Systemic and intrathecal effects of a novel series of phospholipase A₂ inhibitors on hyperalgesia and spinal PGE₂ release

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Phospholipase A₂ inhibition and pain

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Non-Standard Abbreviations
SP: Substance P; IT: Intrathecal; cPLA₂: calcium dependent phospholipase A₂; iPLA₂: calcium independent phospholipase A₂; sPLA₂: secretory phospholipase A₂.
PGE₂: Prostaglandins E₂.

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ABSTRACT

Phospholipase A2 (PLA2) forms are expressed in spinal cord and inhibiting spinal PLA2 induces a potent antihyperalgesia. Here we examined the antihyperalgesic effects after systemic and intrathecal (IT) delivery of four compounds constructed with a common motif consisting of a 2-oxoamide with a hydrocarbon tail and a four carbon tether. These molecules were characterized for their ability to block Group IVA calcium dependent PLA2 (Group IVA cPLA2) and Group VIA calcium independent iPLA2 (Group VIA iPLA2) in inhibition assays using human recombinant enzyme. The rank ordering of potency in blocking Group IVA cPLA2 was AX048, AX006, AX057 > AX010; and for inhibiting Group VIA iPLA2 was AX048, AX057 > AX006, AX010. No agent altered recombinant cyclooxygenase activity. In vivo, intrathecal (30 µg) and systemic (0.2-3 mg/kg, intraperitoneal, IP) AX048 blocked carrageenan hyperalgesia, and after systemic delivery in a model of spinally mediated hyperalgesia induced by IT substance P (SP). The other agents were without activity. In rats prepared with lumbar intrathecal loop dialysis catheters, SP evoked spinal prostaglandin E2 (PGE2) release. AX048 alone inhibited PGE2 release. IT- SR 141617, a cannabinoid CB1 inhibitor at doses which blocked the effects of IT anandamide had no effect upon IT AX048. These results suggest that AX048 is the first systemically bioavailable compound with a significant affinity for Group IVA cPLA2, which produces a potent anti-hyperalgesia. The other agents, though demonstrating enzymatic activity in cell-free assays, appear unable to gain access to the intracellular PLA2 towards which their action is targeted.
INTRODUCTION

Tissue injury and inflammation lead to the development of an evident facilitation in the sensitivity to moderately aversive stimuli, e.g. hyperalgesia. It has been long appreciated that this phenomenon is diminished by agents that block cyclooxygenase (COX) activity (Vane, 1971). While early work suggested that this action resulted from a peripheral effect (Ferreira, 1972), it was subsequently found that inhibition of spinal COX also led to reversal of the facilitated state (Yaksh, 1982; Taiwo and Levine, 1988). These initial findings have been widely confirmed (Yamamoto and Nozaki-Taguchi, 1996; Turnbach and Randich, 2001) Consistent with this action, persistent small afferent input, as arises from tissue injury, was shown to evoke a significant spinal release of prostanoids in vivo in a manner that was blocked by spinally-delivered COX inhibitors (Yaksh, 1982; Malmberg and Yaksh, 1992; Malmberg and Yaksh, 1995; Southall, et al, 1998; Ebersberger, et al., 1999, Samad et al., 2001, Yaksh et al., 2001). An important element of prostaglandin (PG) synthesis is phospholipase A2 (PLA2), as it is required to generate arachidonic acid, which is the substrate for COX-mediated prostanoid formation. In recent work we have shown the presence of constitutive mRNA and protein in the spinal cord for Group IVA calcium-dependent PLA2 (Group IVA cPLA2) and Group VIA calcium-independent iPLA2 (Group VIA iPLA2) and secretory (Group II and V sPLA2) forms (Lucas et al., 2005, Svensson et al., 2005b). Inhibition of Group IV cPLA2 but not Group VI iPLA2 isoforms using IT-delivered agents suggested a role for Group IV cPLA2, but not Group VI iPLA2 (Lucas et al., 2005) in inflammation-evoked hyperalgesia.
We have recently reported the discovery of a novel structural series of 2-oxoamides that inhibit Group IVA cPLA₂ in vitro and in vivo (Kokotos, et al., 2002; Kokotos, et al., 2004). In initial work, 2-oxoamides were observed to inhibit inflammation in the rat paw carrageenan-induced edema assay (Kokotos et al., 2004). In the present work, we have focused on the in vivo activity of four related analogues of this series, AX006, AX010, AX048 and AX057. These molecules were examined for their inhibitory effects on Group IV cPLA₂ and Group VI iPLA₂ as well as on COX activity in in vitro assays. Their actions were then characterized after systemic and intrathecal delivery on thermal hyperalgesia induced by peripheral inflammation (intraplantar carrageenan). In addition, we have previously shown that spinal sensitization can be directly initiated in the absence of peripheral inflammation by spinal delivery of substance P (SP). Substance P, acting through the spinal neurokinin 1 receptor, will evoke the spinal release of PGE₂ and subsequent thermal hyperalgesia. Both of these events are antagonized by spinal cyclooxygenase inhibition (Malmberg and Yaksh, 1992; Yaksh et al., 2001). Based on these observations, we examined the effects of the PLA₂ inhibitors on the hyperalgesia and PGE₂ release evoked by spinally-delivered SP. We report here that one of these agents, after systemic delivery, displays significant antihyperalgesic effects in models of both centrally- and peripherally-initiated hyperalgesia, and in an effective systemic dose blocks the spinally-evoked release of spinal PGE.
METHODS

All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of University of California, San Diego.

In Vivo Studies

Animals

Male Holtzman Sprague-Dawley rats (300–350 g; Harlan Industries) were individually housed and maintained on a 12-hr light/dark cycle with free access to food and water.

Intrathecal catheter implantation

For spinal drug injections, lumbar catheters were implanted in rats under isoflurane anesthesia according to a modification of the procedure described by Yaksh (Yaksh and Rudy, 1976). A polyethylene catheter (PE-5; Spectranetics, 0.014 in OD) was inserted into the intrathecal space and advanced to the rostral edge of the lumbar enlargement through an incision in the atlanto-occipital membrane. Five days after implantation rats were entered into the study. In separate experiments to assess spinal prostaglandins release, rats were prepared with lumbar loop dialysis catheters with three lumens, as previously described, see (Yaksh et al., 2001). In brief, the outer two lumens were connected to a length of dialysis tubing (10 kDa cut off). The catheter was then implanted intrathecally using the same technique as described above for the intrathecal catheter. A three-day interval was allowed to elapse prior to including the animal in a study. In
all cases, the exclusion criteria were i) presence of any neurological sequelae, ii) 20% weight loss after implantation, or iii) catheter occlusion.

Behavioral analysis

*Thermal hyperalgesia.* Two approaches were employed to initiate a hyperalgesic state. An inflammation-evoked thermal hyperalgesia was induced by subcutaneous injection of 2 mg of carrageenan (Sigma, St. Louis, MO, 100 µl of 20% solution (w/v) in physiological saline) into the plantar surface of the left hind paw. The thermally-evoked paw-withdrawal response was assessed (Dirig et al., 1997). In brief, the device consists of a glass surface (maintained at 25°C) on which the rats are placed individually in Plexiglas cubicles (9 x 22 x 25 cm). The thermal nociceptive stimulus originates from a focused projection bulb positioned below the glass surface. The stimulus is delivered separately to either hind paw of each test subject with the aid of an angled mirror mounted on the stimulus source. A timer is actuated with the light source, and latency is defined as the time required for the paw to show a brisk withdrawal as detected by photodiode motion sensors that stop the timer and terminate the stimulus. Paw withdrawal latencies (PWL) are assessed prior to any treatment (control) and at intervals after treatment. Left (injured) and right (uninjured) paw withdrawal latencies are assessed and plotted versus time. In addition, difference latency scores (uninjured–injured) are calculated and the average withdrawal latency over the post-injection observation intervals are calculated for comparison between treatment groups. In addition to the use of a peripheral inflammation, a thermal hyperalgesia is also initiated by the intrathecal injection of SP (20 nmol/10 µL). The mean PWL of the left and
right paws is assessed at each time point. The mean difference between the Pre-IT SP and the Post-IT SP response latency scores is calculated for analysis.

Intrathecal dialysis and PGE$_2$ assay

Spinal dialysis experiments to define the spinal release of PGE$_2$ were conducted in unanesthetized rats 3 days after dialysis catheter implantation. A syringe pump (Harvard, Natick, MA) was connected and dialysis tubing was perfused with artificial cerebrospinal fluid (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$_3^-$, 2.5 HPO$_4^{2-}$ and 3.5 dextrose and 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 NMDA (0.6 µg). 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). The antibody is selective for PGE$_2$ with less than 2.0 % cross-reactivity to PGF$_1\alpha$, PGF$_2\alpha$, 6-ketoPGF$_1\alpha$, PGA$_2$ or PGB$_2$, but cross-reacts with PGE$_1$ and PGE$_3$.

Drug delivery

Drugs were delivered systemically (IP) or spinally (IT). Intraperitoneal drugs were delivered uniformly in doses prepared in volumes of 0.5 ml/kg. Drugs injected IT were administered in a total volume of 10 µl followed by a 10 µl flush using vehicle.
Enzyme assays

*In vitro* Group IV cPLA2 and Group VI iPLA2 assays were done as previously described (Kokotos et al., 2002). Briefly, 100 µM lipid substrate and 100,000 cpm radiolabeled analog were dried down under N2 and dissolved in assay buffer containing 400 µM Triton X-100 to yield a mixed micelle substrate solution. Inhibitors dissolved in DMSO were added to the reaction tubes and allowed to incubate with substrate for five minutes at 40°C. Pure enzyme was added to yield a final volume of 500 µl, and digestion was carried out at 40°C for 30 minutes. Reactions were quenched and extracted using the Dole method and products were quantified by liquid scintillation counting (Dole, 1956). Percent inhibition was determined at a range of inhibitor mole fraction concentrations for Xf(50) calculations.

Inhibition of cyclooxygenase-1 and cyclooxygenase-2 was tested *in vitro* using the COX Activity Assay kit (catalog 760151) from Cayman Chemical. Assays were performed in 96 well plates using 10 µl supplied COX standard (catalog 760152) that contained COX-1 and COX-2 proteins. Activity was detected colorimetrically at 595 nm by the appearance of oxidized N, N, N’, N’-tetramethylphenylenediamine (TMPD), which has an absorption maximum of 611 nm (Kulmacz and Lands, 1983). Inhibitors dissolved in DMSO (study compounds) or ethanol (indomethacin) were added to 50 µM final concentration and allowed to incubate with the assay mixture including enzyme for 5 minutes. After addition of TMPD and arachidonic acid, samples were mixed and allowed to incubate 5 minutes at room temperature before reading absorbance at 595 nm to determine results. Results were calculated and percent inhibition values derived.
Drugs

PLA2 inhibitors employed in these studies were synthesized (see below). These agents were prepared for delivery in a vehicle of 5% Tween 80. Other agents used in these studies, included the cannabinoid agonist anandamide, the CB1 antagonist, SR141716A (supplied courtesy of Benjamin Cravatt, Scripps Institute, La Jolla, CA). Anandamide was prepared in 100% DMSO and SR141716A in ethanol Emulphor and saline (1:1:18). Control studies were run with the respective vehicles.

Drug Synthesis

AX006 and AX010 were prepared as previously described (Kokotos et al., 2002; Kokotos et al., 2004). The synthesis and the characterization of the novel agents AX048 and AX057 are described here in detail. Figure 1 summarizes the synthesis schema.

Coupling of 2-hydroxy-hexadecanoic acid with esters of 4-amino-butanoate

To a stirred solution of 2-hydroxy-hexadecanoic acid (2.0 mmol) and the ester of 4-amino-butanoate (2.0 mmol) in CH₂Cl₂ (20 mL), Et₃N (6.2 ml, 4.4 mmol) and subsequently WSCI (0.42 g, 2.2 mmol) and HOBt (0.32 g, 2.0 mmol) were added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. The solvent was evaporated under reduced pressure and EtOAc (20 mL) was added. The organic layer was washed consecutively with brine, 1 N HCl, brine, 5% NaHCO₃, and brine, dried over Na₂SO₄ and evaporated under reduced
pressure. The residue was purified by column chromatography using CHCl₃-MeOH (95:5) as the eluent.

Ethyl 4-[(2-hydroxyhexadecanoyl)amino]butanoate  Yield 72%; ¹H NMR: δ 6.68 (1H, t, J = 7 Hz, NH), 4.13 (3H, m, CH, COOCH₂CH₃), 3.34 (2H, m, CH₂NH), 2.68 (1H, b, OH), 2.32 (2H, t, J = 7 Hz, CH₂COO), 1.80-1.58 (4H, m, CH₂CH₂COO, CH₂CH), 1.45-1.23 (27H, m, 12×CH₂, COOCH₂CH₃), 0.85 (3H, t, J = 7 Hz, CH₃); ¹³C NMR: δ 174.0, 173.8, 72.2, 60.6, 38.5, 34.9, 31.9, 31.7, 31.4, 29.7, 29.6, 29.5, 29.4, 29.3, 25.0, 24.6, 22.7, 14.1. Anal. calcd. for C₂₂H₄₃NO₄ (385.58): C, 68.53; H, 11.24, N, 3.63. Found: C, 68.12; H, 11.32; N, 3.48.

tert-Butyl 4-[(2-hydroxyhexadecanoyl)amino]butanoate  Yield 64%; ¹H NMR: δ 6.49 (1H, t, J = 7 Hz, NH), 4.12 (1H, m, CH), 3.34 (2H, m, CH₂NH), 2.73 (1H, b, OH), 2.27 (2H, t, J = 7 Hz, CH₂COO), 1.82-1.49 (4H, m, CH₂CH₂COO, CH₂CH), 1.45 [9H, s, C(CH₃)₃], 1.38-1.15 (24H, m, 12×CH₂), 0.89 (3H, t, J = 7 Hz, CH₃); ¹³C NMR: δ 173.9, 173.7, 80.1, 72.3, 38.3, 35.4, 31.9, 31.8, 31.4, 29.7, 29.6, 29.5, 29.4, 29.3, 28.7, 25.1, 24.5, 22.8, 14.1. Anal. calcd. for C₂₄H₄₇NO₄ (413.63): C, 69.69; H, 11.45, N, 3.39. Found: C, 69.42; H, 11.61; N, 3.27.

Oxidation of 2-hydroxy-amides

To a solution of a 2-hydroxy-amide (1.00 mmol) in a mixture of toluene-EtOAc (15 mL), a solution of NaBr (0.11 g, 1.05 mmol) in water (1.3 mL) was added, followed by AcNH-TEMPO (2 mg, 0.01 mmol). To the resulting biphasic system, which was cooled at -5 °C, an aqueous solution of 0.35 M NaOCl (3.1 mL, 1.10 mmol) containing NaHCO₃ (0.25 g, 3 mmol) was added dropwise while stirring vigorously at -5 °C over a period of 1 h. After the mixture had been stirred for a further 15 min at 0 °C, EtOAc (15 mL) and H₂O (5 mL) were added. The aqueous
layer was separated and washed with EtOAc (10 mL). The combined organic layers were washed consecutively with 5% aqueous citric acid (15 mL) containing KI (0.04 g), 10% aqueous Na$_2$S$_2$O$_3$ (6 mL), and brine and dried over Na$_2$SO$_4$. The solvents were evaporated under reduced pressure, and the residue was purified by column chromatography [EtOAc-petroleum ether 1:9 (bp 40-60 °C)].

**Ethyl 4-{(2-oxohexadecanoyl)amino}butanoate (AX048)** Yield 86%; white solid; mp 63-64 °C; $^1$H NMR: δ 7.16 (1H, m, NH), 4.12 (2H, q, $J = 7$ Hz, COOC$_2$H$_5$), 3.33 (2H, m, CH$_2$NH), 2.89 (2H, t, $J = 7$ Hz, CH$_2$COCO), 2.34 (2H, t, $J = 7$ Hz, CH$_2$COO), 1.87 (2H, m, CH$_2$CH$_2$COO), 1.57 (2H, m, CH$_2$CH$_2$COCO), 1.40-1.15 (25H, m, 11 × CH$_2$, COOCH$_2$C$_3$H$_7$), 0.85 (3H, t, $J = 7$ Hz, CH$_3$); $^{13}$C NMR: δ 199.0, 172.7, 160.2, 60.4, 38.5, 36.5, 31.7, 31.4, 29.5, 29.4, 29.3, 29.2, 28.9, 24.2, 23.0, 22.5, 14.0, 13.9; MS (FAB) m/z (%) 384 (100) [M$^+$ + H]. Anal. calcd. for C$_{22}$H$_{41}$NO$_4$ (383.57): C, 68.89; H, 10.77, N, 3.65. Found: C, 68.71; H, 10.88; N, 3.54.

**tert-Butyl 4-{(2-oxohexadecanoyl)amino}butanoate (AX057)** Yield 95%; white solid; mp 61-62 °C; $^1$H NMR: δ 7.11 (1H, m, NH), 3.33 (2H, m, CH$_2$NH), 2.91 (2H, t, $J = 7$ Hz, CH$_2$CO), 2.28 (2H, t, $J = 7$ Hz, CH$_2$COO), 1.84 (2H, m, CH$_2$CH$_2$COO), 1.60 (2H, m, CH$_2$CH$_2$COCO), 1.45 (9H, s, C(CH$_3$)$_3$), 1.38-1.23 (22H, m, 11 × CH$_2$), 0.89 (3H, t, $J = 7$ Hz, CH$_3$); $^{13}$C NMR: δ 198.6, 171.6, 159.7, 80.0, 38.1, 36.1, 32.2, 31.3, 29.0, 28.9, 28.8, 28.7, 28.4, 27.4, 23.8, 22.5, 22.0, 13.5; MS (FAB) m/z (%) 412 (17) [M$^+$ + H], 356 (100). Anal. calcd. for C$_{24}$H$_{45}$NO$_4$ (411.62): C, 70.03; H, 11.02, N, 3.40. Found: C, 69.89; H, 11.32; N, 3.47.
Statistics

Escape latency data are presented as the mean ± SEM. For carrageenan and IT SP analysis of thermal escape latencies were carried out over time and compared with one-way ANOVA. For carrageenan, difference scores between control and injured paws over time were calculated for each group. Comparison of drug with vehicle treatment was performed using an unpaired t-test. For dose response analyses, least squares linear regression was performed and the drug dose required to produce a 50% reduction in the hyperalgesia otherwise observed in the vehicle-treated control animals was estimated. For release studies, release was expressed as percent of baseline and the area under the release curve following IT SP was calculated. Group comparisons were carried out using nonparametric statistics for repeated measures over time and (Friedman analysis) across treatment groups with post hoc analyses being undertaken with Dunns Multiple Comparison analysis. Analyses were performed using Prism statistical software (GraphPad Prism version 4.02 for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com).
RESULTS

Synthesis and physical properties of test agent

Ethyl and tert-butyl 4-amino-butanoates were coupled with 2-hydroxy-hexadecanoic acid using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCl) as a condensing agent in the presence of 1-hydroxylbenzotriazole (HOBt). The 2-hydroxyamides synthesized were oxidized with NaOCl in the presence of a catalytic amount of 4-acetamido-2,2,6,6-tetramethylpiperidine-1-yl-oxy free radical (AcNH-TEMPO) to produce compounds AX048 and AX057.

Characterization of PLA\textsubscript{2} inhibitory activity

Enzymatic assay. The inhibitory effects of AX006, AX010, AX048 and AX057 on pure Group IVA PLA\textsubscript{2} and Group VIA PLA\textsubscript{2} were examined and the results are presented in Table 1 as $X_I(50)$. The $X_I(50)$ is the mole fraction of inhibitor in the total substrate interface required to inhibit the enzyme by 50%. The reason that $X_I(50)$ is used instead of the more common IC\textsubscript{50} or K\textsubscript{i} is that PLA\textsubscript{2} is active only on phospholipid surfaces such as cell membranes, phospholipid vesicles, or phospholipid micelles, where its substrate phospholipids reside. Almost all inhibitors of PLA\textsubscript{2}s partition at least to some degree into the phospholipid surface, because they usually have a hydrophobic portion that complements the hydrophobic active site of the PLA\textsubscript{2}. When these inhibitors partition into the surface, an important physical effect called surface dilution comes into play. In this case, the affinity of the PLA\textsubscript{2} for the inhibitor depends not on the three-dimensional (bulk) concentration of the inhibitor in molar units, but on the two-
dimensional (surface) concentration of the inhibitor in mole fraction units. As indicated (Figure 2, 3, Table 1), AX048 and AX057 were potent against Group IVA PLA2 and Group VIA PLA2, AX006 was potent against Group IVA PLA2 alone, and AX010 was less effective against both.

Characterization of COX inhibitory activity

Incubation with indomethacin produced a near complete inhibition of the COX activity in the assay. In contrast, incubation with the AX compounds at concentrations that had significant effects upon PLA2 (50µM) had no inhibitory effects upon COX activity.

In vivo Behavioral studies

Intraperitoneal delivery and carrageenan-induced thermal hyperalgesia.

Control. Prior to induction of hyperalgesia, baseline thermal escape latencies were on the order of 10-12 sec in all groups. Intraplantar injection of carrageenan induced inflammation of the injected hind paw as well as a corresponding thermal hyperalgesia that was detectable after 60 min lasting throughout the study. The thermal escape latency in animals treated with IP or IT vehicle was significantly reduced to approximately 3-5 seconds within 90-120 minutes (Figures 5 and 6).
Intraperitoneal delivery. Pretreatment (30 min) with 3 mg/kg (IP) of the four agents prior to the carrageenan injection revealed that AX048, but not AX006, AX010, or AX057, reduced the thermal hyperalgesia otherwise observed in the inflamed paw (Figure 5). Importantly, there was no change in the thermal escape latency of the uninjured paw in either the vehicle- or drug-treated animal, e.g., the agent was behaving functionally as an anti-hyperalgesic agent. Comparison of the mean group difference between response latencies of uninjured and injured paws revealed a significant reduction in the AX048-treated group as compared to the vehicle-treated group.

Dose dependency: The effects of IP AX048 were observed to be dose-dependent over the range of 0.2-3 mg/kg. (Slope; p < 0.0004) (Figure 6). The ED50 was defined as the dose that reduced the hyperalgesia observed in a vehicle-treated animal by 50%. On this basis, the estimated IP ED50 value for IP AX048 was 1.2 mg/kg (95% CI: -0.5572 to 0.7713).

Time Course of action. To determine the time course of the drug action, IP delivery of AX048 (3 mg/kg) was undertaken at -15 min, -30 min and -180 min (Figure 7). As indicated, peak effects were noted at 30 min and minimal effects observed at 15 min. The effects persisted through for 180 min but were no different from the control by 360 min.
IT delivery and Carrageenan-induced thermal hyperalgesia

Control. In animals receiving intrathecal injections of vehicle the intraplantar injection of carrageenan resulted in a significant unilateral thermal hyperalgesia as compared to the uninjected paw (Figure 8).

Drug effect. Pretreatment with 30 µg/10 µL of the four agents 15 min prior to the delivery of carrageenan revealed that AX048, but not AX006, AX010, or AX 057, attenuated the thermal hyperalgesia (Figure 8). Again, after intrathecal delivery, there was no change in the thermal escape latency of the uninjured paw in either the vehicle- or drug-treated animal. Comparison of the mean group difference between response latencies of uninjured and injured paws also revealed a significant reduction in the AX 048-treated group in comparison to the vehicle-treated group.

Intrathecal substance P-induced thermal hyperalgesia

Control. Baseline thermal escape latencies were on the order of 10-12 sec. In systemic vehicle-treated animals, the intrathecal injection of SP (20 nmol/10 µl) evoked a significant reduction in thermal escape latency as early as 15 min after injection, which persisted through the 45 min test interval, returning to baseline by 60 min. (Figure 9.)
**Drug effect.** Pretreatment with 3 mg/kg (IP) of the four agents 30 min prior to the intrathecal delivery of SP revealed that AX048, but not AX006, AX010, or AX 057, completely prevented the spinally-evoked thermal hyperalgesia (Figure 9). As in the carrageenan study, there was no evidence that AX048 increased the post-treatment latency to values greater than baseline, e.g. the agent was behaving functionally as an anti-hyperalgesic agent.

**Side effect profile**

After delivery of the highest systemic dose (3 mg/kg) or intrathecal dose (20 µg) of any of the compounds, there were no changes in any assessed reflex end points including eye blink, pinnae, placing or stepping. The animals showed no change in righting response, symmetric ambulation or spontaneous activity.

**Spinal prostaglandin release.**

**Control.** Overall baseline dialysate concentrations after the initial washout and prior to drug treatment were determined to be 555 ± 75 pg / 100µl perfusate. Intrathecal injection of SP (20 µg) but not vehicle (saline, not shown) resulted in a statistically significant increase in PGE2 concentrations in spinal dialysate as compared to the vehicle-treated control (Figure 10.)

**Drug effect.** Pretreatment with the four agents 15 min prior to the delivery of IT SP (20 µg/10 µL) revealed that the evoked release of PGE2 was reduced only in the AX048-treated group. Thus, of the four agents only AX048 exerted a significant inhibitory effect upon PGE2 synthesis and release (Figure 10).
Effects of CB1 inhibition.

To determine whether the effects of the active agent AX048 might be acting directly or indirectly through a central cannabinoid CB-1 receptor, rats were pretreated with IT vehicle or IT SR141716, a CB1 receptor antagonist, followed after 15 min by IT AX048 (30µg) or IT anandamide (100µg). IT SR141716 had no effect when delivered alone (data not shown). As shown in Table 2, in vehicle pretreated animals, IT anandamide resulted in a significant increase in the thermal escape latency of the uninjured paw and that of the injured paw. Both effects were prevented by pretreatment with IT SR141716. IT AX048 significantly reversed the respective hyperalgesia, but had no effect upon the thermal escape latency of the un-injured paw. The antihyperalgesic effects of IT AX048 were not altered by IT SR141716. These observations suggest that IT anandamide, but not IT AX048, were interacting with a spinal CB1 receptor to alter thermal escape latency.
DISCUSSION

AX048, but not the three structurally-related analogues AX006, AX010 and AX057, exerted a significant effect upon centrally (IT-SP) and peripherally-(intraplantar carrageenan) initiated hyperalgesia. As the effective IT dose was 100 times less than required after systemic delivery, we conclude that the IT effect represented a central action. In addition, systemic AX048 blocked the hyperalgesia evoked by IT-SP in the absence of any peripheral injury. This suggests that the activity of the systemically-delivered compound was mediated by a central action. Parallel in vitro characterization of the selectivity of these agents in reversibly blocking Group IVA cPLA$_2$ and Group VIA iPLA$_2$ revealed that AX010 had at best a weak effect, AX006 was Group IVA PLA$_2$ preferring, while AX048 and AX057 were Group IVA cPLA$_2$ and Group VIA iPLA$_2$ preferring. The profile of activity observed here suggests the importance of both Group IVA cPLA$_2$ and Group VIA iPLA$_2$. We showed that IT delivery of methyl arachidonyl fluorophosphonate (MAFP) and arachidonyl trifluoromethylketone (AACOCF$_3$), mixed inhibitors of Group IVA cPLA$_2$ and Group VI iPLA$_2$, produced a dose-dependent inhibition of hyperalgesia induced by intraplantar carrageenan as well as formalin-induced flinching. Moreover, IT injection of AACOCF$_3$ at anti-hyperalgesic doses decreased PGE$_2$ release into spinal dialysate evoked by IT NMDA (Lucas et al., 2005). In contrast, in those studies, an irreversible Group VIA iPLA$_2$ inhibitor (Bromoenol lactone, BEL), given IT, failed to show any antihyperalgesic effects at doses that did not produce motor dysfunction and, at a higher dose, failed to block evoked spinal PGE$_2$ release. Yeo and colleagues (Yeo et al., 2004) reported that intracerebroventricular injection of AACOCF$_3$ or BEL produced antihyperalgesia as measured
using facial carrageenan in mice (Yeo et al., 2004). Burke and colleagues reported that BMS-229724, a Group IVA cPLA₂-inhibitor, was orally active in inhibiting edema and neutrophil infiltration (Burke et al., 2001). The present data thus continue to leave the issue open regarding the relative contribution of Group IVA cPLA₂ and Group VIA iPLA₂.

**Role of Spinal PLA₂ Isoforms in Cascade**

Western blotting and RT-PCR have shown that Group IVA cPLA₂, Group VIA iPLA₂ and sPLA₂ (Groups IIA and V) are constitutively expressed in the spinal cord (Sapirstein and Bonventre, 2000; Lucas et al., 2005; Svensson et al., 2005b). The role of these respective isoforms has been difficult to assess given the lack of potent and selective inhibitors. Based on our earlier work noted above, we have had a particular interest in Group IVA cPLA₂. In the present work, AX048 displayed a dose-dependent suppression of both centrally- and peripherally-evoked thermal hyperalgesia. Importantly, the comparable antihyperalgesic action of AX048 after IT delivery with 20µg versus the dose of 3 mg/kg, given IP, emphasizes an important spinal action. The effects of systemic delivery showed an onset of approximately 30 min and a duration of action that exceeded 180 min. Importantly, this dosing was shown to have a significant effect upon intrathecal SP-evoked spinal PGE₂ release, a downstream biomarker believed to be essentially dependent upon PLA₂ activity (Svensson and Yaksh, 2002).

While the primary target of these molecules examined in the present study is PLA₂, we note that other possibilities may also be relevant including a direct effect upon cyclooxygenase or the endocannabinoid system, both of which may lead to a change in pain behavior in a hyperalgesic...
The present studies, however, showed no effects at the highest concentrations on either COX-1 or COX-2 activity. Recent work suggested that agents interacting with the COX cascade may exert effects through an endocannabinoid pathway (Seidel, et al., 2003). We, however, do not think that an effect through the cannabinoid-1 receptor is likely. The effect upon the centrally-mediated hyperalgesia excludes a peripheral cannabinoid-2 receptor action. Moreover, after IT delivery, anandamide elevated the thermal escape latency of the normal paw, an effect not mimicked by the AX048. Finally, IT SR141716A, a potent CB1 antagonist (Shire, et al., 1999) given intrathecally at a dose which reversed the intrathecal effect of anandamide, failed to alter the effects of AX048. These data suggest an effect of spinal AX048 which is independent of an action upon either endogenous cannabinoid release or upon the receptor itself. These experiments provide supportive evidence consistent with the assertion that AX048 was indeed acting through a PLA2 enzyme. We recognize that these are complex systems and other potential targets might be considered in future studies and include a variety of upstream enzymes (such as mitogen activated protein kinase) (Svensson, et al., 2005a) as well as downstream effects (such as inhibition of prostaglandin synthases or receptors) (Guay, et al., 2004, Reinold et al., 2005).

Factors Governing Central Bioavailability and Activity

These compounds are constructed based on a 2-oxoamide with a hydrocarbon tail and four carbon tether. An important consideration in the functionality of these agents is their high cLog P values, in the range of 6-8. It is widely considered that agents with log P values greater than 5 may not be “druggable” (Lipinski et al., 2001). It is important to note that in the present systems,
the target of drug action is within the cytosol. This requires that the molecule have a lipophilicity that allows it to readily cross the cell membrane to interact with PLA₂. In the present work, we found that three of the molecules, AX048, AX 057 and AX006, possessed appropriate enzyme inhibitory activity in a cell-free *in vitro* assay. Yet only AX048 was observed to show *in vivo* activity. We suspect that the dissociation between *in vitro* and *in vivo* activity that these agents display may well depend on the complex issue of distribution that these molecules face. At present, we believe that AX048 may be acting as a prodrug. The most common prodrug moiety in marketed drugs is the esterification of an acid group with a simple alkyl alcohol. A variety of ester prodrugs, in particular ethyl esters, are summarized in a recent review (Beaumont, et al., 2003). A number of ethyl ester ACE inhibitors, for example enalapril, exhibit greater oral activity than would be expected purely from the increased lipophilicity due to the conversion to ethyl ester. Furthermore, there is evidence that this ethyl ester is actively absorbed by a carrier mechanism (Swan and Tukker, 1997). These data could explain why only ethyl ester (AX048) out of the four agents is active *in vivo*, while the other three agents are inactive at a dose of 3 mg/kg. Nevertheless, the observation that AX048 was able to produce an antihyperalgesic effect indicates that this molecule has properties that allow penetration of cellular membranes. Further work will be required to define the critical physical chemistry that defines the ability of AX048 to gain access to the to the CNS and inhibit intracellular PLA₂.

**Multiple effects of PLA₂ Inhibition**

In the face of peripheral inflammation and tissue injury, an exaggerated processing of nociceptive stimuli ensues. This facilitation reflects in part an afferent-evoked cascade leading to
enhanced nociceptive processing at the spinal level. An important component of this cascade is associated with the actions of spinally-released prostanoids. Support for this thesis arises from the observation that the spinal delivery of prostaglandins will induce hyperalgesia and that these lipidic acids are released into the spinal extracellular space after tissue injury (see references in Introduction). In addition, intrathecal COX inhibitors reduce prostaglandins release and the facilitated state induced by peripheral injury or by the direct activation of these circuits with IT SP and/or glutamate (see Svensson and Yaksh, 2002). This cascade suggests the relevance of pursuing the upstream PLA₂ linkages which precede those mediated by cyclooxygenase. We note, however, that there is substantial evidence that other products of PLA₂ activity are important in nociceptive processing. i) Arachidonic acid generated by PLA₂ isoforms can directly augment NMDA ionophore function (Richards et al., 2003). The NMDA receptor is believed to play an important role in pre- and post-synaptic facilitation at the spinal level (L'Hirondel et al., 1999; Richards et al., 2003). ii) Arachidonic acid formed by the action of PLA₂s also provides the essential substrate necessary for the cyclooxygenase-independent synthesis of isoprostanes. Spinal isoprostanes initiate facilitated transmitter release and neuronal discharge, and their spinal delivery will lead to hyperalgesia (Evans et al., 2000). iii) Platelet-activating factor (PAF), an alkyl-phospholipid, arises from the membrane lipid hydrolysis by PLA₂. PAF produces a prominent allodynia after spinal delivery (Morita et al., 2004). This lipid mediator is present in the spinal cord and is released from stimulated microglia cells (Jaranowska et al., 1995). iv) PLA₂ activity forms lysophosphates. These products have been implicated in facilitated states of pain processing (Inoue et al., 2004; Seung Lee et al., 2005). In short, we hypothesize that a more pronounced effect on spinal nociceptive processing might arise by
blocking these linkages upstream to COX₂. Finally, the present studies showing the development
of systemically bioavailable PLA₂-selective agents may be relevant to therapeutic targets other
than pain. A variety of neuroninflammatory processes may also be mediated through their
activation of neuraxial PLA₂ isoforms.
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REFERENCES


Svensson CI, Lucas KK, Hua XY, Powell HC, Dennis EA and Yaksh TL (2005b) Spinal phospholipase A(2) in inflammatory hyperalgesia: Role of the small, secretory phospholipase A(2). *Neuroscience* 133:543-553.


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LEGENDS FOR FIGURES

Figure 1. Schema indicating the synthetic sequence for these AX compounds.

Figure 2. *In vitro* dose response inhibition curves of AX006 (circles), AX010 (squares), AX048 (up triangles), AX057 (down triangles) for Group IVA cPLA₂. Curves represent a fit to a logarithmic function.

Figure 3. *In vitro* dose response inhibition curves of AX010 (squares), AX048 (up triangles), AX057 (down triangles) for Group iVI iPLA₂. Curves represent a fit to a logarithmic function.

Figure 4. Effects of agents on *in vitro* cyclooxygenase activity expressed as percent inhibition. Figure presents the mean ± SD for drug treated samples versus control. As indicated, indomethacin (Indo, 50 µM) but not AX006 (50 µM), AX010 (50 µM), AX048 (50 µM) or AX057 (50 µM) served to inhibit cyclooxygenase activity at the doses employed.

Figure 5. Effects of AX006, AX010, AX048 and AX057 (3 mg/kg, IP) on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan. Drug or vehicle was delivered at 30 min prior to intraplantar injection of carrageenan and thermal escape latency was measured immediately before and at intervals afterwards up to 180 min. Each set of graphs shows the mean ± SEM of the response latency (sec) over time for the injured (*Inj*) and uninjured (*Uninj*) paw for drug and vehicle treated animals. As indicated in the legend for each graph, in control treated groups, the carrageenan paw displayed a significant decline in latency from baseline (1
way ANOVA). This decline was prevented only by AX048. The histogram inset displays the mean group cumulative difference in response latencies between uninjured and injured paw over the test interval (90-180 min). As indicated, this measure of hyperalgesia was significantly reduced only by AX048 (unpaired t-test).

**Figure 6.** Dose response curve for the anti-hyperalgesic effects of IP AX048 on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan. Each point presents the mean and SEM (N = 5) of the summed difference in response latencies between injured and uninjured paw (*Slope : p<0.0004). The horizontal solid and dashed line presents the mean ± SEM of the vehicle treated control animals). Studies were carried out as described in Figure 4. Graph presents the mean ± SEM of the group cumulative difference in response latencies between uninjured and injured paw over the test interval (90-180 min) as a function of dose. The horizontal solid and dashed line presents the mean ± SEM of the thermal hyperalgesia observed in vehicle treated rats after carrageenan. The ED50 dose of AX048 (50% reduction in the thermal escape latency.

**Figure 7.** Effects of pretreatment interval on antihyperalgesic effects of AX048 (3 mg/kg, IP) on carrageenan evoked thermal hyperalgesia. Drug was delivered at 15, 30, 180 or 360 min prior to the delivery of intraplantar carrageenan and thermal escape was measured immediately before carrageenan and at intervals afterwards up to three hours. Data are expressed as the cumulative latency difference between injured and uninjured paw. Maximum effects were observed at 30
min and persisted through 3 hrs. 1 way ANOVA (p = 0.0006) followed by post hoc Bonferroni’s Multiple Comparison Test (n = 4-12 / treatment group). ** p<0.05 as compared to Control.

**Figure 8.** Effects of AX006, AX010, AX048 and AX057 (IT 30 µg/10 µL) on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan. Drug or vehicle was delivered at 15 min prior to intraplantar injection of carrageenan and thermal escape was measured immediately before and at intervals afterwards up to 180 min. Each set of graphs shows the mean ± SEM of the response latency (sec) over time for the injured (Inj) and uninjured (Uninj) paw for drug and vehicle treated animals. As indicated in the legend for each graph, in control treated groups, the carrageenan paw displayed a decline in latency from baseline (1 way ANOVA). This decline was prevented only by AX048. The histogram inset displays the mean group cumulative difference in response latencies between uninjured and injured paw over the test interval (90-180 min). As indicated, this measure of hyperalgesia was significantly reduced only by AX048 (unpaired t-test).

**Figure 9.** Effects of AX006, AX010, AX048 and AX057 (3 mg/kg, IP) on intrathecal SP evoked thermal hyperalgesia. Drug or vehicle was delivered at 30 prior to the intrathecal delivery of substance P (IT-SP: 30 nmol) and thermal escape was measured immediately before IT SP and at intervals afterwards up to 60 min. Data are expressed as the response latency (sec) over time. As indicated in the legend for each graph, 1 way ANOVA showed significant thermal hyperalgesia reversal from vehicle for AX048, but not the other agents.
Figure 10. Unanesthetized rats prepared with spinal dialysis catheters received IP injections of vehicle or AX006, AX010, AX048 and AX057 (3 mg/kg, IP) followed 20 min later by an intrathecal injections of substance P (IT-SP: 20 nmol). (Top) Time course of PGE2 release. determined in sequential 15 min samples out through 45 min following IT SP in animals pretreated with IP vehicle or IP AX048 (3 mg/kg). IT SP evoked a time dependent increase in release following IP vehicle but not following IP AX048 (* p < 05). (Bottom) Area under the time effect curve for PGE2 release from 0-45 min in rats receiving vehicle, AX006, AX010, AX048 or AX057). As indicated, after IP AX006, AX010 or AX057, IT SP evoked a significant increase as compared to vehicle only. (Kruskall Wallace p <0.008. * p<0.05; ** p<0.01, Dunns Multiple Comparison versus vehicle (VEH). In contrast, following IP AX048 there was no difference between release as compared to IP vehicle alone (p>0.05).
**Table 1.** List of compounds including physical characteristics, *in vitro* $X_t(50)$ values for Group IVA and Group VIA PLA₂. N.D. denotes 25% inhibition or less at 0.091 mole fraction, L.D. indicates between 25% and 50% inhibition at 0.091 mole fraction.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Mol. Wt.</th>
<th>CLog P</th>
<th>Group IVA $X_t(50)$ (mole fraction)</th>
<th>Group VIA $X_t(50)$ (mole fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX006</td>
<td><img src="image" alt="AX006 Structure" /></td>
<td>355.52</td>
<td>6.6</td>
<td>0.024 ± 0.015</td>
<td>N.D.</td>
</tr>
<tr>
<td>AX010</td>
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<td>369.54</td>
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<td>L.D.</td>
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<tr>
<td>AX048</td>
<td><img src="image" alt="AX048 Structure" /></td>
<td>383.57</td>
<td>7.6</td>
<td>0.022 ± 0.009</td>
<td>0.027 ± 0.009</td>
</tr>
<tr>
<td>AX057</td>
<td><img src="image" alt="AX057 Structure" /></td>
<td>411.62</td>
<td>8.3</td>
<td>0.031 ± 0.017</td>
<td>0.026 ± 0.014</td>
</tr>
</tbody>
</table>
**Table 2**  
Effects of Intrathecal SR141716A on the effects of Intrathecal Anandamide and AX048 at 2 hrs post carrageenan on thermal escape latency of the uninjured and injured paw.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Un-injured paw</th>
<th>Injured Paw</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT Vehicle + IT Vehicle</td>
<td>10.4 ± 1.5</td>
<td>3.3 ± 1.1*</td>
</tr>
<tr>
<td>IT Vehicle + IT Anandamide (100µg)</td>
<td>18 ± 3.2#</td>
<td>15.2 ± 4.2#</td>
</tr>
<tr>
<td>IT SR141716A (20µg) + IT Anandamide (100µg)</td>
<td>10.8 ± 2.4</td>
<td>3.1 ± 1.3*</td>
</tr>
<tr>
<td>IT Vehicle + IT AX048 (30µg)</td>
<td>10.3 ± 2.0</td>
<td>7.8± 1.8 #</td>
</tr>
<tr>
<td>IT SR141716A + IT AX048 (30µg)</td>
<td>11.7 ±2.9</td>
<td>7.1 ± 1.9#</td>
</tr>
</tbody>
</table>

*: p<0.05 vs uninjured paw; two tailed paired t-test.

#: p<0.05 vs respective vehicle treated paw: two tailed unpaired t-test.

N= 4-6 rats / treatment group.
\[
\text{OH} \quad \text{OH} + H_2N \quad \text{AC} \quad \text{AC} \quad R \quad \text{WSCl} \quad \text{HOBr} \quad \text{NaOCl, AcNH}_{2} \quad \text{NaBr, NaHCO}_3
\]

AX048  R: CH\_2CH\_3
AX057  R: C(CH\_3)\_3
Figure 2

Inhibition (%) vs [Inhibitor] (mole fraction)
Figure 5.

**AX 006**
- AX006 Inj n=5
- AX006 Uninj n=5
- Control Inj n=5
- Control Uninj n=5

**AX 010**
- AX010 Inj n=5
- AX010 Uninj n=5
- Control Inj n=5
- Control Uninj n=5

**AX 048**
- AX048, Inj n=17
- AX048, Uninj n=17
- Cont, Inj n=13
- Cont, Uninj n=13

**AX 057**
- AX057 Inj n=18
- AX057 Uninj n=18
- Control Inj n=16
- Control Uninj n=16

**ESCAPE LATENCY (Sec)**
- Pre 30 60 90 120 150 180

**TIME (Min)**
- Pre Inj 30 60 90 120 150 180

*p-values and significant differences indicated.*
Figure 6.

\[ Y = -3.41X + 0.84 \]

\[ r^2 = 0.85 \]

Control ± SEM

50% of Control
Figure 7

![Graph showing Δ LATENCY (Sec) over time](image-url)
Figure 8.

**AX 006-IT**

- AX006 Inj., n=5
- AX006 Uninj., n=5
- Control Inj., n=5
- Control Uninj., n=5

- p=0.005
- p<0.001

- ESCAPE LATENCY (Sec.)
- TREATMENT

**AX 010-IT**

- AX010 Inj., n=4
- AX010 Uninj., n=4
- Control Inj., n=4
- Control Uninj., n=4

- p=0.008
- p<0.001

- ESCAPE LATENCY (Sec.)
- TREATMENT

**AX 048-IT**

- AX048 Inj., n=15
- AX048 Uninj., n=15
- Control Inj., n=17
- Control Uninj., n=17

- p=0.053
- p<0.001

- ESCAPE LATENCY (Sec.)
- TREATMENT

**AX 057-IT**

- AX057 Inj., n=5
- AX057 Uninj., n=5
- Control Inj., n=5
- Control Uninj., n=5

- p=0.003
- p<0.001

- ESCAPE LATENCY (Sec.)
- TREATMENT
Figure 9.

**AX 006**
- p = 0.1836
- AX006 n=5
- Cont n=7

**AX 010**
- p = 0.8625
- AX010 n=6
- Control n=7

**AX 048**
- p = 0.0319
- AX048, n=9
- Control, n=10

**AX 057**
- p = 0.4615
- AX057 n=16
- Control n=17

Y-axis: Escape Latency (Sec)
X-axis: Time (Min)
AUC (% BASELINE)

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>0</th>
<th>1000</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP VEH</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IT SP:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH</td>
<td></td>
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<tr>
<td>AX006</td>
<td>*</td>
<td></td>
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<tr>
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<tr>
<td>AX057</td>
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<tr>
<td>AX048 VEH</td>
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</tr>
<tr>
<td>SP</td>
<td>*</td>
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</tr>
</tbody>
</table>

% BASELINE RELEASE

- Vehicle + SP
- AX048 + SP

TIME AFTER IT SP

- 0
- 15
- 30
- 45

Figure 10: SPINAL PGE2 RELEASE