,9-tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione 3-oxime) [51] was used to determine whether the effect of KA on sPLA₂-IIA secretion was mediated through the GluR5- or GluR6-containing receptors. 20 μM UBP302 abolished KA-induced vesicular exocytosis and release of sPLA₂-IIA ($p < 0.05$; fig. 5a, b). On the other hand, NS 102 did not significantly suppress sPLA₂-IIA secretion. These results indicate that KA-induced exocytosis and release of sPLA₂-IIA in SH-SY5Y cells are mediated by receptors containing the GluR5 subunit.

KA-Induced sPLA₂-IIA Secretion Is Ca²⁺ Dependent

The number of spontaneous fusion events was significantly reduced when extracellular Ca²⁺ was depleted by the calcium chelator EGTA (2 mM), or when the intracellular Ca²⁺ pool was depleted by thapsigargin (1 μM, 15 min pretreatment) [52] (fig. 6a). In addition, 10 μM KA-induced sPLA₂-IIA secretion was suppressed when external or internal Ca²⁺ was depleted (fig. 6a, b). The results indicate that Ca²⁺ is required for exocytosis of sPLA₂-IIA in SH-SY5Y cells under basal conditions or after KA stimulation.

KA-Induced sPLA₂-IIA Secretion Is PKC Dependent

KA receptors especially those containing GluR5 could signal through a metabotropic action involving phospholipase C and activation of PKC [33, 36]. The latter is known to regulate exocytosis via phosphorylation of key proteins in the exocytotic cascade [53]. Bisindolylmaleimides (BIS, selective and potent inhibitors of PKC) were used to block PKC activity [54, 55] in SH-SY5Y cells to determine a possible role of PKC in spontaneous and KA-induced sPLA₂-IIA secretion. Inhibition of PKC by either of the BIS compounds Ro-32-0432 (BIS-XI) [56] or Ro-31-8220 (BIS-IX) [57, 58] reduced the number of fusion events under basal and KA-stimulated conditions (fig. 7a).

BIS compounds did not affect basal sPLA₂-IIA release as detected by Western blots (fig. 7b) but significantly inhibited KA-induced sPLA₂-IIA release. In addition 100 nM phorbol-12-myristate-13-acetate (PMA), a potent diacylglycerol (DAG) analog that activates PKC, induced exocytosis and release of sPLA₂-IIA, while this effect was largely blunted in the presence of PKC inhibitor (fig. 7). These findings indicate a role of PKC in KA-induced exocytosis of sPLA₂-IIA.

Signal Peptide Is Required for Vesicular Localization and Exocytosis of sPLA₂-IIA

We next examined the role of signal peptide of sPLA₂-IIA in vesicular localization and exocytosis using

Fig. 3. Glutamate analogs differentially modulate exocytosis and release of sPLA₂-IIA from SH-SY5Y cells stably expressed sPLA₂-IIA-GFP (full-length sPLA₂-IIA). **a** Cells were incubated for 30 min without (control, Con) or with 10 μ M each glutamate, KA, NMDA or AMPA, followed by imaging of cells by TIRFM. The number of fusion events during a 2-min time interval was counted. Values represent mean $\pm$ SEM (5 or 6 cells from 3 or 4 separate experiments). **b, c** Top panels show the representative immunoblots of sPLA₂-IIA fusion protein released from cells (5×10^6 cells/ flask) into the serum-free DMEM media during 2 h of incubation at 37°C, without (control, Con) or with 10 μ M each

glutamate, KA, NMDA or AMPA (**b**), or without (control, Con) or with KA (10 nM to 100 μ M) (**c**). The lower panels show the statistics (mean $\pm$ SEM, $n = 6$) of optical density of the sPLA₂-IIA-GFP blots under different conditions, normalized by optical density of GAPDH blots, from 3 or 4 separate experiments of each group. ^a $p < 0.05$ vs. control, analyzed by one-way ANOVA and Bonferroni's post hoc test. a.u. = Arbitrary unit.

Fig. 4. CNQX fails to block the KA-induced sPLA₂-IIA secretion from SH-SY5Y cells stably cloned with sPLA₂-IIA-GFP (full-length sPLA₂-IIA). **a** Cells were incubated 30 min without (control, Con), or with KA (10 μ M), or KA plus CNQX (20 μ M), or NMDA (10 μ M), or NMDA plus CNQX, or AMPA (10 μ M), or AMPA plus CNQX, followed by imaging of cells by TIRFM. The number of fusion events during a 2-min time interval was counted. Values represent mean $\pm$ SEM (5 or 6 cells from 3 or 4 separate experiments). **b** The top panel shows the representative immunoblots of sPLA₂-IIA fusion protein released into the serum-free media during 2 h of incubation, without (control, Con), or with KA (10 μ M), or KA plus CNQX (20 μ M), or NMDA (10 μ M), or NMDA plus CNQX, or AMPA (10 μ M), or AMPA plus CNQX. The lower panel shows the statistics (mean $\pm$ SEM, $n = 6$) of optical density of the sPLA₂-IIA-GFP blots, normalized by optical density of GAPDH blots, from 3 or 4 separate experiments. ^a $p < 0.05$ vs. control; ^b $p < 0.05$ vs. AMPA treatment alone, analyzed by one-way ANOVA and Bonferroni's post hoc test. a.u. = Arbitrary unit.

Fig. 5. KA-induced sPLA₂-IIA secretion is through the GluR5-containing receptors. **a** SH-SY5Y cells stably expressed sPLA₂-IIA-GFP (full-length sPLA₂-IIA) were incubated for 30 min without (control, Con) or with KA (10 μ M), KA plus UBP 302 (UBP 302, GluR5-containing KA receptor antagonist, 20 μ M), or KA plus NS 102 (NS 102, GluR6-containing KA receptor antagonist, 20 μ M), followed by imaging of cells by TIRFM. The number of fusion events during a 2-min time interval was counted. Values represent mean $\pm$ SEM (5 or 6 cells from 3 or 4 separate experiments). **b** The top panel shows the representative immunoblots of sPLA₂-IIA fusion protein released into the serum-free medium during 2 h of incubation, without (control, Con) or with KA (10 μ M), KA plus UBP 302 (20 μ M), or KA plus NS 102 (20 μ M). The lower panel shows the statistics (mean $\pm$ SEM, $n = 6$) of optical density of the sPLA₂-IIA-GFP blots, normalized by optical density of GAPDH blots, from 3 or 4 separate experiments. ^a $p < 0.05$ vs. control; ^b $p < 0.05$ vs. KA treatment alone, analyzed by one-way ANOVA and Bonferroni's post hoc test. a.u. = Arbitrary unit.

Fig. 6. KA-induced sPLA₂-IIA secretion is Ca²⁺ dependent. **a** SH-SY5Y cells stably expressed with sPLA₂-IIA-GFP (full-length sPLA₂-IIA) were incubated for 30 min in bath solution, without (control, Con) or with KA (10 μ M), EGTA (2 mM), EGTA plus KA, thapsigargin (1 μ M), or thapsigargin plus KA, followed by imaging of cells by TIRFM. The number of fusion events during a 2-min time interval was counted. Values represent mean $\pm$ SEM (6 or 7 cells from 3 or 4 separate experiments). **b** The top panel shows the representative immunoblots of sPLA₂-IIA fusion protein released into the serum-free medium during 2 h of incubation, without (control, Con) or with KA (10 μ M), EGTA (2 mM), EGTA plus KA, thapsigargin (1 μ M, 15-min pretreatment), or thapsigargin plus KA. The lower panel shows the statistics (mean $\pm$ SEM, $n = 6$) of optical density of the sPLA₂-IIA-GFP blots, normalized by optical density of GAPDH blots, from 3 or 4 separate experiments. ^a $p < 0.05$ vs. control; ^b $p < 0.05$ vs. KA treatment alone, analyzed by one-way ANOVA and Bonferroni's post hoc test. a.u. = Arbitrary unit.

TIRFM. In contrast to the numerous vesicle-like structures in SH-SY5Y cells stably cloned with sPLA₂-IIA-GFP (full-length sPLA₂-IIA) (fig. 1a), cells that stably expressed sPLA₂-IIA without signaling peptide showed homogeneous green fluorescence without any visible vesicle-like structures (fig. 8a).

Exocytosis was not observed in cells that stably expressed sPLA₂-IIA without signal peptide (fig. 8b) and there was very little sPLA₂-IIA in the culture medium of cells expressing sPLA₂-IIA-GFP without signal peptide. Moreover, 10 μ M KA could no longer induce sPLA₂-IIA secretion from these cells (fig. 8c). These findings indicate that the signal sequence of sPLA₂-IIA is essential for its vesicular localization and exocytosis.

KA-Induced sPLA₂-IIA Secretion

The effects of glutamate and its analogs on the secretion of sPLA₂-IIA from SH-SY5Y cells stably transfected with sPLA₂-IIA-IRES-EGFP vector were analyzed by Western blots. Cells were transfected with sPLA₂-IIA-IRES-EGFP vector which produces separate expression of sPLA₂-IIA and EGFP, and sPLA₂-IIA released into the medium was directly detected with a specific antibody to the enzyme. As shown in figure 9a, 10 μ M KA stimulated sPLA₂-IIA secretion (sPLA₂-IIA, about 15 kDa; $p < 0.05$). However, no significant enhancement of sPLA₂-IIA secretion was found after addition of NMDA, AMPA or low doses of glutamate itself. Very low levels of EGFP (EGFP, about 27 kDa) were detected in the culture medium and no increase in EGFP was detected after addition of glutamate analogs (fig. 9b), consistent with regulated secretion of sPLA₂-IIA but not EGFP upon stimulation by KA.

Discussion

The present study was carried out to elucidate the factors that result in sPLA₂ exocytosis in neurons. sPLA₂-IIA was stably expressed as a GFP fusion protein in SH-SY5Y cells which has morphological and biochemical characteristics and proteomic expression of mature neurons [59, 60]. sPLA₂-IIA was found to be contained in fusion-competent vesicles in these cells and released through exocytosis, even under basal conditions. In contrast, cells that stably express sPLA₂-IIA without signal peptide showed homogeneous green fluorescence without visible vesicle-like structures and exocytosis. These results are striking as they demonstrate that sPLA₂-IIA is packaged in secretory vesicles and released, similar to several of the neuropeptide modulators such as substance

Fig. 7. KA-induced sPLA₂-IIA secretion is PKC dependent. **a** SH-SY5Y cells stably expressed with sPLA₂-IIA-GFP (full-length sPLA₂-IIA) were incubated for 30 min without (control, Con) or with KA (10 μ M), KA plus BIS-XI (2.5 μ M), BIS-XI, BIS-IX (2.5 μ M), PMA (100 nM), or PMA plus BIS-XI, followed by imaging of cells by TIRFM. The number of fusion events during a 2-min time interval was counted. Values represent mean $\pm$ SEM (6 or 7 cells from 3 or 4 separate experiments). **b** The top panel shows the representative immunoblots of sPLA₂-IIA fusion protein released into the serum-free medium during 2 h of incubation, without (control, Con) or with KA (10 μ M), KA plus BIS-XI (2.5 μ M, 30-min pretreatment), BIS-XI, BIS-IX (2.5 μ M), PMA (100 nM), or PMA plus BIS-XI. The lower panel shows the statistics (mean $\pm$ SEM, $n = 7$) of optical density of the sPLA₂-IIA-GFP blots, normalized by optical density of GAPDH blots, from 3 or 4 separate experiments. ^a $p < 0.05$ vs. control; ^b $p < 0.05$ vs. KA or PMA treatment alone, analyzed by one-way ANOVA and Bonferroni's post hoc test. a.u. = Arbitrary unit.

P and NPY which are contained in granules at nerve endings [61, 62]. In addition, immunocytochemical analyses showed that sPLA₂-IIA is colocalized with a marker of secretory vesicles, synaptotagmin-1 [48]. The latter is a calcium sensor important for synaptic vesicle release in axons and vesicular exocytosis in dendrites [63, 64].

Fig. 8. Signal peptide of sPLA₂-IIA is required for vesicular localization and exocytosis of sPLA₂-IIA. **a** Typical TIRFM image. **b** Bright-field image of SH-SY5Y cell stably expressed GFP-tagged sPLA₂-IIA (sPLA₂-IIA with no signal peptide) showing homogeneous green fluorescence without any visible vesicle-like structures (**a**). Scale bar = 5 μ m. **c** TIRFM analysis of the vesicular exocytosis (fusion events) from the two cloned cells stably expressed with either GFP-tagged full-length sPLA₂-IIA (FL, full-length sPLA₂-IIA) or GFP-tagged sPLA₂-IIA without signal peptide (NSP, sPLA₂-IIA with no signal peptide). The cloned cells were incubated without or with KA (10 μ M) for 30 min, followed by imaging the live cells. The number of fusion events during a

2-min time interval was counted. Values represent mean $\pm$ SEM (5 or 6 cells from 3 separate experiments). **d** The top panel shows the representative immunoblots of sPLA₂-IIA fusion protein released from the respective cloned cells into the serum-free medium during 2 h of incubation, without or with KA (10 μ M). The lower panel shows the statistics (mean $\pm$ SEM, $n = 6$) of optical density of the blots in arbitrary units (a.u.), normalized by optical density of GAPDH, from 3 or 4 separate experiments. Negative control used the same serum-free medium before 2 h of incubation. ^a $p < 0.05$ vs. NSP (without KA); ^b $p < 0.05$ vs. full length (without KA) analyzed by one-way ANOVA and Bonferroni's post hoc test.

Several studies have reported sPLA₂ in neurons of the CNS [5, 6]. We therefore sought to determine whether sPLA₂ could be released from cells in response to excitatory, glutamatergic stimulation. This was carried out on SH-SY5Y cells, which have been shown to express NMDA, AMPA and KA receptors [65–67] including the GluR5

subunit [59] (see online supplementary figure 1, www.karger.com/doi/10.1159/000330414). Besides direct visualization and quantitation of exocytotic events by TIRFM (fig. 2b), Western blot analyses were carried out to detect secretion of sPLA₂-IIA-EGFP fusion protein into the culture media. The dose of AMPA, KA, NMDA or glutamate

Fig. 9. KA stimulates the release of sPLA₂-IIA (not GFP-tagged fusion protein) from SH-SY5Y cells stably cloned with sPLA₂-IIA-IRES-EGFP (full-length sPLA₂-IIA). The top panels show the representative immunoblots of sPLA₂-IIA (**a**) or EGFP (**b**) released from the cloned cells, which stably and separately expressed both full-length sPLA₂-IIA and EGFP, into the serum-free medium during 2 h of incubation, without (control, Con) or with 10 μ M each of glutamate, KA, NMDA, or AMPA. The lower panels show the statistics (mean $\pm$ SEM, $n = 6$) of optical density of the blots, normalized by optical density of GAPDH, from 3 or 4 separate experiments. Immunoblots of sPLA₂-IIA and EGFP were obtained from the same sample sets. ^a $p < 0.05$ vs. control, analyzed by one-way ANOVA and Bonferroni's post hoc test. a.u. = Arbitrary unit.

used in these experiments was 10 μ M, which is less than the toxic doses of 40 μ M for AMPA [68]; 25–200 μ M for KA [67, 69]; 0.1–5 mM for NMDA [69, 70], and 2–100 mM for glutamate [71, 72] for human neuroblastoma SH-SY5Y cells. External application of KA or AMPA resulted

in increased exocytosis and release of sPLA₂-IIA whereas NMDA did not show any significant effect (fig. 2). In contrast, no exocytosis was observed after AMPA or KA stimulation in cells expressing sPLA₂-IIA without signal peptide (fig. 8a, b). These observations confirm the requirement of this peptide in sPLA₂ vesicular localization and exocytosis, and importantly, demonstrate that AMPA- or KA-induced exocytosis of sPLA₂-IIA was not the result of general toxicity or lysis of cells, due to excessive glutamatergic stimulation under our experimental conditions (fig. 9a). Together, the results indicate that AMPA and KA receptors are important for regulated secretion of sPLA₂-IIA from neuron-like cells.

The relative contributions of AMPA and KA receptors to sPLA₂-IIA exocytosis were verified using antagonists to these receptors. Cells that express sPLA₂-IIA-GFP fusion protein were treated with AMPA or KA in the presence of receptor antagonists. AMPA receptors are composed of GluR1–4 subunits, whilst KA receptors consist of GluR5–7 and KA1–2 subunits [32–34]. These subunits probably exist as heteromers in vivo [73]. KA can activate AMPA receptors, and AMPA can bind to KA receptors, suggesting overlapping binding affinity for AMPA and KA receptors [74]. CNQX is an antagonist at AMPA receptors but has some activity against KA receptors. 10–20 μ M CNQX blocks KA receptor-mediated excitatory postsynaptic currents by 40–80% [75]. Addition of CNQX partially blocked AMPA- but not KA-induced sPLA₂-IIA exocytosis. This is expected, since CNQX acts mainly at AMPA receptors. UBP 302 and NS102 are selective antagonists of GluR5 and GluR6 KA receptor subunits, respectively, at 10 μ M [76, 77]. Application of UBP 302 (a GluR5 subunit-specific KA receptor antagonist) abolished KA-induced sPLA₂-IIA secretion, whereas addition of NS102 (GluR6 subunit-specific KA receptor antagonist) did not show such inhibition. These observations indicate that AMPA and KA receptors are important for stimulating exocytosis of sPLA₂-IIA although the majority of the effect appears to be mediated by GluR5 receptors.

The above experiments were carried out on SH-SY5Y cells that stably express sPLA₂-IIA-GFP fusion protein. As controls against any unforeseen effects of the GFP tag on sPLA₂-IIA enzyme function, we conducted further studies using cell lines that express full-length sPLA₂-IIA, independent of the GFP tag. This was carried out using the sPLA₂-IIA-IRES-EGFP vector, i.e. sPLA₂-IIA and EGFP were cotransfected in the same cell using a single vector but not as a fusion protein. Stable cell lines expressing sPLA₂-IIA were selected based on the EGFP

fluorescence label, and enzyme release from these cells was detected using an antibody to sPLA₂-IIA. The findings confirm that sPLA₂-IIA protein is released from the SH-SY5Y cells by KA stimulation.

Possible mechanisms of KA action on sPLA₂-IIA release were also elucidated. AMPA, KA and NMDA receptors are typically described as ligand-gated ionotropic glutamate receptors [31]. Activation of these receptors increases free intracellular Ca²⁺ in neurons and SH-SY5Y cells [65]. Significantly reduced spontaneous or KA-induced fusion events and release of sPLA₂-IIA were detected in cells treated with the Ca²⁺ chelator EGTA or after depletion of intracellular calcium by thapsigargin, consistent with a requirement for Ca²⁺ in vesicular exocytosis [78]. Exocytosis was also induced by high potassium depolarization of cells (online suppl. fig. 2). In several CNS regions including the hippocampus and spinal cord, reports have indicated that KA receptors could be metabotropically coupled with G-protein signaling cascades that involve the activation of phospholipase C generation of DAG and subsequent activation of PKC [33, 36, 39]. We observed that KA-induced sPLA₂-IIA exocytosis was inhibited by a PKC inhibitor, whereas activation of PKC by PMA triggered vesicular secretion of sPLA₂-IIA. This may involve several proteins implicated in exocytosis which are PKC substrates, including SNAP-25 (synaptosome-associated protein) and Munc-18 (essential component of the synaptic vesicle fusion protein complex) which potentiate vesicle recruitment and sensitize vesicle release [53]. Moreover GAP-43, a specific PKC substrate that is important in priming of exocytosis [79] is upregulated in hippocampal granule neurons treated with KA [80, 81]. PKC may in turn phosphorylate KA receptors, leading to changes in channel conductance [82, 83]. Together, the results indicate PKC-dependent KA-induced vesicular exocytosis and release of sPLA₂-IIA.

The link between glutamate receptors and sPLA₂-IIA exocytosis may be functionally important. Since sPLA₂ is present in the brain under basal conditions, this could implicate a link between glutamate signaling, sPLA₂ secretion, and neurotransmitter exocytosis. sPLA₂ is detected in different parts of the brain including the hippocampus [6, 7] and is involved in synaptic transmission and LTP [84, 85]. KA receptor subunits GluR5 and KA2 also form the predominant heteromeric pair in postsynaptic KA receptors of the hippocampus [33]. KA-mediated excitatory postsynaptic currents in the hippocampus are generated mainly by GluR5-containing KA receptors [77]. In addition, postsynaptic KA-mediated excitatory postsynaptic current onto second-order neurons in the

spinal cord has been reported [86]. Electron microscopy reveals that the majority of KA receptors, GluR5, 6, 7, are postsynaptic to substance P-containing axon terminals in the trigeminal dorsal horn [87]. Together with electron-microscopic observations that sPLA₂-IIA is localized in a postsynaptic location in the spinal cord [6], we hypothesize that release of glutamate at a glutamatergic synapse may activate GluR5-containing KA receptors to induce sPLA₂-IIA exocytosis from storage vesicles in dendrites. Previous electrophysiological study indicates that sPLA₂-IIA could stimulate the release of neurotransmitters from PC12 neuroendocrine cells and cultured neurons [40]; hence, it is conceivable that sPLA₂-IIA could diffuse across the synaptic cleft to induce glutamate release from axon terminals. In this scenario, sPLA₂-IIA is released from dendrites as a result of AMPA or KA receptor activation, and acts as a retrograde signal to amplify neurotransmission.

Our findings of high-level expression of sPLA₂-IIA in the dorsal horn and spinal trigeminal nucleus [6] and the present link with KA receptors are consistent with a role of these receptors and sPLA₂ in nociceptive transmission. Intraperitoneal injection of the KA receptor antagonist SYM 2081 reduced mechanical allodynia and thermal hyperalgesia after chronic-constriction nerve injury [88] or freeze injury [89]. The GluR5 antagonist LY 382884 produced antinociceptive responses to formalin in rats [90]. Moreover, responses to capsaicin or inflammatory pain were significantly reduced in mice lacking GluR5 but not GluR6 subunits [91]. Similarly, intrathecal administration of the sPLA₂ inhibitor, LY311727 reduced inflammatory hyperalgesia in rats [20], and intracerebroventricular injection of the sPLA₂ inhibitor 12-episcalaradial decreased nociceptive responses after facial carrageenan injection in mice [21].

In conclusion, the present findings show that sPLA₂-IIA enzymes are packed into fusion-competent vesicles in human neuroblastoma SH-SY5Y cells and are released through exocytosis by glutamate analogs. Together, these results provide evidence of a link between glutamate receptors and sPLA₂-regulated secretion in neurons that may play an important role in the normal physiology of the CNS, nociception and neurodegenerative diseases.

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References

- Hanasaki K, Arita H: Purification and characterization of a high-affinity binding protein for pancreatic-type phospholipase A₂. *Biochim Biophys Acta* 1992;1127:233–241.
- Dennis EA: The growing phospholipase A₂ superfamily of signal transduction enzymes. *Trends Biochem Sci* 1997;22:1–2.
- Tischfield JA: A reassessment of the low molecular weight phospholipase A₂ gene family in mammals. *J Biol Chem* 1997;272:17247–17250.
- Murakami M, Kudo I: Phospholipase A₂. *J Biochem* 2002;131:285–292.
- Wang G, Zhou D, Wang C, Gao Y, Zhou Q, Qian G, DeCoster MA: Hypoxic preconditioning suppresses group III secreted phospholipase A₂-induced apoptosis via JAK2-STAT3 activation in cortical neurons. *J Neurochem* 2010;114:1039–1048.
- Ma MT, Nevalainen TJ, Yeo JF, Ong WY: Expression profile of multiple secretory phospholipase A₂ isoforms in the rat CNS: enriched expression of sPLA₂-IIA in brainstem and spinal cord. *J Chem Neuroanat* 2010;39:242–247.
- Kolko M, Christoffersen NR, Barreiro SG, Miller ML, Pizza AJ, Bazan NG: Characterization and location of secretory phospholipase A₂ groups IIE, V, and X in the rat brain. *J Neurosci Res* 2006;83:874–882.
- Thwin MM, Ong WY, Fong CW, Sato K, Kodama K, Farooqui AA, Gopalakrishnakone P: Secretory phospholipase A₂ activity in the normal and kainate injected rat brain, and inhibition by a peptide derived from python serum. *Exp Brain Res* 2003;150:427–433.
- Massicotte G, Baudry M: Triggers and substrates of hippocampal synaptic plasticity. *Neurosci Biobehav Rev* 1991;15:415–423.
- Fujita S, Ikegaya Y, Nishikawa M, Nishiyama N, Matsuki N: Docosahexaenoic acid improves long-term potentiation attenuated by phospholipase A₂ inhibitor in rat hippocampal slices. *Br J Pharmacol* 2001;132:1417–1422.
- Bliss TV, Collingridge GL: A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 1993;361:31–39.
- Dumuis A, Sebben M, Haynes L, Pin JP, Bockaert J: NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature* 1988;336:68–70.
- Bliss TV, Errington ML, Lynch MA, Williams JH: Presynaptic mechanisms in hippocampal long-term potentiation. *Cold Spring Harb Symp Quant Biol* 1990;55:119–129.
- Williams JH, Errington ML, Lynch MA, Bliss TV: Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission in the hippocampus. *Nature* 1989;341:739–742.
- Ma MT, Yeo JF, Farooqui AA, Zhang J, Chen P, Ong WY: Differential effects of lysophospholipids on exocytosis in rat PC12 cells. *J Neural Transm* 2010;117:301–308.
- Nomura T, Nishizaki T, Enomoto T, Itoh H: A long-lasting facilitation of hippocampal neurotransmission via a phospholipase A₂ signaling pathway. *Life Sci* 2001;68:2885–2891.
- Massicotte G, Oliver MW, Lynch G, Baudry M: Effect of bromophenacyl bromide, a phospholipase A₂ inhibitor, on the induction and maintenance of LTP in hippocampal slices. *Brain Res* 1990;537:49–53.
- Okada D, Yamagishi S, Sugiyama H: Differential effects of phospholipase inhibitors in long-term potentiation in the rat hippocampal mossy fiber synapses and Schaffer/commissural synapses. *Neurosci Lett* 1989;100:141–146.
- Molloy GY, Rattray M, Williams RJ: Genes encoding multiple forms of phospholipase A₂ are expressed in rat brain. *Neurosci Lett* 1998;258:139–142.
- Svensson CI, Lucas KK, Hua XY, Powell HC, Dennis EA, Yaksh TL: Spinal phospholipase A₂ in inflammatory hyperalgesia: role of the small, secretory phospholipase A₂. *Neuroscience* 2005;133:543–553.
- Yeo JF, Ong WY, Ling SF, Farooqui AA: Intracerebroventricular injection of phospholipase A₂ inhibitors modulates allodynia after facial carrageenan injection in mice. *Pain* 2004;112:148–155.
- Lin TN, Wang Q, Simonyi A, Chen JJ, Cheung WM, He YY, Xu J, Sun AY, Hsu CY, Sun GY: Induction of secretory phospholipase A₂ in reactive astrocytes in response to transient focal cerebral ischemia in the rat brain. *J Neurochem* 2004;90:637–645.
- Lauritzen I, Heurteaux C, Lazdunski M: Expression of group II phospholipase A₂ in rat brain after severe forebrain ischemia and in endotoxic shock. *Brain Res* 1994;651:353–356.
- Titworth WL, Cheng X, Ke Y, Deng L, Burckhardt KA, Pendleton C, Liu NK, Shao H, Cao QL, Xu XM: Differential expression of sPLA₂ following spinal cord injury and a functional role for sPLA₂-IIA in mediating oligodendrocyte death. *Glia* 2009;57:1521–1537.
- Moses GS, Jensen MD, Lue LF, Walker DG, Sun AY, Simonyi A, Sun GY: Secretory PLA₂-IIa: A new inflammatory factor for Alzheimer's disease. *J Neuroinflammation* 2006;3:28.
- Adibhatla RM, Hatcher JF: Secretory phospholipase A₂ IIA is up-regulated by TNF- α and IL-1 α/β after transient focal cerebral ischemia in rat. *Brain Res* 2007;1134:199–205.
- Thomas G, Bertrand F, Saunier B: The differential regulation of group IIA and group V low molecular weight phospholipases A₂ in cultured rat astrocytes. *J Biol Chem* 2000;275:10876–10886.
- Yagami T, Ueda K, Asakura K, Hata S, Kuroda T, Sakaeda T, Takasu N, Tanaka K, Gemba T, Hori Y: Human group IIA secretory phospholipase A₂ induces neuronal cell death via apoptosis. *Mol Pharmacol* 2002;61:114–126.
- Kolko M, de Turco EB, Diemer NH, Bazan NG: Secretory phospholipase A₂-mediated neuronal cell death involves glutamate ionotropic receptors. *Neuroreport* 2002;13:1963–1966.
- Yagami T, Ueda K, Asakura K, Nakazato H, Hata S, Kuroda T, Sakaeda T, Sakaguchi G, Itoh N, Hashimoto Y, Hori Y: Human group IIA secretory phospholipase A₂ potentiates Ca²⁺ influx through L-type voltage-sensitive Ca²⁺ channels in cultured rat cortical neurons. *J Neurochem* 2003;85:749–758.
- Madden DR: The structure and function of glutamate receptor ion channels. *Nat Rev Neurosci* 2002;3:91–101.
- Bettler B, Mülle C: Review: neurotransmitter receptors. II. AMPA and kainate receptors. *Neuropharmacology* 1995;34:123–139.
- Huettnner JE: Kainate receptors and synaptic transmission. *Prog Neurobiol* 2003;70:387–407.
- Keinanen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH: A family of AMPA-selective glutamate receptors. *Science* 1990;249:556–560.
- Iremonger KJ, Benediktsson AM, Bains JS: Glutamatergic synaptic transmission in neuroendocrine cells: basic principles and mechanisms of plasticity. *Front Neuroendocrinol* 2010;31:296–306.
- Lerma J: Roles and rules of kainate receptors in synaptic transmission. *Nat Rev Neurosci* 2003;4:481–495.
- Poli A, Contestabile A, Migani P, Rossi L, Rondelli C, Virgili M, Bissoli R, Barnabei O: Kainic acid differentially affects the synaptosomal release of endogenous and exogenous amino acid neurotransmitters. *J Neurochem* 1985;45:1677–1686.
- Caruso C, Durand D, Watanobe H, Lasaga M: NMDA and group I metabotropic glutamate receptors activation modulates substance P release from the arcuate nucleus and median eminence. *Neurosci Lett* 2006;393:60–64.
- Rodriguez-Moreno A, Sihra TS: Kainate receptors with a metabotropic modus operandi. *Trends Neurosci* 2007;30:630–637.
- Wei S, Ong WY, Thwin MM, Fong CW, Farooqui AA, Gopalakrishnakone P, Hong W: Group IIA secretory phospholipase A₂ stimulates exocytosis and neurotransmitter release in pheochromocytoma-12 cells and cultured rat hippocampal neurons. *Neuroscience* 2003;121:891–898.

- 41 Matsuzawa A, Murakami M, Atsumi G, Imai K, Prados P, Inoue K, Kudo I: Release of secretory phospholipase A₂ from rat neuronal cells and its possible function in the regulation of catecholamine secretion. *Biochem J* 1996;318:701–709.
- 42 Suzuki N, Ishizaki J, Yokota Y, Higashino K, Ono T, Ikeda M, Fujii N, Kawamoto K, Hanasaki K: Structures, enzymatic properties, and expression of novel human and mouse secretory phospholipase A₂s. *J Biol Chem* 2000;275:5785–5793.
- 43 Nyman KM, Ojala P, Laine VJ, Nevalainen TJ: Distribution of group II phospholipase A₂ protein and mRNA in rat tissues. *J Histochem Cytochem* 2000;48:1469–1478.
- 44 Holz RW, Axelrod D: Secretory granule behaviour adjacent to the plasma membrane before and during exocytosis: total internal reflection fluorescence microscopy studies. *Acta Physiol (Oxf)* 2008;192:303–307.
- 45 Ye F, Than A, Zhao Y, Goh KH, Chen P: Vesicular storage, vesicle trafficking, and secretion of leptin and resistin: the similarities, differences, and interplays. *J Endocrinol* 2010;206:27–36.
- 46 Zhang J, Xue R, Ong WY, Chen P: Roles of cholesterol in vesicle fusion and motion. *Biophys J* 2009;97:1371–1380.
- 47 Than A, Ye F, Xue R, Ong JW, Poh CL, Chen P: The crosstalks between adipokines and catecholamines. *Mol Cell Endocrinol* 2011;332:261–270.
- 48 Marqueze B, Boudier JA, Mizuta M, Inagaki N, Seino S, Seagar M: Cellular localization of synaptotagmin I, II, and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat. *J Neurosci* 1995;15:4906–4917.
- 49 Walch-Solimena C, Takei K, Marek KL, Midyett K, Sudhof TC, De Camilli P, Jahn R: Synaptotagmin: a membrane constituent of neuropeptide-containing large dense-core vesicles. *J Neurosci* 1993;13:3895–3903.
- 50 More JC, Nistico R, Dolman NP, Clarke VR, Alt AJ, Ogden AM, Buelens FP, Troop HM, Kelland EE, Pilato F, Bleakman D, Bortolotto ZA, Collingridge GL, Jane DE: Characterisation of UBP296: a novel, potent and selective kainate receptor antagonist. *Neuropharmacology* 2004;47:46–64.
- 51 Verdoorn TA, Johansen TH, Drejer J, Nielsen EO: Selective block of recombinant GluR6 receptors by NS-102, a novel non-NMDA receptor antagonist. *Eur J Pharmacol* 1994;269:43–49.
- 52 Foster DJ, Vitvitsky VM, Banerjee R, Heacock AM, Fisher SK: Muscarinic receptor regulation of osmosensitive taurine transport in human SH-SY5Y neuroblastoma cells. *J Neurochem* 2009;108:437–449.
- 53 Morgan A, Burgoyne RD, Barclay JW, Craig TJ, Prescott GR, Ciuflo LF, Evans GJ, Graham ME: Regulation of exocytosis by protein kinase C. *Biochem Soc Trans* 2005;33:1341–1344.
- 54 Wilkinson SE, Parker PJ, Nixon JS: Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem J* 1993;294:335–337.
- 55 Xue R, Zhao Y, Chen P: Involvement of PKC α in PMA-induced facilitation of exocytosis and vesicle fusion in PC12 cells. *Biochem Biophys Res Commun* 2009;380:371–376.
- 56 Hatori K, Nagai A, Heisel R, Ryu JK, Kim SU: Fractalkine and fractalkine receptors in human neurons and glial cells. *J Neurosci Res* 2002;69:418–426.
- 57 Murphy NP, McCormack JG, Ball SG, Vaughan PF: The effect of protein kinase C activation on muscarinic-M3- and K⁺-evoked release of [³H]noradrenaline and increases in intracellular Ca²⁺ in human neuroblastoma SH-SY5Y cells. *Biochem J* 1992;282:645–650.
- 58 Leonaritis G, Petrikos L, Mangoura D: Regulation of the Ras-GTPase activating protein neurofibromin by C-tail phosphorylation: implications for protein kinase C/Ras/extracellular signal-regulated kinase 1/2 pathway signaling and neuronal differentiation. *J Neurochem* 2009;109:573–583.
- 59 Gatta V, Drago D, Fincati K, Valenti MT, Dalle Carbonare L, Sensi SL, Zatta P: Microarray analysis on human neuroblastoma cells exposed to aluminum, β (1–42)-amyloid or the β (1–42)-amyloid aluminum complex. *PLoS One* 2011;6:e15965.
- 60 Agholme L, Lindstrom T, Kagedal K, Marcusson J, Hallbeck M: An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. *J Alzheimers Dis* 2010;20:1069–1082.
- 61 Pickel VM, Joh TH, Reis DJ, Leeman SE, Miller RJ: Electron microscopic localization of substance P and enkephalin in axon terminals related to dendrites of catecholaminergic neurons. *Brain Res* 1979;160:387–400.
- 62 Jarvi R, Helen P, Peltö-Huikko M, Hervonen A: Neuropeptide Y (NPY)-like immunoreactivity in rat sympathetic neurons and small granule-containing cells. *Neurosci Lett* 1986;67:223–227.
- 63 Schwab Y, Mouton J, Chasserot-Golaz S, Marty I, Maulet Y, Jover E: Calcium-dependent translocation of synaptotagmin to the plasma membrane in the dendrites of developing neurones. *Brain Res Mol Brain Res* 2001;96:1–13.
- 64 Maher BJ, Mackinnon RL 2nd, Bai J, Chapman ER, Kelly PT: Activation of postsynaptic Ca²⁺ stores modulates glutamate receptor cycling in hippocampal neurons. *J Neurophysiol* 2005;93:178–188.
- 65 Naarala J, Nykvist P, Tuomala M, Savolainen K: Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells. *FEBS Lett* 1993;330:222–226.
- 66 Nair VD, Niznik HB, Mishra RK: Interaction of NMDA and dopamine D2L receptors in human neuroblastoma SH-SY5Y cells. *J Neurochem* 1996;66:2390–2393.
- 67 Cannarsa R, Landuzzi D, Cavina C, Candelletti S, Romualdi P: Kainic acid down-regulates NOP receptor density and gene expression in human neuroblastoma SH-SY5Y cells. *J Mol Neurosci* 2008;35:171–177.
- 68 Kim SJ, Han Y: Insulin inhibits AMPA-induced neuronal damage via stimulation of protein kinase B (AKT). *J Neural Transm* 2005;112:179–191.
- 69 Tetich M, Kutner A, Leskiewicz M, Budziszewska B, Lason W: Neuroprotective effects of (24R)-1,24-dihydroxycholecalciferol in human neuroblastoma SH-SY5Y cell line. *J Steroid Biochem Mol Biol* 2004;89–90:365–370.
- 70 Singh J, Kaur G: Neuroprotection mediated by subtoxic dose of NMDA in SH-SY5Y neuroblastoma cultures: activity-dependent regulation of PSA-NCAM expression. *Brain Res Mol Brain Res* 2005;137:223–234.
- 71 Gao M, Zhang WC, Liu QS, Hu JJ, Liu GT, Du GH: Pinoscembrin prevents glutamate-induced apoptosis in SH-SY5Y neuronal cells via decrease of bax/bcl-2 ratio. *Eur J Pharmacol* 2008;591:73–79.
- 72 Sun ZW, Zhang L, Zhu SJ, Chen WC, Mei B: Excitotoxicity effects of glutamate on human neuroblastoma SH-SY5Y cells via oxidative damage. *Neurosci Bull* 2010;26:8–16.
- 73 Paternain AV, Herrera MT, Nieto MA, Lerma J: GluR5 and GluR6 kainate receptor subunits coexist in hippocampal neurons and coassemble to form functional receptors. *J Neurosci* 2000;20:196–205.
- 74 Sommer B, Burnashev N, Verdoorn TA, Keinänen K, Sakmann B, Seeburg PH: A glutamate receptor channel with high affinity for domoate and kainate. *EMBO J* 1992;11:1651–1656.
- 75 Vignes M, Collingridge GL: The synaptic activation of kainate receptors. *Nature* 1997;388:179–182.
- 76 Jakobsen B, Tasker A, Zimmer J: Domoic acid neurotoxicity in hippocampal slice cultures. *Amino Acids* 2002;23:37–44.
- 77 Wondolowski J, Frerking M: Subunit-dependent postsynaptic expression of kainate receptors on hippocampal interneurons in area CA1. *J Neurosci* 2009;29:563–574.
- 78 Burgoyne RD, Morgan A: Secretory granule exocytosis. *Physiol Rev* 2003;83:581–632.
- 79 Misonou H, Ohara-Imaizumi M, Murakami T, Kawasaki M, Ikeda K, Wakai T, Kumakura K: Protein kinase C controls the priming step of regulated exocytosis in adrenal chromaffin cells. *Cell Mol Neurobiol* 1998;18:379–390.
- 80 Bendotti C, Baldessari S, Samanin R, De Biasi S: Ultrastructural immunolocalization of GAP-43 in the sprouted mossy fibres of kainic acid lesioned rats. *Neuroreport* 1994;5:2645–2648.

- 81 McNamara RK, Routtenberg A: NMDA receptor blockade prevents kainate induction of protein F1/GAP-43 mRNA in hippocampal granule cells and subsequent mossy fiber sprouting in the rat. *Brain Res Mol Brain Res* 1995;33:22–28.
- 82 Melyan Z, Wheal HV, Lancaster B: Metabotropic-mediated kainate receptor regulation of IsAHP and excitability in pyramidal cells. *Neuron* 2002;34:107–114.
- 83 Cho K, Francis JC, Hirbec H, Dev K, Brown MW, Henley JM, Bashir ZI: Regulation of kainate receptors by protein kinase C and metabotropic glutamate receptors. *J Physiol* 2003;548:723–730.
- 84 Massicotte G, Vanderklish P, Lynch G, Baudry M: Modulation of DL- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/quisqualate receptors by phospholipase A₂: a necessary step in long-term potentiation? *Proc Natl Acad Sci USA* 1991;88:1893–1897.
- 85 Wolf MJ, Izumi Y, Zorumski CF, Gross RW: Long-term potentiation requires activation of calcium-independent phospholipase A₂. *FEBS Lett* 1995;377:358–362.
- 86 Li P, Wilding TJ, Kim SJ, Calejesan AA, Huettner JE, Zhuo M: Kainate-receptor-mediated sensory synaptic transmission in mammalian spinal cord. *Nature* 1999;397:161–164.
- 87 Hegarty DM, Mitchell JL, Swanson KC, Aicher SA: Kainate receptors are primarily postsynaptic to SP-containing axon terminals in the trigeminal dorsal horn. *Brain Res* 2007;1184:149–159.
- 88 Sutton JL, Maccacchini ML, Kajander KC: The kainate receptor antagonist 2S,4R-4-methylglutamate attenuates mechanical allodynia and thermal hyperalgesia in a rat model of nerve injury. *Neuroscience* 1999;91:283–292.
- 89 Ta LE, Dionne RA, Friction JR, Hodges JS, Kajander KC: Sym-2081 a kainate receptor antagonist reduces allodynia and hyperalgesia in a freeze injury model of neuropathic pain. *Brain Res* 2000;858:106–120.
- 90 Simmons RM, Li DL, Hoo KH, Deverill M, Ornstein PL, Iyengar S: Kainate GluR5 receptor subtype mediates the nociceptive response to formalin in the rat. *Neuropharmacology* 1998;37:25–36.
- 91 Ko S, Zhao MG, Toyoda H, Qiu CS, Zhuo M: Altered behavioral responses to noxious stimuli and fear in glutamate receptor 5 (GluR5)- or GluR6-deficient mice. *J Neurosci* 2005;25:977–984.