Title

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Journal

Neuroscience, 133(2)

ISSN

0306-4522

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Publication Date

2005

DOI

10.1016/j.neuroscience.2005.01.024

Peer reviewed

Neuroscience 133 (2005) 543–553

SPINAL PHOSPHOLIPASE A_2 IN INFLAMMATORY HYPERALGESIA: ROLE OF THE SMALL, SECRETORY PHOSPHOLIPASE A_2

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Abstract—Current work emphasizes that peripheral tissue injury and inflammation results in a heightened sensitivity to subsequent noxious input (hyperalgesia) that is mediated in large part by the spinal synthesis and release of eicosanoids, in particular prostaglandins. Secreted phospholipase A_2s ($sPLA_2$s) form a class of structurally related enzymes that release arachidonic acid from cell membranes that is further processed to produce eicosanoids. We hypothesized that spinal $sPLA_2$s may contribute to inflammation-induced hyperalgesia. Spinal cord tissue and cerebrospinal fluid were collected from rats for assessment of $sPLA_2$ protein expression and $sPLA_2$ activity. A basal $sPLA_2$ protein expression and activity was detected in spinal cord homogenate (87±17 pmol/min/mg), though no activity could be detected in cisternal cerebrospinal fluid, of naive rats. The $sPLA_2$ activity did not change in spinal cord tissue or cerebrospinal fluid assessed over 8 h after injection of carrageenan into the hind paw. However, the $sPLA_2$ activity observed in spinal cord homogenates was suppressed by addition of LY311727, a selective $sPLA_2$ inhibitor. To determine the role of this spinal $sPLA_2$ in hyperalgesia, we assessed the effects of lumbar intrathecal (IT) administration of LY311727 in rats with chronic IT catheters in three experimental models of hyperalgesia. IT LY311727 (3–30 μg) dose-dependently prevented intraplantar carrageenan-induced thermal hyperalgesia and formalin-induced flinching, at doses that had no effect on motor function. IT LY311727 also suppressed thermal hyperalgesia induced by IT injection of substance P (30 nmol). Using *in vivo* spinal microdialysis, we found that IT injection of LY311727 attenuated prostaglandin E_2 release into spinal dialysate otherwise evoked by the IT injection of substance P. Taken together, this work points to a role for constitutive $sPLA_2$s in spinal nociceptive processing. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: phospholipase A_2, substance P, formalin, carrageenan, PGE_2, spinal cord.

The phospholipase A_2 (PLA_2) superfamily of enzymes is defined by its ability to cleave fatty acids from the *sn-2* position of phospholipids, producing free fatty acids and lysophospholipids. These enzymes are divided into 14 groups that comprise three major types: the cytosolic, calcium-dependent PLA_2s ($cPLA_2$), the cytosolic, calcium-independent PLA_2s ($iPLA_2$) and the secreted, calcium-dependent PLA_2 ($sPLA_2$) (Six and Dennis, 2000; Balsinde et al., 2002). $sPLA_2$s form a class of structurally related enzymes that have been implicated in a variety of physiological and pathological effects such as modification of cell proliferation, cell adhesion, inflammation, rheumatoid arthritis, atherosclerosis, and cancer (Kudo and Murakami, 2002). A number of mammalian $sPLA_2$s have been identified to date: groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, XII (Gelb et al., 2000; Six and Dennis, 2000; Ho et al., 2001). In general, different $sPLA_2$ isoforms exhibit tissue-specific and species-specific expression, which suggests that their cellular behaviors and functions differ (Valentin et al., 1999). Group IB $sPLA_2$ is abundant in pancreas and its main function is thought to be digestion of dietary phospholipids (Kramer et al., 1989). Both group IIA and group V $sPLA_2$ have been shown to release arachidonic acid (AA) from cell membranes that is further processed to produce eicosanoids (Balsinde and Dennis, 1996; Naraba et al., 1998) and are believed to be key $sPLA_2$ enzymes in the inflammatory cascade. However, the physiological function of most of the $sPLA_2$ enzymes remains to be determined.

Tissue injury and inflammation results in a heightened sensitivity to subsequent noxious input (hyperalgesia) where eicosanoids, in particular prostaglandins (PG), are known to play an important role. It is thought that PG augment nociceptive processing by actions both at the peripheral site of injury where they sensitize afferent terminals, as well as in the spinal cord, where they increase evoked excitability (Nicol et al., 1992; Baba et al., 2001). In previous work (Lucas et al., 2004) we observed that intrathecal (IT) injection of inhibitors blocking both group IVA $cPLA_2$, and group VI $iPLA_2$ activity attenuated inflammation-induced hyperalgesia as well as stimulus-evoked spinal prostaglandin E_2 (PGE_2) release. Specific inhibition of group VI $iPLA_2$ had no effect on hyperalgesia or PGE_2 release, suggesting that group IVA $cPLA_2$ mediates spinal

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Abbreviations: AA, arachidonic acid; ACSF, artificial cerebrospinal fluid; AUC, area under the curve; COX, cyclooxygenase; $cPLA_2$, calcium-dependent phospholipase A_2; CSF, cerebrospinal fluid; DPPC, 1-palmitoyl-2-palmitoyl-diacyl-*sn*-glycero-3-phosphatidylcholine; HI, hyperalgesic index; $iPLA_2$, calcium-independent phospholipase A_2; IT, intrathecal; MAFP, methyl arachidonoyl fluorophosphonate; PAPC, 1-palmitoyl-2-arachidonoyl-diacyl-*sn*-glycero-3-phosphatidylcholine; PG, prostaglandins; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA_2, phospholipase A_2; PLPE, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl -*sn*-glycero-3-phosphatidylserine; PWL, paw withdrawal latency; SP, substance P; $sPLA_2$, secretory phospholipase A_2; SUVs, small unilamellar vesicles.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.01.024

sensitization. This indicates that when the availability of cyclooxygenase (COX) substrate (AA) is diminished, there is a reduction in PG synthesis and a following modulation of nociception. The functional importance of spinal PG in this process has been shown in behavioral models of injury- and inflammation-induced hyperalgesia, where inhibition of spinal COX normalizes the otherwise enhanced pain sensitivity (Malmberg and Yaksh, 1992; Kumar et al., 1997; Yamamoto and Nozaki-Taguchi, 1997; Yaksh et al., 1998; Samad et al., 2001). Accumulating evidence further suggests that group IVA cPLA$_2$ together with group IIA and V sPLA$_2$ are functionally coupled with COX-1 and COX-2 pathways, for immediate and delayed PG biosynthesis (Kuwata et al., 1998). For example, it has been demonstrated that carrageenan- and carrageenin-induced inflammation and PGE$_2$ production is blocked by group II sPLA$_2$ inhibitors (Miyake et al., 1993; Garcia-Pastor et al., 1999). Findings indicating that expression of group IIA sPLA$_2$ is markedly induced upon exposure to proinflammatory stimuli in several different cell types, including vascular smooth muscle cells (Nakano and Arita, 1990), astrocytes (Oka and Arita, 1991), macrophages (Arbibe et al., 1997), mesangial cells (Vervoordeldonk et al., 1996), and fibroblasts (Kuwata et al., 1998), further supports a role for sPLA$_2$ in inflammatory responses. We hypothesize that sPLA$_2$ activity participates in the facilitation of spinal pain processing through modulation of PGE$_2$ synthesis. The focus of the present work is to measure sPLA$_2$ activity in the spinal cord prior to and after peripheral inflammation and to determine if inhibition of spinal sPLA$_2$ affects inflammation-induced hyperalgesia and/or PGE$_2$ synthesis.

EXPERIMENTAL PROCEDURES

Animal experiments

These were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (La Jolla, CA, USA) and were in compliance with AALAC guidelines for animal use. All studies were performed in such a manner as to minimize group size. Male Holtzman Sprague–Dawley rats (250–350 g; Harlan Industries, Madison, WI, USA) were housed pair-wise in cages and maintained on a 12-h light/dark cycle with free access to food and water at all times.

Tissue preparation and collection of cerebrospinal fluid (CSF)

Prior to killing, the rats were deeply anesthetized and after decapitation the spinal cords were ejected from the spinal column by a saline-filled syringe. The lumbar part of the spinal cord was frozen on dry ice and stored at −70 °C. Frozen spinal cords were pulverized using a BioPulverizor (Biospec Products, Bartlesvill, OK, USA) pre-chilled on dry ice. Pulverized tissue was then transferred to a microcentrifuge tube and mixed with 750 μl lysis buffer: 10 mM HEPES, pH 7.5, 1 mM EDTA, and 0.34 M sucrose. Twenty microliters mammalian protease inhibitor cocktail (Sigma, St. Louis, MO, USA) was added immediately. Samples were vortexed and sonicated until homogenous and then centrifuged at 16,000×*g*, 4 °C, 40 min. Supernatants were transferred to a fresh Eppendorf tube and the pellet was discarded. Cerebrospinal fluid (CSF) was withdrawn from the cisterna magna. Rats were anesthetized and a microcapillary tube with a pulled tip was used for puncture of the cisternal membrane; and 50–75 μl CSF was collected from each rat and immediately frozen on dry ice and stored at −70 °C.

Western blot

Prior to killing, rats were deeply anesthetized and after decapitation the spinal cords were ejected from the vertebral column by a saline-filled syringe. The lumbar part of the spinal cord was immediately homogenized in extraction buffer (50 mM Tris buffer, containing 0.5% Triton X-100, 150 mM NaCl, 10 mM DTT, 1 mM EDTA and protease inhibitors) by sonication. The tissue extracts were subjected to denaturing NuPAGE 10% Bis–Tris gel electrophoresis (50 μg total protein loaded per well) and then electrophoretically transferred to nitrocellulose membranes (Micronic Separation Inc. Westborough, MA, USA). After blocking nonspecific binding sites with 5% low-fat milk in PBS containing 0.1% Tween 20 for 1 h in room temperature, the membranes were probed with antibodies (group IIA sPLA$_2$ and group V sPLA$_2$, 1:500; Cayman Chemical, Ann Arbor, MI, USA) overnight at 4 °C. After washing, the antibody–protein complexes were probed with secondary antibodies labeled with horseradish peroxidase for 1 h at room temperature and detected with chemiluminescent reagents. The nitrocellulose membranes were stripped with Re-Blot Western blot recycling kit (Chemicon, Temecula, CA, USA) and reblotted b-actin (1:10,000, Sigma, St. Louis, MO, USA).

PLA$_2$ activity assays

Following homogenization and centrifugation, the spinal cord supernatants were assayed for sPLA$_2$ activity using a modification of the group specific IIA assay. CSF samples were thawed on ice and assayed without further handling.

Non-radioactive lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA): 1-palmitoyl-2-arachidonoyl-diacyl-*sn*-glycero-3-phosphatidylcholine (PAPC), 1-palmitoyl-2-palmitoyl-diacyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylethanolamine (PLPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS). Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) was purchased from Roche Applied Science (Palo Alto, CA, USA). Radioactive PAPC was purchased from Perkin Elmer Life Sciences (Boston, MA, USA): phosphatidylcholine, L-α-1-palmatoyl-2-arachidonyl, [arachidonyl-1-^{14}C]. Radioactive DPPC and PLPE were purchased from Amersham Biosciences (Piscataway, NJ, USA): phosphatidylcholine, 1-palmitoyl-2-[^{14}C] palmitoyl; 1-palmitoyl-2-[^{14}C] linoleoyl-phosphatidyl ethanolamine. Triton X-100 was purchased from Calbiochem (San Diego, CA, USA). Bromoenolactone and methyl arachidonoyl fluorophosphonate (MAFP) were purchased from Cayman Chemical. LY311727 was a kind gift from Lilly Research Laboratories.

Briefly, the sPLA$_2$ assay conditions were: 100 μM lipid PLPE/POPS (1/1) spiking with 100,000 cpm ^{14}C-labeled PLPE, 100 mM HEPES, pH 7.5, 1 mM Ca^{2+}, 1 mg/ml BSA, and 0.8 μM MAFP. The group VI iPLA$_2$ assay conditions were: 100 μM DPPC spiking with 100,000 cpm ^{14}C-labeled DPPC in 400 μM Triton X-100 mixed micelles, 100 mM HEPES, pH 7.5, 5 mM EDTA, and 1 mM ATP. The group IV cPLA$_2$ assay conditions were: 100 μM lipid PAPC/PIP$_2$ (97/3) spiking with 100,000 cpm ^{14}C-labeled PAPC in 400 μM Triton X-100 mixed micelles, 100 mM HEPES, pH 7.5, 0.08 mM $CaCl_2$, 0.1 mg/ml BSA and 2 mM DTT. The total volume for each assay was 500 μl: 200 μl lipid, 250 μl assay buffer, 50 μl sample. In each case the amount of calcium added was adjusted to account for the addition of EDTA in the lysis buffer to give the final concentration listed above.

Lipid preparation

Lipid was aliquoted, dried under N_2 and lyophilized for at least 1 h to remove all traces of chloroform. For mixed micelles the lipid was then resuspended in 100 mM HEPES and Triton X-100 and mi-

celles were created by repeated vortexing and heating in hot water until the solution clarified. For small unilamellar vesicles (SUVs) the lipid was resuspended in 100 mM HEPES, vortexed for 10 min and then sonicated on ice until the solution cleared. The preparation was then tested by centrifuging an aliquot at 15,000×*g*, R/T for 5 min. The radioactivity of a 5 μl sample of the centrifuged samples as well as a 5 μl sample of the original preparation was determined by scintillation counting and compared. If SUVs do not form properly they will precipitate upon centrifugation and the respective radioactive counts will be significantly different. Substrate was used only if the counts were within 200 cpm of each other.

Modified Dole assay

Samples were incubated with substrate for 1 h at 40 °C in a shaking bath. The assay was then terminated by addition of 2.5 ml Dole reagent (isopropyl alcohol:heptane:0.5 M sulfuric acid 400:100:20, V:V:V). Silica gel (0.1–0.2 mg) was added to each tube followed by 1.5 ml heptane and 1.5 ml deionized water. Each tube was vortexed for 15 s. One milliliter of the organic phase was removed and passed through a Pasteur pipette filled with silica gel (0.1–0.2 mg). This column was then washed with 1 ml diethyl ether. Five milliliters of scintillation cocktail (Biosafe II; RPI, Mount Prospect, IL, USA) was then added to the eluent and the radioactivity was determined by scintillation counting.

For the inhibitor studies, the same phospholipid and buffer conditions were used as above with the addition of 0.8 μM (0.8 mol percent) of the $sPLA_2$ inhibitor: LY311727 (dissolved in DMSO). LY311727 was a kind gift from Dr. Jerome Fleisch, Lilly Research Laboratories.

Intrathecal catheter implantation

For IT drug delivery, chronic lumbar catheters were implanted in rats under isoflurane anesthesia according to a modification of the procedure described by Yaksh and Rudy (1976). A polyethylene catheter (PE-5) was inserted through an incision in the atlanto-occipital membrane and advanced caudally to the rostral edge of the lumbar enlargement. Studies involving rats with chronic IT catheters were carried out 5 days after implantation. Rats were housed individually after implantation under the same conditions described above. Exclusion criteria were i) presence of any neurological sequel ii) 20% weight loss after implantation or iii) catheter occlusion.

Drugs and delivery

LY311727 was dissolved in 5% CremophorEL (Sigma, St. Louis, MO, USA) and 5% DMSO (Sigma, St. Louis, MO, USA) in physiological saline and delivered intrathecally in volumes of 10 μl followed by a 10-μl flush using saline. Substance P (SP; 30 nmol, Sigma, St. Louis, MO, USA) was dissolved in physiological saline.

Induction of inflammation and assessment of hyperalgesia

To induce a state of local inflammation, 2 mg of carrageenan (Sigma, St. Louis, MO, USA; 100 μl of 2% solution [w/v] in physiological saline) was injected s.c. into the plantar surface of the left hind paw. To assess the thermally evoked paw-withdrawal response, a device modeled after that described by Hargreaves et al. (1988) was used (Dirig and Yaksh, 1995; Dirig et al., 1997). The device consists of a glass surface (maintained at 25 °C) on which the rats were placed individually in Plexiglas cubicles. The thermal nociceptive stimulus originates from a focused projection bulb positioned below the glass surface. A timer is actuated with the light source, and latency was defined as the time required for the paw to show a brisk withdrawal as detected by photodiode motion sensors that stopped the timer and terminated the stimulus. Basal paw withdrawal latencies (PWL) were assessed at time (t)=−20 min. At t=−10 min the animals received IT vehicle or LY311727 and at t=0 the carrageenan was injected intraplantar. Withdrawal latencies were then assessed at t=60, 90,120, 150, 180 and 240 min and expressed as PWL. The data were also presented as hyperalgesic index (HI). The HI is a calculation, which defines the magnitude of carrageenan-induced sensitization. It represents the area under the time effect-curve after stimulation in which the "percent reduction from baseline (e.g. Pre-carrageenan) response latency" is plotted versus time. The resulting metric is percent change×min. The formula for calculating the percent change is: (baseline latency−post drug latency)×100/(baseline latency) where latency is expressed in seconds. Increasing values show increasing hyperalgesia.

Formalin-induced flinching

Flinching was assessed by an automated detection system (Yaksh et al., 2001). A soft metal band is placed on the hind paw of the animal being tested. Animals are allowed to acclimate in individual Plexiglas chambers for 1 h before being moved to a test chamber. Just before the animal's placement into the test chamber, it is briefly restrained in a cloth towel, and 5% formalin (50 μl) is injected into the dorsal side of the banded paw. Data collection is initiated after the animal is placed inside the test chamber. Pain behavior was quantified by counting the incidences of spontaneous flinching or shaking of the injected paw. The flinches were counted for 1-min periods for 60 min. Two phases of spontaneous flinching of the injected paw were observed after formalin injection and defined as phase 1 (0–9 min) and phase 2 (10–60 min). For analysis, the total flinches for the phase 1 and phase 2 are calculated for each animal and these data are used for statistical comparison.

Intrathecal dialysis and PGE_2 assay

To assess the release of PGE_2 from the lumbar IT space of the unanesthetized rat, animals were prepared with indwelling triple lumen dialysis probes. In brief, each probe consisted of a triple lumen length of polyethylene tubing. The two outer lumens were connected by a loop of dialysis tubing. The middle lumen was used for local drug delivery. The probe was implanted as described above for the IT catheter and externalized on the back of the neck. Details of this probe system and its validation are provided elsewhere (Marsala et al., 1995; Hua et al., 1999; Koetzner et al., 2004). Dialysis experiments were conducted in unanesthetized rats 3 days after the implant. A syringe pump (Harvard, Natick, MA, USA) was connected and dialysis tubing was perfused with artificial CSF (ACSF) at a rate of 10 μl/min. The ACSF contained (mM) 151.1 Na^+, 2.6 K^+, 0.9 Mg^{2+}, 1.3 Ca^{2+}, 122.7 Cl^-, 21.0 HCO_3 and 2.5 HPO_4, and it was bubbled with 95% O_2/5% CO_2 before each experiment to adjust the final pH to 7.2. The efflux (20 min per fraction) was collected in an automatic fraction collector (Eicom, Kyoto, Japan) at 4 °C. Two baseline samples were collected following a 30-min washout, and an additional three fractions after IT injection of SP (30 nmol) through the central lumen of the dialysis catheter. LY311727 was delivered IT 15 min prior to SP in a solution of 5% Cremophor EI, 5% DMSO in saline. The concentration of PGE_2 in spinal dialysate was measured by ELISA using a commercially available kit (Assay Designs 90001; Assay Designs, Ann Arbor, MI, USA). The antibody is selective for PGE_2 with less than 2.0% cross-reactivity to $PGF_{1\alpha}$, $PGF_{2\alpha}$, 6-keto$PGF_{1\alpha}$, PGA_2 or PGB_2, but cross-reacts with PGE_1 and PGE_3.

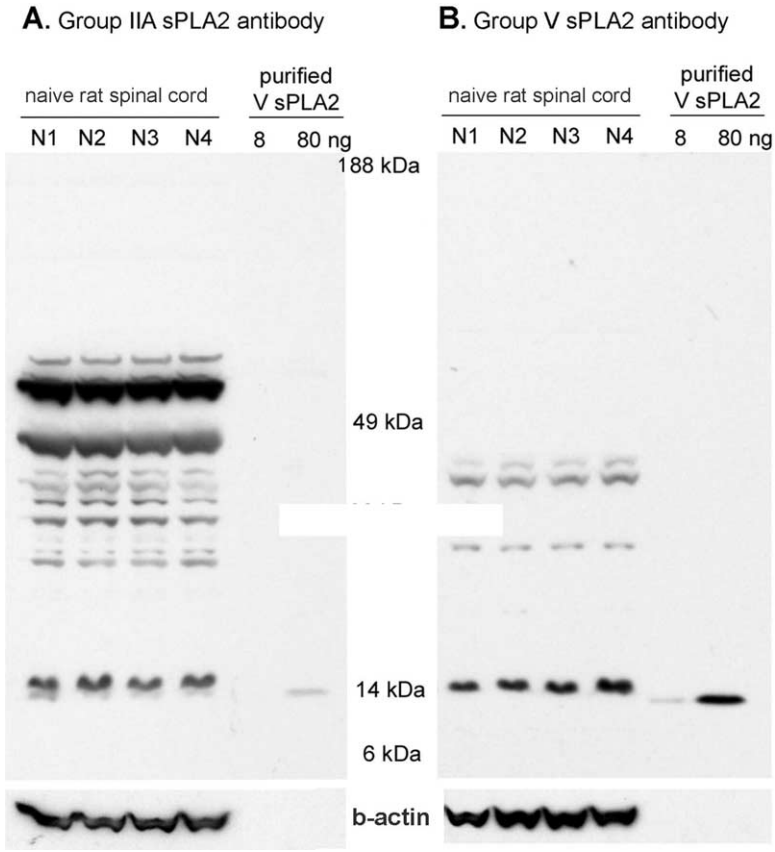

Fig. 1. Two Western blots showing presence of $sPLA_2$ protein expression in naive rat spinal cord using two different antibodies; (A) group IIA $sPLA_2$ antibody and (B) group V $sPLA_2$ antibody. Human purified group V $sPLA_2$ was loaded as control.

Statistics

Five to nine rats were included in each activity assay as is specified and assayed in triplicate. Values represent the average±standard deviation. Differences between groups were compared with one-way ANOVA or Student's *t*-test using Prism statistical software. For behavioral studies and PGE_2 release measurements four to eight animals were included per group and each time point and bar represents mean±standard error of mean. Differences between groups were compared with one-way ANOVA and Tukey post hoc test using Prism statistical software if nothing else is specified.

RESULTS

Spinal $sPLA_2$ protein expression and activity

We have previously shown that groups IB, IIA IIC and V $sPLA_2$ are present in naive rat spinal cord (Lucas et al., 2004). To determine if $sPLA_2$ protein is expressed in rat spinal cord, we performed Western blotting using spinal cord homogenates from four naive rats. Unfortunately, there are few antibodies available that specifically recognize rat $sPLA_2$. Here we used a group IIA and a group V $sPLA_2$ antibody (Cayman Chemical) and both antibodies labeled protein running at 14 kDa (Fig. 1A,B). Group IIA $sPLA_2$ is not available as purified protein so only purified human group V $sPLA_2$ was loaded as control. The group V $sPLA_2$ antibody recognized purified human group V $sPLA_2$ (Fig. 1B), indicating that the immunoreactive bands in the lanes for spinal cord homogenates are constitutively expressed group V $sPLA_2$. The group IIA $sPLA_2$ antibody showed some cross-reactivity with human group V $sPLA_2$, as a weak band was also observed at about 14 kDa in the lane where purified human group V $sPLA_2$ protein was loaded (Fig. 1A). The human recombinant group V sPLA2 positive control ran slightly faster than group V sPLA2 detected in the spinal cord homogenate. The construct used for synthesis of this protein lacks the signal peptide and is therefore 18 amino acids shorter than the endogenous form (Chen et al., 1994; Balsinde et al., 1999b). This resulted in a protein that is about 13.5 kDa and may explain why it runs slightly faster than group V $sPLA_2$ (14 kDa) in the spinal cord homogenate. In addition, the control is purified human group V $sPLA_2$ while the samples are rat group V $sPLA_2$. Accordingly, the different migration rates may be due to species differences. Both antibodies, in particular the group IIA $sPLA_2$ antibody, also gave rise to bands at higher molecular weights. It is possible that sPLA2 forms aggregates that are not disassembled by the denaturing conditions, or that these antibodies possess non-specific binding.

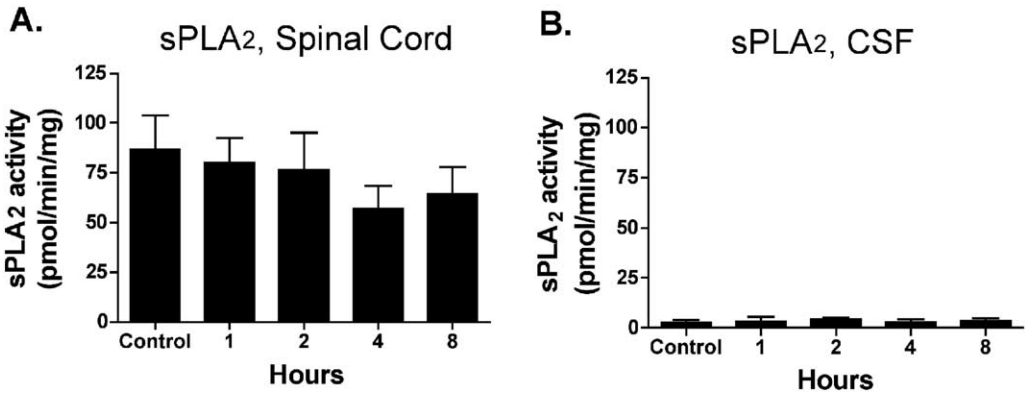

Fig. 2. $sPLA_2$ activity of (A) rat lumbar spinal cord and (B) CSF. Constitutive enzyme activity on PLPE/POPS (1/1) SUVs was observed following carrageenan injection to the hind paw over an 8 h time course in comparison with control. Each bar represents the average and standard deviation of five rats assayed in triplicate.

To test for $sPLA_2$ activity in the spinal cord a modified Dole assay was utilized that favors $sPLA_2$ over $cPLA_2$ or $iPLA_2$ activity (Yang et al., 1999); 0.8 mol% MAFP was added to this assay to eliminate any possible $cPLA_2$ or $iPLA_2$ activity that could contribute to phospholipid hydrolysis, ensuring that the observed activity can be attributed with confidence to $sPLA_2$.

Lumbar spinal homogenates and CSF were assayed from control rats or carrageenan-treated rats killed 1, 2, 4 or 8 h after injection of carrageenan to the hind paw. The lumbar spinal cord homogenate shows significant $sPLA_2$ activity with no measurable change following carrageenan treatment (Fig. 2A). The CSF had no significant $sPLA_2$ at any time point measured (Fig. 2B).

In vitro inhibition of $sPLA_2$ activity

To verify that the $sPLA_2$ inhibitor LY311727 was indeed acting to inhibit spinal $sPLA_2$ activity, spinal homogenates from untreated rats were assayed for $sPLA_2$, $iPLA_2$ and $cPLA_2$ activity in the presence or absence of the inhibitor (Fig. 3A–C). At concentrations above 1 mol% total substrate surface, inhibitors can interfere with the presentation of the lipid substrate, resulting in non-specific inhibition. In order to prevent non-specific effects due to high surface concentrations, the inhibitor was tested at 0.8 μM (0.8 mol% total substrate surface). LY311727 substantially decreased the $sPLA_2$ activity of rat lumbar spinal cord homogenates but has no effect on $iPLA_2$ or $cPLA_2$ activity.

Inhibition of spinal $sPLA_2$ prevents carrageenan-induced thermal hyperalgesia

When carrageenan is injected into the plantar surface of a paw, a characteristic inflammation and associated thermal hyperalgesia is produced. The mean baseline PWL prior to carrageenan injection was 12.7 ± 0.4 s ($n=23$). Two hours after carrageenan injection into the paw a significant reduction in PWL to 3.4 ± 0.6 s was observed in vehicle-pretreated animals, and this hyperalgesia lasted throughout the study (Fig. 4A). Intrathecal pretreatment with LY311727 (3–30 μg) resulted in a dose-dependent blockade of the onset of carrageenan-induced thermal hyperalgesia (Fig. 4A). The HI was calculated for each dose over the time period 0–240 min. As indicated, 10 min pretreatment with LY311727 produced a statistically significant dose-dependent anti-hyperalgesia (Fig. 4B). Importantly, this effect was observed only on the inflamed paw. There were no significant changes, even at the highest dose of either compound, in the response latency of the uninflamed, contralateral paw (Fig. 4B). Thus, the effect of LY311727 is antihyperalgesic rather than analgesic.

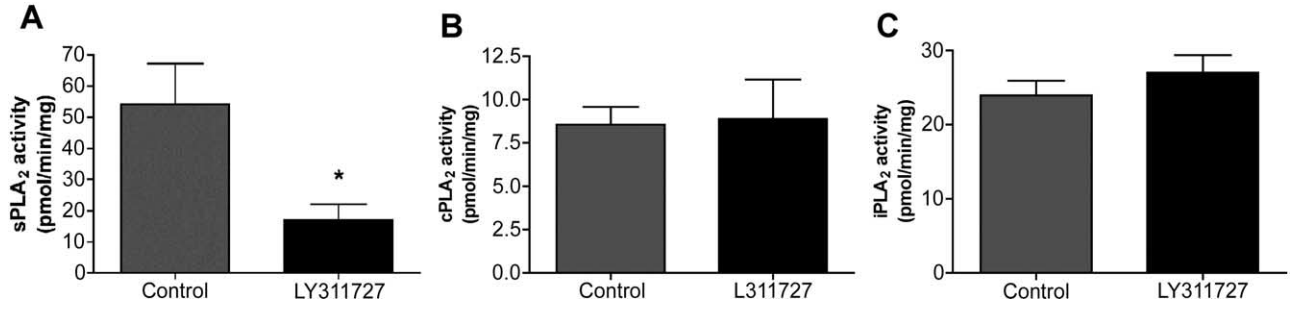

Fig. 3. Inhibition of spinal PLA_2 activity *in vitro*. Rat lumbar spinal cord homogenates were assayed for (A) $sPLA_2$ (B) $cPLA_2$ (C) $iPLA_2$ activity in the presence of 0.8 mol% LY311727 (presented with substrate). Each bar represents the average and standard deviation of nine rats assayed in triplicate and (*) represents $P<0.0001$ versus control conditions.

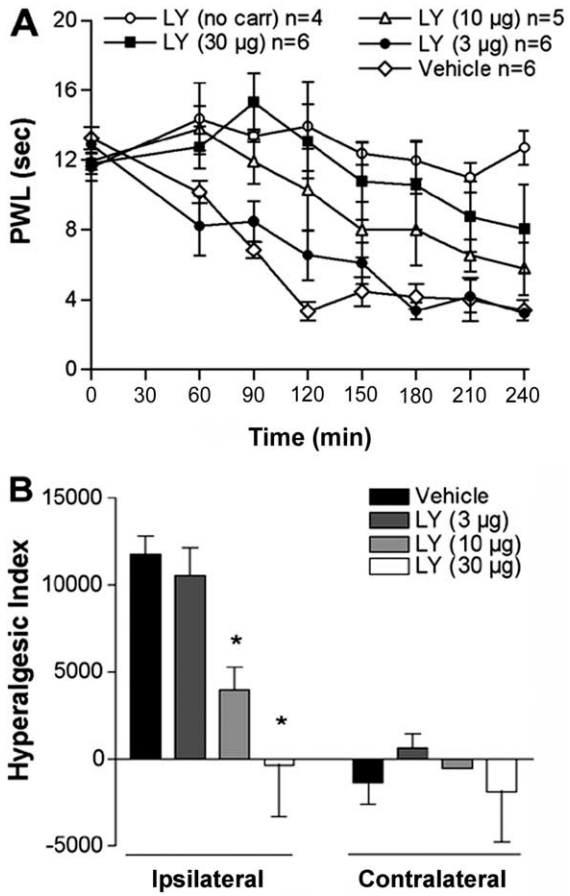

Fig. 4. Effect of $sPLA_2$ inhibition on carrageenan-induced thermal hyperalgesia. (A) PWL plotted versus time after the injection of intraplantar carrageenan in to left hind paw of rats pretreated (−10 min) with IT vehicle or LY311727 (LY; 3–30 μg). One control group received IT LY but no carrageenan. (B) HI (see Experimental Procedures) observed after different doses of IT LY7. (*) Represents $P<0.05$ versus vehicle treated carrageenan-injected group. Each time point and bar represents mean±S.E.M.; $n=4-6$ rats per group.

Inhibition of spinal $sPLA_2$ attenuates formalin-induced hyperalgesia

Injection of formalin into the paw results in a biphasic increase in the activity of dorsal horn wide dynamic range neurons (Haley et al., 1990), and a parallel biphasic appearance of paw flinching where phase two represents spinal sensitization (Yaksh et al., 2001). In order to study the effect of $sPLA_2$ inhibition on formalin-induced hyperalgesia LY311727 was injected IT 10 min prior to injection of formalin into the paw to the hind paw. The significance of $sPLA_2$ activity in spinal pain processing is indicated by the observation that IT injection of LY311727 prior to paw formalin injection resulted in a potent dose-dependent attenuation of the second phase of flinching behavior (Fig. 5A). In Fig. 5B the total number of flinches during phase I (1–9 min) and phase II (10–60 min after formalin injection) is shown. Intrathecal administration of LY311727 did not affect flinching frequency during phase I while the highest dose of LY311727 significantly reduced the total number of flinches during phase II (from 944±114 [IT vehicle] to 477±75 [IT LY311727 30 μg], $P<0.05$).

Inhibition of spinal $sPLA_2$ inhibits both thermal hyperalgesia and spinal PGE_2 release induced by IT injection of SP

To directly induce central sensitization, rats received IT injections of SP (30 nmol). This resulted in a decrease in PWL to thermal stimulation, suggesting hyperalgesia (Fig. 6A). To determine the capacity of LY311727 to antagonize this sensitization 30 μg LY311727 was injected IT 10 min prior to IT injection of SP. As shown in Fig. 6A, IT LY311727 partially blocked SP-induced hyperalgesia. Calculation of HI for 0–60 min post-SP revealed that IT LY311727 (30 μg) partly prevented IT SP thermal hyperalgesia ($P<0.05$; Fig. 6B).

We have previously shown that IT injection of SP gives rise to a pronounced increase of PGE_2 in CSF collected by spinal dialysis (Hua et al., 1999). Here again we show that IT injection of SP (0.6 μg; Fig. 5C)

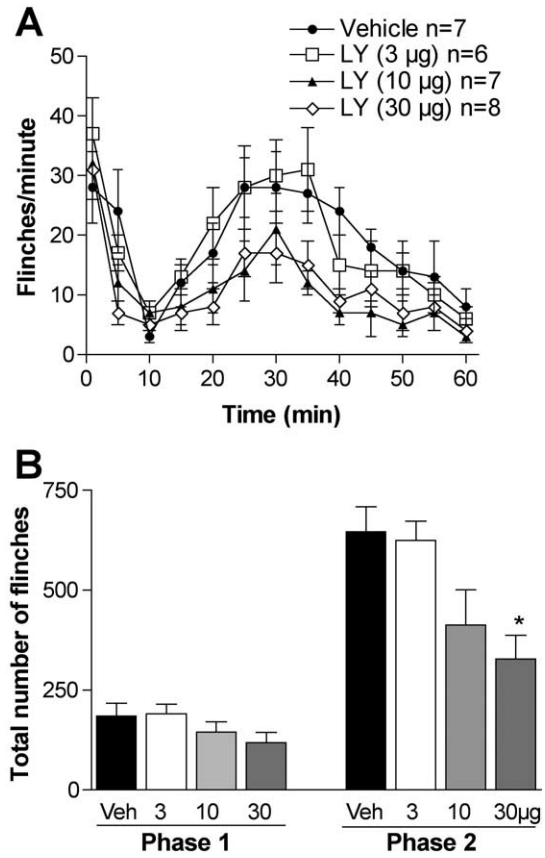

Fig. 5. Effects of $sPLA_2$ inhibition on formalin-induced flinching behavior. (A) Number of flinches per minute (every fifth minute) plotted versus time following injection of formalin into the dorsal side of the right hind paw. The flinching behavior was significantly attenuated in the group pretreated (−10 min) with Ly 311272 (LY; 30 μg) as compared with vehicle-injected group. (B) Cumulative number of flinches during phase 1 (1–9 min, total number) and phase 2 (10–60 min, total number) observed after different doses of IT LY (*) Represents $P<0.05$ versus vehicle-treated formalin-injected group). Each time point and bar represents mean±S.E.M., $n=6-8$ rats per group.

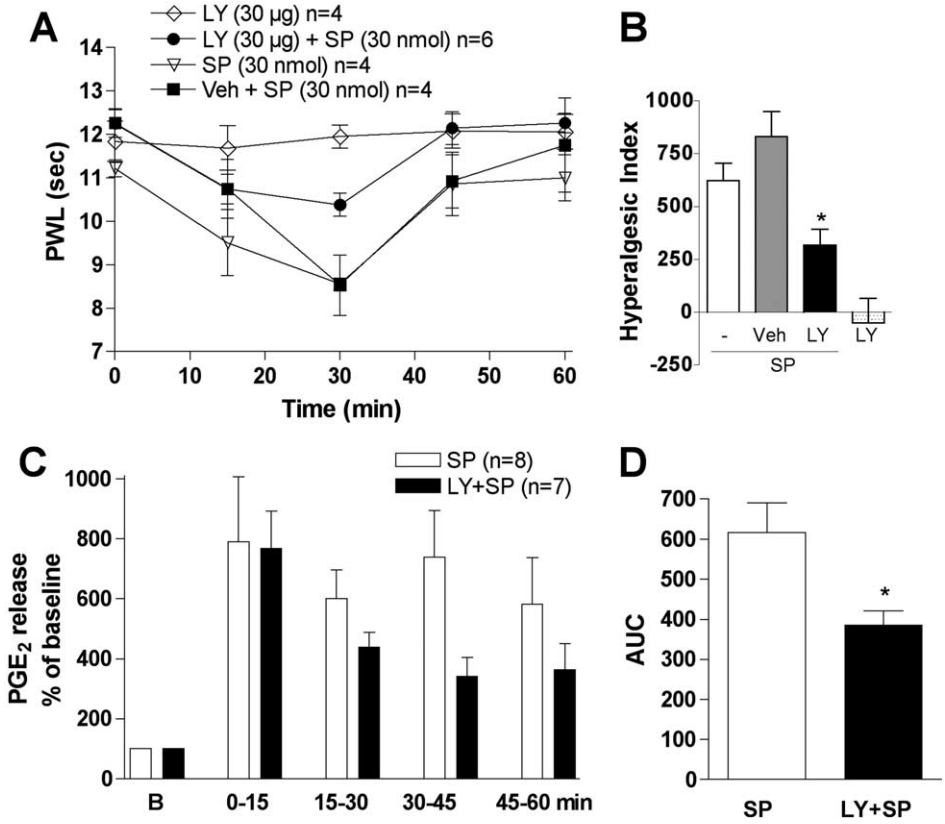

Fig. 6. Effect of inhibition of spinal $sPLA_2$ on thermal hyperalgesia and PGE_2 release evoked by IT SP. (A) IT injection of SP (30 nmol) resulted in a transient decrease in PWL that was partially blocked by IT pretreatment with LY311727 (LY; 30 μg). (B) Behavioral response presented as HI (see Experimental Procedures). (*) Indicates $P<0.05$ as compared with IT SP-injected groups. Each time point represents the mean±S.E.M. ($n=4–6$ rats per group). (C) Percentage changes over baseline of PGE_2 release in CSF after IT injection of SP (30 nmol) or LY (30 μg) followed by SP (30 nmol). (D) PGE_2 release presented as AUC calculated during 60 min after IT injection of SP. Each time point and bar represents the mean±S.E.M. ($n=7–8$ per group).

but not vehicle (data not shown) resulted in a significant increase of PGE_2 concentrations in spinal dialysate. Baseline release of PGE_2 was determined after a washout period of 30 min. In the absence of pretreatment, baseline dialysate concentrations were determined to be 202±12 femtomol/100 μl perfusate ($N=19$). Pretreatment with IT LY311727 (30 μg; Fig. 6C), but not vehicle (data not shown) 15 min prior to SP partially prevented IT SP-evoked PGE_2 release. Comparisons of area under the curve (AUC) between groups showed that SP-evoked release of PGE2 was significantly lower in the group that received LY311727 prior to SP ($P<0.05$; Fig. 6D).

DISCUSSION

Spinally mediated hyperalgesia is a frequent component of the pain experienced following tissue injury (Yaksh et al., 1999). It is widely believed that this spinal sensitization reflects a cascade of events that is initiated in part by persistent sensory input generated at the site of injury and inflammation, and the subsequent spinal primary afferent release of excitatory amino acids (glutamate) and peptides (SP). The present study details three essential points in support of the hypothesis that spinal $sPLA_2$ activity is one factor involved in regulation of spinal changes that leads to a behaviorally defined hyperalgesia. First, we show the presence of $sPLA_2$ protein and activity in lumbar spinal cord homogenates of naive rats. This indicates that even in the absence of a peripheral inflammatory stimulus, $sPLA_2$ is present and active. Second, inhibition of spinal $sPLA_2$, using intrathecally delivered LY311727, results in attenuation of nociceptive behavior in two models of inflammation-induced hyperalgesia. Importantly, even after direct activation of second order dorsal horn neurons by IT injection of SP, in the absence of peripheral inflammation, inhibition of spinal $sPLA_2$ leads to a reduction in hypersensitivity. This emphasizes that the IT effects do not depend upon the peripheral inflammation, rather on the central activation that arises secondary to such peripheral stimuli. Last, we link $sPLA_2$ activity to PGE_2 formation. Inhibition of spinal $sPLA_2$ attenuates IT SP-evoked PGE_2 release suggesting that $sPLA_2$ may be an important factor in regulation of formation of eicosanoids involved in facilitation of spinal pain processing.

Several $sPLA_2$ isoforms have been reported to be constitutively expressed in a number of different tissues and they display distinct yet overlapping tissue distribution (Kramer et al., 1989; Nevalainen et al., 2000; Kudo and Murakami, 2002). Group IIA $sPLA_2$ has been demon-

strated in brain (Lauritzen et al., 1994) and astrocytes (Lin et al., 2004), where a marked increase in group IIA $sPLA_2$ mRNA levels were observed after global cerebral ischemia. In contrast, Sawada et al. (1999) reported the absence of both group IIA and V $sPLA_2$ prior to and after stimulation with systemic LPS. Our data support the constitutive presence of $sPLA_2$ in the spinal cord. In two independent experiments using RT-PCR and real-time quantitative PCR, we detected groups IB, IIA, IIC, and V $sPLA_2$ mRNA in naive spinal cord tissue (Lucas et al., 2004). In addition, within the constraints of antibody specificity, we demonstrate the presence of $sPLA_2$ protein in naive rat spinal cord. The antibodies employed in this study are raised against groups IIA and V $sPLA_2$ respectively. However, based on the weak cross-reactivity detected for the groups IIA $sPLA_2$ and group V $sPLA_2$, we do not claim to have irrefutably shown the presence of one or the other $sPLA_2$ isoform specifically, but feel that our data indicate the presence of $sPLA_2$s in general in naive rat spinal cord.

Of the $sPLA_2$ isozymes, the group IIA enzyme has been studied most extensively because of its involvement in inflammatory processes in peripheral systems. The group IIA $sPLA_2$ enzyme is found in trace amounts in a variety of mammalian tissues, but is found at very high levels in various inflamed tissues (Lambeau and Lazdunski, 1999). Group IIA $sPLA_2$ mRNA is expressed *in vitro*, in cultured astrocytes (Oka and Arita, 1991; Tong et al., 1995; Xu et al., 2003), supporting our finding of group IIA $sPLA_2$ in the spinal cord, and both mRNA levels and activity can be increased in response to proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1β. In order to investigate whether peripheral inflammation promotes increases in spinal $sPLA_2$ activity we assessed $sPLA_2$ activity in spinal homogenates of animals that had received injection of carrageenan to one hind paw. The $sPLA_2$ assay utilized in this study is known to favor the group IIA $sPLA_2$ over the group V $sPLA_2$. However, the activity of the group IB and IIC $sPLA_2$ has not been determined on this substrate and could be contributing to the observed activity. No increase in activity was observed following injection of carrageenan to the hind paw. This is consistent with data reported by Samad et al. (2001), where a high basal $sPLA_2$ activity was measured in control spinal cord homogenates with no change in activity 12 h after a unilateral injection of Freud's complete adjuvant to the hind paw. There are a number of limitations of *in vitro* PLA_2 assays which limit comparison of *in vitro* to *in vivo* results. *In vivo* the only subgroup of $sPLA_2$s that is able to contribute to the release of AA must be 1) in an oxidizing environment (outside the cell), 2) located at the phospholipid membrane, and 3) have plentiful free calcium available. The assays used in all of these studies are unable to separate this subgroup from the $sPLA_2$s which *in vivo* would not be active. This is because: 1) the assay conditions are set to create an oxidizing environment, 2) the samples are homogenized to release all $sPLA_2$s from the tissue, whether the enzymes were inside the cell, outside the cell floating freely or outside the cell actively hydrolyzing phospholipid at the membrane, and 3) calcium concentrations are fixed in the assay buffer. As a result the activity assays are only useful as a measure of the amount of $sPLA_2$ enzyme available in a given sample and cannot reflect changes *in vivo* that would result in increased AA release. The assays are also limited in their ability to mimic the concentration of inhibitor added *in vivo*. Because inhibition is determined by the percent concentration of inhibitor in relation to substrate surface and the surface concentration inside the spinal cord cannot be determined, it is not possible to compare concentrations used *in vitro* to concentrations of inhibitor administered intrathecally.

We hypothesized that the $sPLA_2$s may be secreted into the CSF, but our CSF sampling studies indicate that this does not occur to a measurable degree. However, it is important to note that it is possible that we, by sampling at the cisterna magna, failed to assess changes in $sPLA_2$ activity that may have occurred at the lumbar level. We believe that $sPLA_2$s are released into the extracellular matrix where they facilitate the local release of AA that leads to a local segmental sensitization of the spinal cord. Thus, while the tissue $sPLA_2$ activity assays do not indicate whether there was any change in activity *in situ*, this work along with the Western blot data supports a constitutive expression of $sPLA_2$ in the spinal cord. Moreover, in parallel with the activity study we also examined the effect of LY311727 on $sPLA_2$ activity in spinal cord homogenates and found that addition of LY311727 resulted in a profound decrease of $sPLA_2$ activity while having no effect on $cPLA_2$ or $iPLA_2$ activity. This suggests that the agent indeed inhibits rat spinal $sPLA_2$ activity and that the observed decrease in hyperalgesic behavior following IT LY311727 is in fact due to decreased spinal $sPLA_2$ activity.

In recent work we have observed that inhibition of spinal group IVA $cPLA_2$, but not group VI $iPLA_2$, attenuates carrageenan and formalin-induced hyperalgesia as well as SP-evoked PGE_2 release (Lucas et al., 2004). Here we demonstrate that inhibition of spinal $sPLA_2$ has similar effects. This is in agreement with other studies indicating that both group IV $cPLA_2$ and group IIA and V $sPLA_2$ contribute to AA release. It has been suggested that functional group IV $cPLA_2$ is crucial for group IIA and group V $sPLA_2$ activity (Murakami et al., 1998). It is still unclear exactly how these enzymes interact with each other; however, it is clear that they can be coupled with COX-dependent PGE_2 generation. Given the absence of specific $sPLA_2$ inhibitors, we cannot assert which of the $sPLA_2$ isozymes are involved. The inhibitor used in this study, LY311727, is widely accepted as being specific for $sPLA_2$ over $cPLA_2$ or $iPLA_2$ as is supported by numerous publications (Schevitz et al., 1995; Chen and Dennis, 1998; Balsinde et al., 1999a) as well as our own findings; however its efficacy against each $sPLA_2$ type has not been well determined. It has, however, been reported that LY311727 significantly decreases the activity of human and mouse group IIA, but that it is not as effective against human or mouse group IB, mouse group IIC or human or mouse group V (Singer et al., 2002). Studies in our laboratory indicate that LY311727 is a very effective inhibitor against human group V $sPLA_2$ (K. K Lucas, unpublished observations). Thus, because of the lack of documented selectivity of LY311727, we cannot

specify the contribution of each $sPLA_2$ subgroup in this study. However, the fact that LY311727 readily attenuated hyperalgesic behavior in three experimental models of hyperalgesia as well as stimuli-evoked PGE_2 release, suggests that at least one of these isozymes is involved in the release of AA to initiate the production of PG in the spinal cord. Another possible approach one could undertake in order to study the role of specific $sPLA_2$s in spinal pain processing would be to knock down the protein expression of specific $sPLA_2$ forms in the spinal cord. This has successfully been performed in macrophage-like cell line (Balboa et al., 1996; Shinohara et al., 1999), fibroblasts (Kuwata et al., 1998) and human placenta explants (Lappas et al., 2001) and the results from these studies indeed indicate a role for group II and V $sPLA_2$ in eicosanoid biosynthesis.

The mechanism by which $sPLA_2$ contributes to spinal sensitization is not certain. Here we show that inhibition of $sPLA_2$ partially prevents IT SP-evoked PGE_2 release. Other mechanisms are possible. For example, aside from its role in membrane lipolysis, previous work has suggested the presence of a membrane receptor for $sPLA_2$, the M-type receptor (Lambeau et al., 1995). However, it still remains to be determined if the M-type receptor is present in the spinal cord and if its biological function can be linked to spinal facilitation of nociception. Recent work suggests that $sPLA_2$ may play both indirect and direct roles in neurotransmission. It has been suggested that group IIA $sPLA_2$ facilitates neurotransmission. For example, the secretion of group IIA $sPLA_2$ is coupled to catecholamine release in PC12 cells (Kudo et al., 1996) and glutamate release in ischemic rat cerebral cortex (O'Regan et al., 1995). In addition, injection of $sPLA_2$ into the brain causes epileptic seizure (Dorandeu et al., 1998) as well as neuronal apoptosis (Yagami et al., 2002). Some of these effects have been attributed to the ability of $sPLA_2$ to increase Ca^{2+} influx through stimulation of an L-type voltage-sensitive Ca^{2+} channel (Yagami et al., 2003). Although our studies focused on the role of $sPLA_2$ in AA release and subsequent PG production it is possible that the observed $sPLA_2$ activity is involved in additional mechanisms that lead to a facilitation of pain processing.

In summary, we have presented evidence that there is $sPLA_2$ mRNA, protein expression and enzyme activity in the spinal cord. Inhibition of spinal $sPLA_2$ suppresses nociceptive behavior in three established experimental models of hyperalgesia. Consistent with the presumed actions of these inhibitors, inhibition of spinal $sPLA_2$ at behaviorally effective doses prevented stimulus-evoked spinal PGE_2 release. These studies provide support for the role of constitutively expressed spinal $sPLA_2$ in post-tissue injury nociceptive processing.

Acknowledgments—This work was supported by National Institute of Health grants NS 16541 (T.L.Y.), GM 20,501 (E.A.D.), GMOB4611 (E.A.D.) and by United States Department of Education training grant GAANN (K.K.L.). The authors wish to thank Bethany Fitzsimmons and Alan Moore for technical assistance and Dr. Jerome Fleisch, Lilly Research Laboratories, for the generous gift of LY311727.

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(Accepted 21 January 2005)
(Available online 10 May 2005)