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Systemic and Intrathecal Effects of a Novel Series of Phospholipase A_2 Inhibitors on Hyperalgesia and Spinal Prostaglandin E_2 Release

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ABSTRACT

Phospholipase A_2 (PLA_2) forms are expressed in spinal cord, and inhibiting spinal PLA_2 induces a potent antihyperalgesia. Here, we examined the antihyperalgesic effects after systemic and i.t. 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 PLA_2 ($cPLA_2$) and group VIA calcium-independent PLA_2 ($iPLA_2$) in inhibition assays using human recombinant enzyme. The rank ordering of potency in blocking group IVA $cPLA_2$ was AX048 (ethyl 4-[(2-oxohexadecanoyl)amino]butanoate), AX006 (4-[(2-oxohexadecanoyl)amino]butanoic acid), and AX057 (*tert*-butyl 4-[(2-oxohexadecanoyl)amino]butanoate) > AX010 (methyl 4-[(2-oxohexadecanoyl)amino]butanoate) and for inhibiting group VIA $iPLA_2$ was AX048, AX057 > AX006, and AX010. No agent altered recombinant cyclooxygenase activity. In vivo, i.t. (30 μg) and systemic (0.2–3 mg/kg i.p.) AX048 blocked carrageenan hyperalgesia and after systemic delivery in a model of spinally mediated hyperalgesia induced by i.t. substance P (SP). The other agents were without activity. In rats prepared with lumbar i.t. loop dialysis catheters, SP evoked spinal prostaglandin E_2 (PGE_2) release. AX048 alone inhibited PGE_2 release. Intrathecal SR141617, a cannabinoid CB1 inhibitor at doses that blocked the effects of i.t. anandamide had no effect upon i.t. AX048. These results suggest that AX048 is the first systemically bioavailable compound with a significant affinity for group IVA $cPLA_2$, which produces a potent antihyperalgesia. The other agents, although demonstrating enzymatic activity in cell-free assays, appear unable to gain access to the intracellular PLA_2 toward which their action is targeted.

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). Although 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, 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 A_2 (PLA_2) because it is required to generate arachidonic acid, which is the substrate for COX-mediated

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ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; PLA_2, phospholipase A_2; $cPLA_2$, calcium-dependent PLA_2; $iPLA_2$, calcium-independent PLA_2; AX006, 4-[(2-oxohexadecanoyl)amino]butanoic acid; AX010, methyl 4-[(2-oxohexadecanoyl)amino]butanoate; AX048, ethyl 4-[(2-oxohexadecanoyl)amino]butanoate; AX057, *tert*-butyl 4-[(2-oxohexadecanoyl)amino]butanoate; SP, substance P; NMDA, *N*-methyl-D-aspartate; DMSO, dimethyl sulfoxide; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; ANOVA, analysis of variance; $AACOCF_3$, arachidonyl trifluoromethylketone; BMS-229724, 4-[4-[2-[2-[bis(4-chlorophenyl)methoxy]ethylsulfonyl]ethoxy]phenyl]-1,1,1-trifluoro-2-butanone.

prostanoid formation. In recent work, we have shown the presence of constitutive mRNA and protein in the spinal cord for group IVA calcium-dependent PLA_2 ($cPLA_2$) and group VIA calcium-independent PLA_2 (iPLA2) and groups II and V secretory PLA_2 forms (Lucas et al., 2005; Svensson et al., 2005b). Inhibition of group IV $cPLA_2$ but not group VI $iPLA_2$ isoforms using i.t.-delivered agents suggested a role for group IV $cPLA_2$, but not group VI $iPLA_2$ (Lucas et al., 2005), in inflammation-evoked hyperalgesia.

We have reported recently the discovery of a novel structural series of 2-oxoamides that inhibit group IVA $cPLA_2$ in vitro and in vivo (Kokotos et al., 2002, 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 analogs of this series, AX006, AX010, AX048, and AX057. These molecules were examined for their inhibitory effects on group IV $cPLA_2$ and group VI $iPLA_2$ as well as on COX activity in in vitro assays. Their actions were then characterized after systemic and i.t. delivery on thermal hyperalgesia induced by peripheral inflammation (intraplantar carrageenan). In addition, we have shown previously 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 PGE_2 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 PLA_2 inhibitors on the hyperalgesia and PGE_2 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.

Materials and 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, Indianapolis, IN) were individually housed and maintained on a 12-h 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 and Rudy (1976). A polyethylene catheter (PE- 5, 0.014 in outside diameter; Spectranetics, Colorado Springs, CO) was inserted into the i.t. 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 i.t. using the same technique as described above for the i.t. catheter. A 3-day interval was allowed to elapse prior to including the animal in a study. In all cases, the exclusion criteria were the presence of any neurological sequelae, 20% weight loss after implantation, or 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-Aldrich, 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 × 22 × 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 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 latencies over the postinjection 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 i.t. injection of SP (20 nmol/10 μl). The mean paw withdrawal latency of the left and right paws is assessed at each time point. The mean difference between the pre- and post-i.t. 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 Apparatus Inc., Holliston, MA) was connected, and dialysis tubing was perfused with artificial cerebrospinal fluid at a rate of 10 μl/min. The artificial cerebrospinal fluid contained 151.1 mM Na^+, 2.6 mM K^+, 0.9 mM Mg^{2+}, 1.3 mM Ca^{2+}, 122.7 mM Cl^-, 21.0 mM HCO_3, 2.5 mM HPO_4, and 3.5 mM 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/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 i.t. injection of NMDA (0.6 μg). The concentration of PGE_2 in spinal dialysate was measured by enzyme-linked immunosorbent assay 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-keto$PGF_{1\alpha}$, PGA_2, or PGB_2 but cross-reacts with PGE_1 and PGE_3.

Drug Delivery. Drugs were delivered systemically (i.p.) or spinally (i.t.). Intraperitoneal drugs were delivered uniformly in doses prepared in volumes of 0.5 ml/kg. Drugs injected i.t. were administered in a total volume of 10 μl followed by a 10-μl flush using vehicle.

Enzyme Assays. In vitro group IV $cPLA_2$ and group VI $iPLA_2$ 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 N_2 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 5 min 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 min. 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 $X_I(50)$ calculations.

Inhibition of cyclooxygenase-1 and cyclooxygenase-2 was tested in vitro using the COX Activity Assay kit (catalog no. 760151) from Cayman Chemical (Ann Arbor, MI). Assays were performed in 96-

well plates using 10 μl of supplied COX standard (catalog no. 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, 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 min. After addition of *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine and arachidonic acid, samples were mixed and allowed to incubate for 5 min at room temperature before reading absorbance at 595 nm to determine results. Results were calculated, and percent inhibition values were 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 and 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, 2004). The synthesis and the characterization of the novel agents AX048 and AX057 are described here in detail. Figure 1 summarizes the synthesis schema.

For 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 CH_2Cl_2 (20 ml), Et_3N (6.2 ml, 4.4 mmol) and subsequently 2-hydroxy-hexadecanoic acid using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (0.42 g, 2.2 mmol) and 1-hydroxylbenzotriazole (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% $NaHCO_3$, and brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by column chromatography using $CHCl_3$/MeOH (95:5) as the eluent.

Ethyl 4-[(2-hydroxyhexadecanoyl)amino]butanoate. Yield 72%; ^{1}H NMR. δ 6.68 (1H, t, J = 7 Hz, NH), 4.13 (3H, m, CH, $COOCH_2CH_3$), 3.34 (2H, m, CH_2NH), 2.68 (1H, b, OH), 2.32 (2H, t, J = 7 Hz, CH_2COO), 1.80–1.58 (4H, m, CH_2CH_2COO, CH_2CH), 1.45–1.23 (27H, m, 12×CH_2, $COOCH_2CH_3$), 0.85 (3H, t, J = 7 Hz, CH_3); ^{13}C 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 $C_{22}H_{43}NO_4$ (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%; ^{1}H NMR. δ 6.49 (1H, t, J = 7 Hz, NH), 4.12 (1H, m, CH), 3.34 (2H, m, CH_2NH), 2.73 (1H, b, OH), 2.27 (2H, t, J = 7 Hz, CH_2COO), 1.82–1.49 (4H, m, CH_2CH$_2$COO, CH_2CH), 1.45 [9H, s, C(CH$_3$)$_3$], 1.38–1.15 (24H, m, 12×CH_2), 0.89 (3H, t, J = 7 Hz, CH_3); ^{13}C 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 $C_{24}H_{47}NO_4$ (413.63): C, 69.69; H, 11.45, N, 3.39. Found: C, 69.42; H, 11.61; N, 3.27.

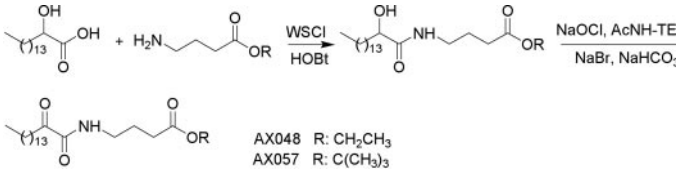


Fig. 1. Scheme indicating the synthetic sequence for these AX compounds.

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 4-acetamido-2,2,6,6-tetramethylpiperidine-1-yloxy free radical (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 $NaHCO_3$ (0.25 g, 3 mmol) was added drop wise 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 H_2O (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 $Na_2S_2O_3$ (6 ml), and brine and dried over Na_2SO_4. The solvents were evaporated under reduced pressure, and the residue was purified by column chromatography [EtOAc-petroleum ether 1:9 (b.p., 40–60°C)].

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, $COOCH_2CH_3$), 3.33 (2H, m, CH_2NH), 2.89 (2H, t, J = 7 Hz, CH_2COCO), 2.34 (2H, t, J = 7 Hz, CH_2COO), 1.87 (2H, m, CH_2CH$_2$COO), 1.57 (2H, m, CH_2CH$_2$COCO), 1.40–1.15 (25H, m, 11×CH_2, $COOCH_2CH_3$), 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.

AX057. Yield 95%; white solid; mp 61–62°C; ^{1}H NMR. δ 7.11 (1H, m, NH), 3.33 (2H, m, CH_2NH), 2.91 (2H, t, J = 7 Hz, CH_2CO), 2.28 (2H, t, J = 7 Hz, CH_2COO), 1.84 (2H, m, CH_2CH$_2$COO), 1.60 (2H, m, CH_2CH$_2$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

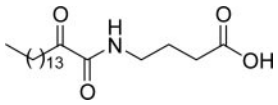
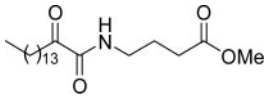
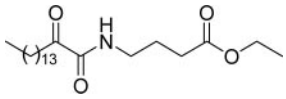
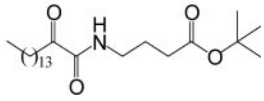
Escape latency data are presented as the mean ± S.E.M. For carrageenan and i.t. SP analysis of thermal escape, latencies were carried out over time and compared with one-way ANOVA. For carrageenan, difference scores between control and injured paws over time were calculated for each group. Comparison of drug with vehicle treatment was performed using an unpaired Student's *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 percentage of baseline, and the area under the release curve following i.t. 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 Inc., San Diego, CA).

Results

Synthesis and Physical Properties of Test Agent

Ethyl and *tert*-butyl 4-amino-butonate were coupled with 2-hydroxy-hexadecanoic acid using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide as a condensing agent in the presence of 1-hydroxylbenzotriazole. The 2-hydroxyamides synthesized were oxidized with NaOCl in the presence of a catalytic amount of 4-acetamido-2,2,6,6-tetramethylpiperi-

TABLE 1
List of compounds including physical characteristics, in vitro $X_I(50)$ values for groups IVA and VIA PLA_2
N.D., 25% inhibition or less at 0.091 mole fraction; L.D., between 25 and 50% inhibition at 0.091 mole fraction.

Compound	Structure	Mol. Wt.	CLogP	Group IVA $X_I(50)$ (mole fraction)	Group VIA $X_I(50)$
AX006		355.52	6.6	0.024 ± 0.015	N.D.
AX010		369.54	7.1	N.D.	L.D.
AX048		383.57	7.6	0.022 ± 0.009	0.027 ± 0.009
AX057		411.62	8.3	0.031 ± 0.017	0.026 ± 0.014

dine-1-yloxy free radical to produce compounds AX048 and AX057.

Characterization of PLA_2 Inhibitory Activity: Enzymatic Assay. The inhibitory effects of AX006, AX010, AX048, and AX057 on pure group IVA PLA_2 and group VIA PLA_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_{50} or K_I is that PLA_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_2s 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_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_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 (Figs. 2 and 3; Table 1), AX048 and AX057 were potent against group IVA PLA_2 and group VIA PLA_2, AX006 was potent against group IVA PLA_2 alone, and AX010 was less effective against both.

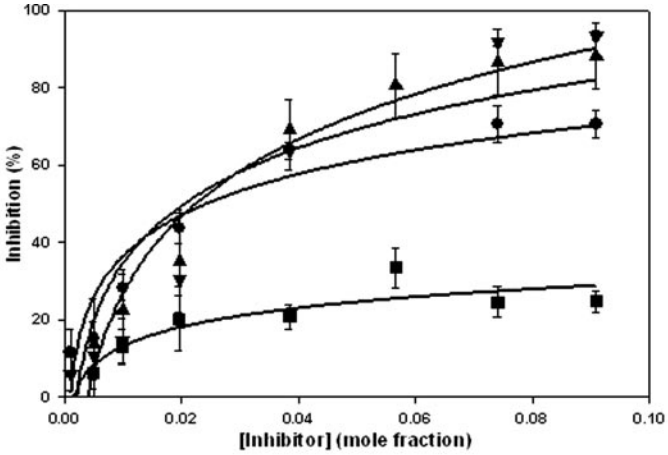


Fig. 2. In vitro dose-response inhibition curves of AX006 (circles), AX010 (squares), AX048 (up triangles), and AX057 (down triangles) for group IVA $cPLA_2$. Curves represent a fit to a logarithmic function.

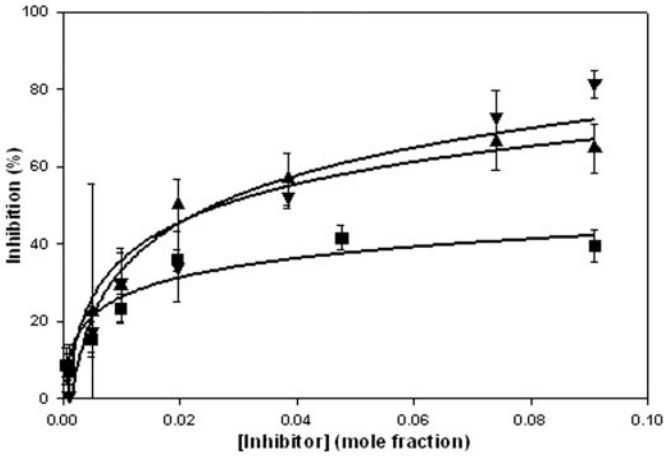


Fig. 3. In vitro dose-response inhibition curves of AX010 (squares), AX048 (up triangles), and AX057 (down triangles) for group iVI $iPLA_2$. Curves represent a fit to a logarithmic function.

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 PLA_2 (50 μM) had no inhibitory effects upon COX activity (Fig. 4).

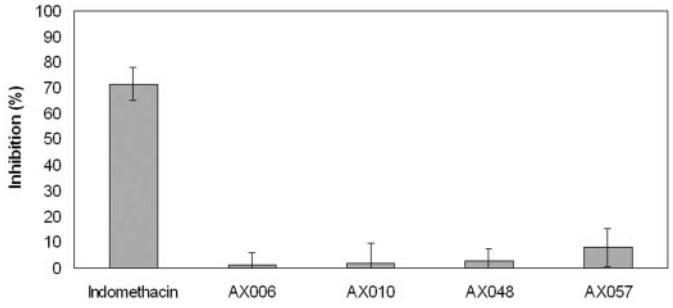


Fig. 4. Effects of agents on in vitro cyclooxygenase activity expressed as percent inhibition. Figure presents the mean ± S.D. for drug-treated samples versus control. As indicated, indomethacin (Indo, 50 μM) but not AX006 (50 μM), AX010 (50 μM), AX048 (50 μM), or AX057 (50 μM) served to inhibit cyclooxygenase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

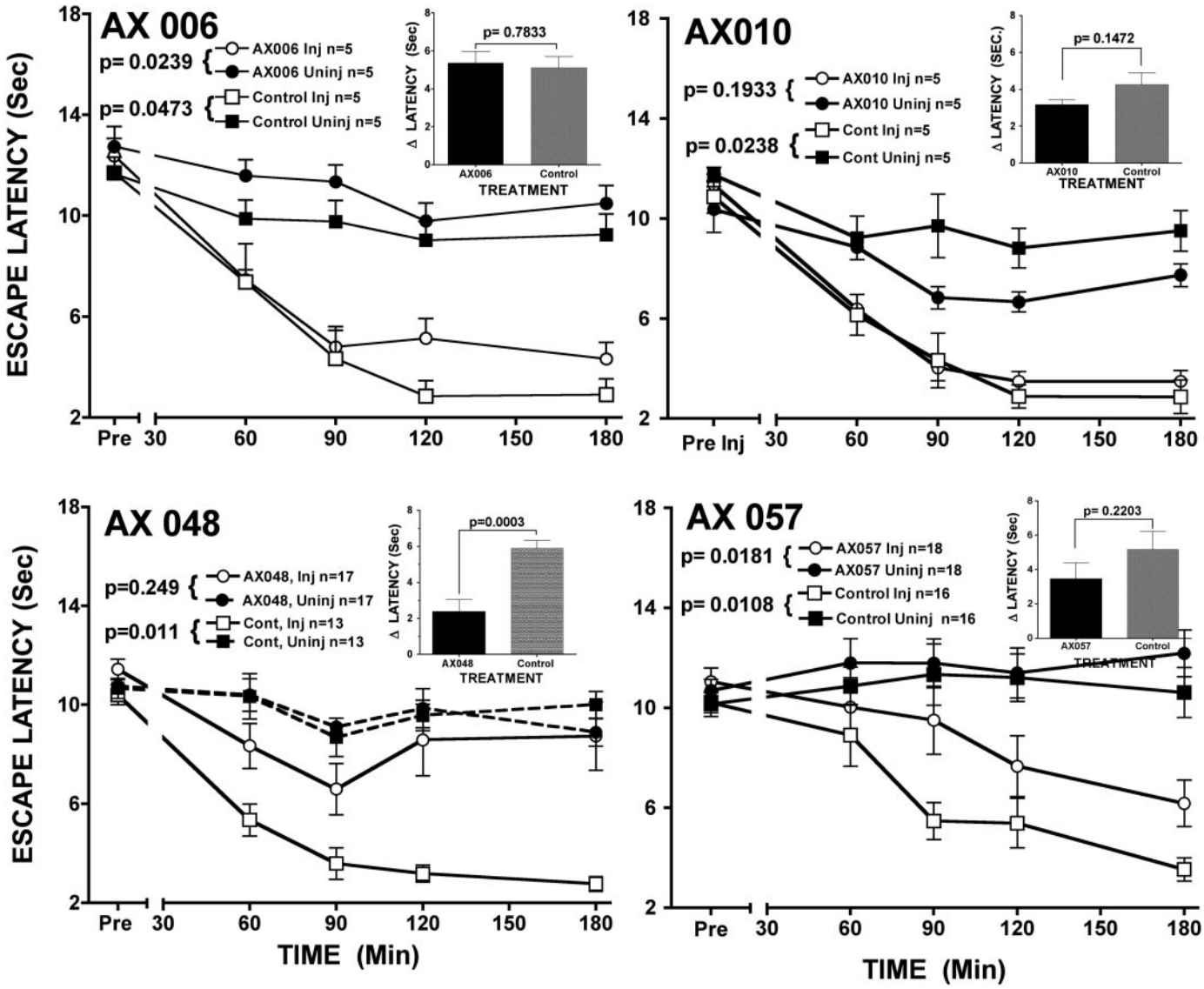


Fig. 5. Effects of AX006, AX010, AX048, and AX057 (3 mg/kg i.p.) on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan. Drug or vehicle was delivered at 30 min prior to intraplantar injection of carrageenan, and thermal escape latency was measured immediately before and at intervals afterward up to 180 min. Each set of graphs shows the mean ± S.E.M. of the response latency (seconds) 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 (one-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 paws over the test interval (90–180 min). As indicated, this measure of hyperalgesia was significantly reduced only by AX048 (unpaired Student's *t* test).

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 to 12 s 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 i.p. or i.t. vehicle was significantly reduced to approximately 3 to 5 s within 90 to 120 min (Figs. 5 and 6).

Intraperitoneal Delivery. Pretreatment (30 min) with 3 mg/kg (i.p.) 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 (Fig. 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 antihyperalgesic 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 with the vehicle-treated group.

Dose Dependence. The effects of i.p. AX048 were observed to be dose-dependent over the range of 0.2 to 3 mg/kg (slope; $P < 0.0004$) (Fig. 6). The ED_{50} was defined as the dose that reduced the hyperalgesia observed in a vehicle-treated animal by 50%. On this basis, the estimated i.p. ED_{50} value for i.p. AX048 was 1.2 mg/kg (95% confidence interval, −0.5572 to 0.7713).

Time Course of Action. To determine the time course of the drug action, i.p. delivery of AX048 (3 mg/kg) was under-

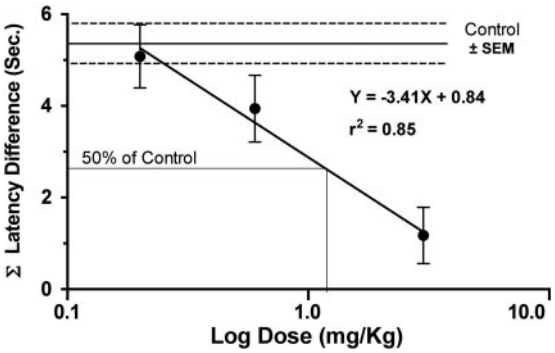


Fig. 6. Dose-response curve for the antihyperalgesic effects of i.p. AX048 on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan. Each point presents the mean and S.E.M. ($n = 5$) of the summed difference in response latencies between injured and uninjured paw (*, slope, $P < 0.0004$). The horizontal solid and dashed line represents the mean ± S.E.M. of the vehicle-treated control animals. Studies were carried out as described in Fig. 4. Graph presents the mean ± S.E.M. of the group cumulative difference in response latencies between uninjured and injured paws over the test interval (90–180 min) as a function of dose. The horizontal solid and dashed line presents the mean ± S.E.M. of the thermal hyperalgesia observed in vehicle-treated rats after carrageenan. The ED_{50} dose of AX048 (50% reduction in the thermal escape latency) was 1.2 mg/kg.

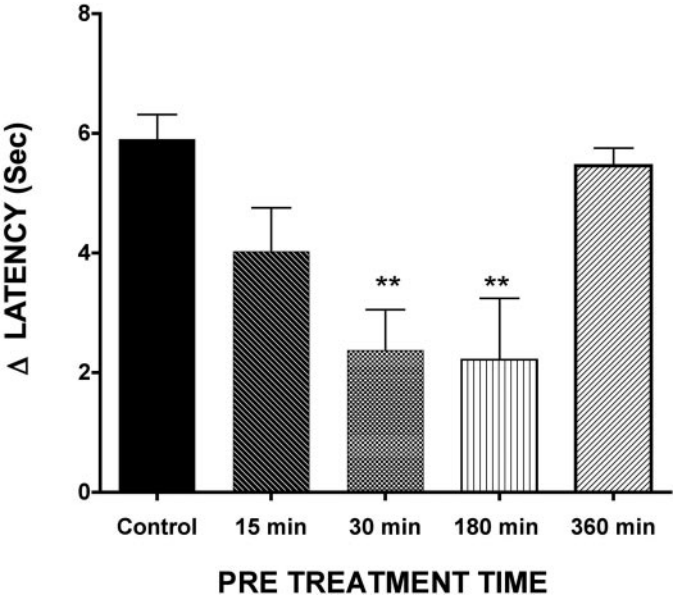


Fig. 7. Effects of pretreatment interval on antihyperalgesic effects of AX048 (3 mg/kg i.p.) on carrageenan-evoked thermal hyperalgesia. Drug was delivered at 15, 30, 180, or 360 min prior to the delivery of intraplantar carrageenan, and thermal escape was measured immediately before carrageenan and at intervals afterward up to 3 h. Data are expressed as the cumulative latency difference between injured and uninjured paws. Maximum effects were observed at 30 min and persisted for 3 h. One-way ANOVA ($P = 0.0006$) followed by post hoc Bonferroni's multiple comparison test ($n = 4$–12 / treatment group). **, $P < 0.05$ as compared with control.

taken at −15, −30, and −180 min (Fig. 7). As indicated, peak effects were noted at 30 min, and minimal effects were observed at 15 min. The effects persisted through for 180 min but were no different from the control by 360 min.

Intrathecal Delivery and Carrageenan-Induced Thermal Hyperalgesia

Control. In animals receiving i.t. injections of vehicle, the intraplantar injection of carrageenan resulted in a significant unilateral thermal hyperalgesia as compared with the uninjected paw (Fig. 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 AX057, attenuated the thermal hyperalgesia (Fig. 8). Again, after i.t. 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 AX048-treated group in comparison with the vehicle-treated group.

Intrathecal Substance P-Induced Thermal Hyperalgesia

Control. Baseline thermal escape latencies were on the order of 10 to 12 s. In systemic vehicle-treated animals, the i.t. 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 (Fig. 9).

Drug Effect. Pretreatment with 3 mg/kg (i.p.) of the four agents 30 min prior to the i.t. delivery of SP revealed that AX048, but not AX006, AX010, or AX057, completely prevented the spinally evoked thermal hyperalgesia (Fig. 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 antihyperalgesic agent.

Side Effect Profile. After delivery of the highest systemic dose (3 mg/kg) or i.t. 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/100 μl perfusate. Intrathecal injection of SP (20 μg) but not vehicle (saline, not shown) resulted in a statistically significant increase in PGE_2 concentrations in spinal dialysate as compared with the vehicle-treated control (Fig. 10).

Drug Effect. Pretreatment with the four agents 15 min prior to the delivery of i.t. SP (20 μg/10 μl) revealed that the evoked release of PGE_2 was reduced only in the AX048-treated group. Thus, of the four agents only AX048 exerted a significant inhibitory effect upon PGE_2 synthesis and release (Fig. 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 i.t. vehicle or i.t. SR141716, a CB1 receptor antagonist, followed after 15 min by i.t. AX048 (30 μg) or i.t. anandamide (100 μg). Intrathecal SR141716 had no effect when delivered alone (data not shown). As shown in Table 2, in vehicle-pretreated animals, i.t. 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 i.t. SR141716. Intrathecal AX048 significantly reversed the respective hyperalgesia but had no effect upon the thermal escape latency of the

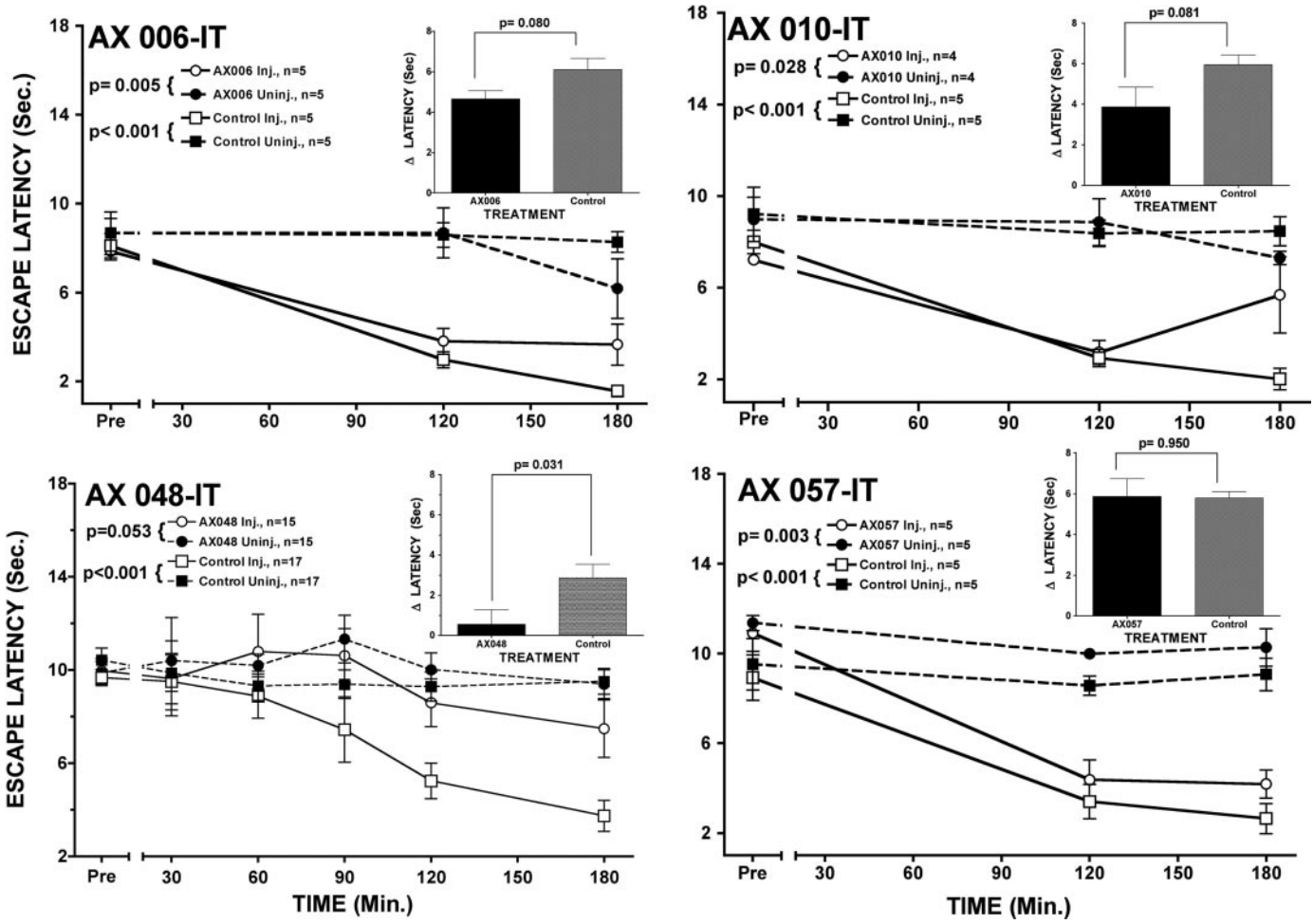


Fig. 8. Effects of AX006, AX010, AX048, and AX057 (i.t. 30 μg/10 μl) on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan. Drug or vehicle was delivered at 15 min prior to intraplantar injection of carrageenan and thermal escape was measured immediately before and at intervals afterward up to 180 min. Each set of graphs shows the mean ± S.E.M. of the response latency (seconds) 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 (one-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 Student's *t* test).

uninjured paw. The antihyperalgesic effects of i.t. AX048 were not altered by i.t. SR141716. These observations suggest that i.t. anandamide, but not i.t. AX048, were interacting with a spinal CB1 receptor to alter thermal escape latency.

Discussion

AX048, but not the three structurally related analogs AX006, AX010, and AX057, exerted a significant effect upon centrally (i.t. SP) and peripherally (intraplantar carrageenan) initiated hyperalgesia. Because the effective i.t. dose was 100 times less than required after systemic delivery, we conclude that the i.t. effect represented a central action. In addition, systemic AX048 blocked the hyperalgesia evoked by i.t. 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, whereas 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 i.t. delivery of methyl arachidonyl fluorophosphonate 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, i.t. injection of $AACOCF_3$ at antihyperalgesic doses decreased PGE_2 release into spinal dialysate evoked by i.t. NMDA (Lucas et al., 2005). In contrast, in those studies, an irreversible group VIA $iPLA_2$ inhibitor (bromoenol lactone), given i.t., 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 et al. (2004) reported that i.c.v. injection of $AACOCF_3$ or bromoenol lactone produced antihyperalgesia as measured using facial carrageenan in mice (Yeo et al., 2004). Burke et al. (2001) reported that BMS-229724, a group IVA $cPLA_2$ inhibitor, was orally active in inhibiting edema and neutrophil infiltration. The present data thus continue to leave the issue open regarding

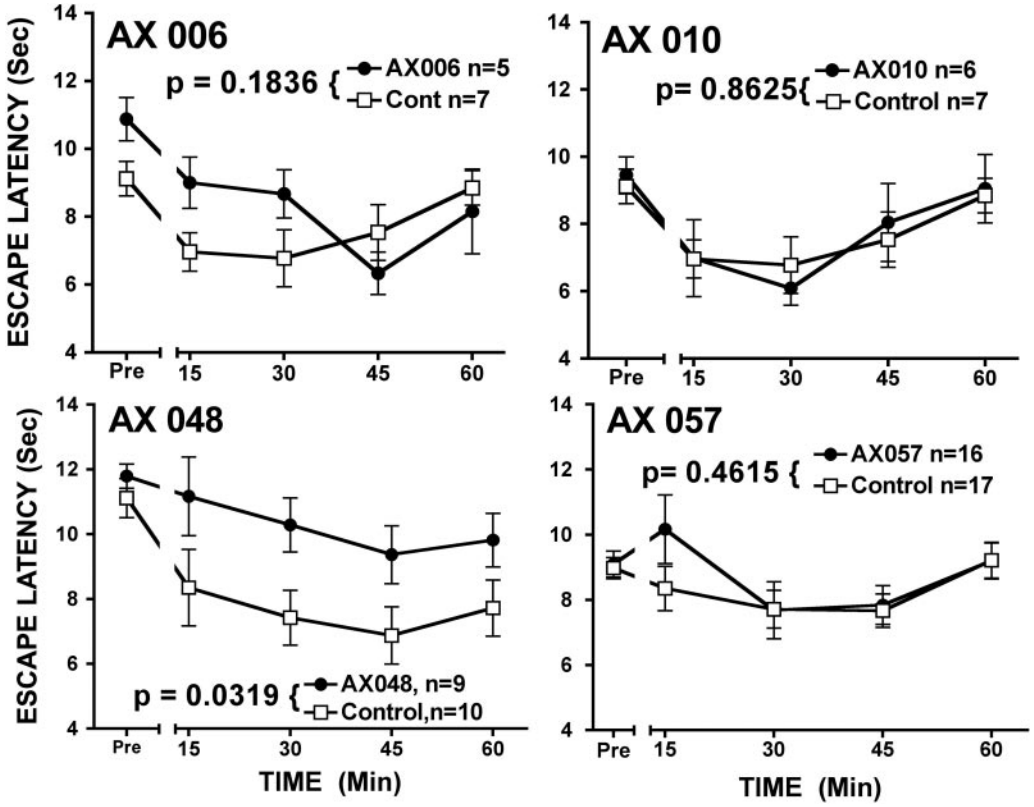


Fig. 9. Effects of AX006, AX010, AX048, and AX057 (3 mg/kg i.p.) on i.t. SP-evoked thermal hyperalgesia. Drug or vehicle was delivered at 30 prior to the i.t. delivery of substance P (i.t. SP, 30 nmol), and thermal escape was measured immediately before i.t. SP and at intervals afterward up to 60 min. Data are expressed as the response latency (seconds) over time. As indicated in the legend for each graph, one-way ANOVA showed significant thermal hyperalgesia reversal from vehicle for AX048, but not the other agents.

the relative contribution of group IVA $cPLA_2$ and group VIA $iPLA_2$.

Role of Spinal PLA_2 Isoforms in Cascade. Western blotting and reverse transcription-polymerase chain reaction have shown that group IVA $cPLA_2$, group VIA $iPLA_2$, and secretory PLA_2 (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 $cPLA_2$. 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 i.t. delivery with 20 μg versus the dose of 3 mg/kg, given i.p., 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 i.t. SP-evoked spinal PGE_2 release, a downstream biomarker believed to be essentially dependent upon PLA_2 activity (Svensson and Yaksh, 2002).

Although the primary target of these molecules examined in the present study is PLA_2, 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 state (see Rice et al., 2002; Svensson and Yaksh, 2002). 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 though 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 i.t. delivery, anandamide elevated the thermal escape latency of the normal paw, an effect not mimicked by the AX048. Finally, SR141716A, a potent CB1 antagonist (Shire et al., 1999), given i.t. at a dose that reversed the i.t. effect of anandamide, failed to alter the effects of AX048. These data suggest an effect of spinal AX048 that 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 though 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 to 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 PLA_2. In the present work, we found that three of the molecules, AX048, AX057, and AX006, possessed appropriate enzyme inhibitory activity in a cell-free in vitro assay. Yet, only AX048 was observed to show in vivo activity.

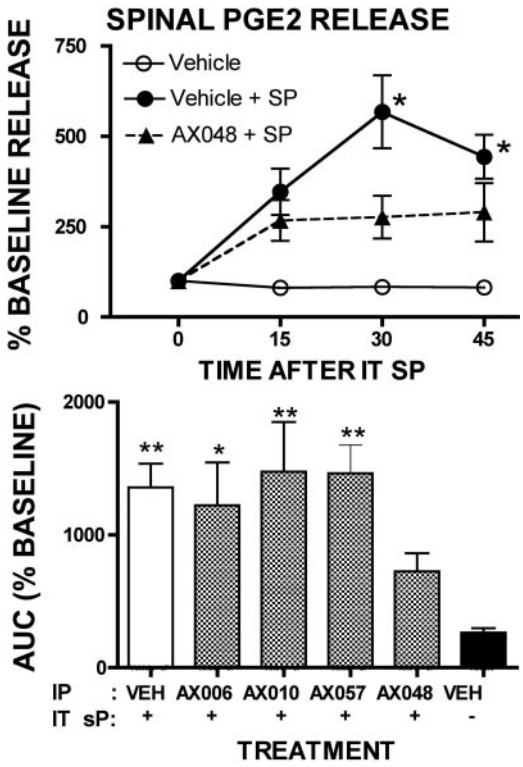


Fig. 10. Unanesthetized rats prepared with spinal dialysis catheters received i.p. injections of vehicle or AX006, AX010, AX048, and AX057 (3 mg/kg i.p.) followed 20 min later by i.t. injections of substance P (i.t. SP, 20 nmol). Top, time course of PGE_2 release determined in sequential 15-min samples out through 45 min following i.t. SP in animals pretreated with i.p. vehicle or i.p. AX048 (3 mg/kg). Intrathecal SP evoked a time-dependent increase in release following i.p. vehicle but not following i.p. AX048 (*, $P < 0.05$). Bottom, area under the time effect curve for PGE_2 release from 0 to 45 min in rats receiving vehicle, AX006, AX010, AX048, or AXO57. As indicated, after i.p. AX006, AX010, or AX057, i.t. SP evoked a significant increase as compared with vehicle only [Kruskall Wallace, $P < 0.008$; *, $P < 0.05$; **, $P < 0.01$, Dunns multiple comparison versus vehicle (VEH)]. In contrast, following i.p. AXO48, there was no difference between release as compared with i.p. vehicle alone ($P > 0.05$).

TABLE 2

Effects of i.t. SR141716A on the effects of i.t. anandamide and AX048 at 2 h postcarrageenan on thermal escape latency of the uninjured and injured paws

n = 4 to 6 rats/treatment group.

Treatment	Uninjured Paw	Injured Paw
i.t. Vehicle + i.t. vehicle	10.4 ± 1.5	3.3 ± 1.1*
i.t. Vehicle + i.t. anandamide (100 μg)	18 ± 3.2**	15.2 ± 4.2**
i.t. SR141716A (20 μg) + i.t. anandamide (100 μg)	10.8 ± 2.4	3.1 ± 1.3*
i.t. vehicle + i.t. AX048 (30 μg)	10.3 ± 2.0	7.8 ± 1.8**
i.t. SR141716A + i.t. AX048 (30 μg)	11.7 ± 2.9	7.1 ± 1.9**

* $P < 0.05$ vs. uninjured paw; two-tailed paired Student's t test.

** $P < 0.05$ vs. respective vehicle-treated paw; two-tailed unpaired Student's t test.

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 angiotensin-converting enzyme 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) of the four agents is active in vivo, whereas 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 central nervous system and inhibit intracellular PLA_2.

Multiple Effects of PLA_2 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, i.t. COX inhibitors reduce prostaglandins release and the facilitated state induced by peripheral injury or by the direct activation of these circuits with i.t. SP and/or glutamate (see Svensson and Yaksh, 2002). This cascade suggests the relevance of pursuing the upstream PLA_2 linkages which precede those mediated by cyclooxygenase. We note, however, that there is substantial evidence that other products of PLA_2 activity are important in nociceptive processing. Arachidonic acid generated by PLA_2 isoforms can directly augment NMDA ionophore function (Richards et al., 2003). The NMDA receptor is believed to play an important role in pre- and postsynaptic facilitation at the spinal level (L'Hirondel et al., 1999; Richards et al., 2003). Arachidonic acid formed by the action of PLA_2s 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). Platelet-activating factor, an alkyl-phospholipid, arises from the membrane lipid hydrolysis by PLA_2. Platelet-activating factor 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). PLA_2 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 COX. Finally, the present studies showing the development of systemically bioavailable PLA_2-selective agents may be relevant to therapeutic targets other than pain. A variety of neuron inflammatory processes may also be mediated through their activation of neuraxial PLA_2 isoforms.

Acknowledgments

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