Systemic and Intrathecal Effects of a Novel Series of Phospholipase A₂ Inhibitors on Hyperalgesia and Spinal Prostaglandin E₂ Release

Tony L. Yaksh, George Kokotos, Camilla I. Svensson, Daren Stephens, Christoforos G. Kokotos, Bethany Fitzsimmons, Dimitra Hadjipavlou-Litina, Xiao-Ying Hua, and Edward A. Dennis

Departments of Anesthesiology (T.L.Y., C.I.S., B.F., X.-Y.H.) and Chemistry and Biochemistry (D.S., E.A.D.), School of Medicine, University of California, San Diego, La Jolla, California; Department of Chemistry, University of Athens, Athens, Greece (G.K., C.G.K.); and Department of Pharmaceutical Chemistry, University of Thessaloniki, Thessaloniki, Greece (D.H.-L.)

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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 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 PLA₂ (cPLA₂) and group VIA calcium-independent PLA₂ (iPLA₂) in inhibition assays using human recombinant enzyme. The rank ordering of potency in blocking group IVA cPLA₂ was AX048 (ethyl 4-[(2-oxohexadecanoyl)amino]butanoate), AX006 (4-[(2-oxohexadecanoyl)amino]butanoic acid), and AX057 (tert-butyl 4-[(2-oxohexadecanoyl)amino]butanoate) and for inhibiting group VIA iPLA₂ was AX048, AX057, AX006, and AX010 (methyl 4-[(2-oxohexadecanoyl)amino]butanoate) and for inhibiting group VIA iPLA₂ 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 E₂ (PGE₂) release. AX048 alone inhibited PGE₂ 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 cPLA₂, which produces a potent antihyperalgesia. The other agents, although demonstrating enzymatic activity in cell-free assays, appear unable to gain access to the intracellular PLA₂ 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 prostanooids 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 A₂ (PLA₂) because it is required to generate arachidonic acid, which is the substrate for COX-mediated

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ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; PLA₂, phospholipase A₂; cPLA₂, calcium-dependent PLA₂; iPLA₂, calcium-independent PLA₂; 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-1H-pyrazole-3-carboxamide hydrochloride; ANOVA, analysis of variance; AACOCF₃, arachidonoyl trifluoromethylketone; BMS-229724, 4-[(2-[bis(4-chlorophenyl)methoxy]ethoxy)ethylsulfonyl]ethoxy)phenyl]-1,1,1-trifluoro-2-butanone.
hyperalgesia and PGE2 release evoked by spinally delivered
onized by spinal cyclooxygenase inhibition (Malmberg and
quent thermal hyperalgesia. Both of these events are antag-
2005b). Inhibition of group IV cPLA2 but not group VI iPLA2
(0.6
ins of both centrally and peripherally initiated hyperalgesia
delivery, displays significant antihyperalgesic effects in mod-
IV calcium-independent PLA2 (iPLA2) and groups II and V
secretory PLA2 forms (Lucas et al., 2005; Svensson et al.,
Inhibition of group IV cPLA2 but not group VI iPLA2
isoforms using i.t.-delivered agents suggested a role for group
IV cPLA2, but not group VI iPLA2 (Lucas et al., 2005), in
inflammation-evoked hyperalgesia.

We have reported recently the discovery of a novel struc-
tural series of 2-oxoamides that inhibit group IVA cPLA2 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 inhibi-
tory effects on group IV cPLA2 and group VI iPLA2 as well as
on COX activity in vitro assays. Their actions were then
characterized after systemic and i.t. delivery on thermal hyper-
algesia induced by peripheral inflammation (intraplantar
carrageenan). In addition, we have shown previously that
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 subse-
quently thermal hyperalgesia. Both of these events are antag-
onized by spinal cyclooxygenase inhibition (Malmberg and
Yaksh, 1992; Yaksh et al., 2001). Based on these observa-
tions, 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 anti-hyperalgesic effects in mod-
els 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 Cali-
fornia, San Diego.

In Vivo Studies

Animals. Male Holtzman Sprague-Dawley rats (300–350 g; Har-
lan, 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 diam-
eter; 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 pre-
pared 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 sequela,
20% weight loss after implantation, or catheter occlusion.

Behavioral Analysis

Thermal Hyperalgesia. Two approaches were employed to init-
tiate a hyperalgesic state. An inflammation-evoked thermal hyper-
algesia was induced by subcutaneous injection of 2 mg of carra-
geenan [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 (main-
tained at 25°C) on which the rats are placed individually in Plexiglas
cubes (9 × 22 × 25 cm). The thermal nociceptive stimulus origi-
nates 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 la-
tencies 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 re-
ponse latency scores is calculated for analysis.

Intrathecal Dialysis and PGE2 Assay. Spinal dialysis experi-
ments to define the spinal release of PGE2 were conducted in un-
anesthetized rats 3 days after dialysis catheter implantation. A
syringe pump (Harvard Apparatus Inc., Holliston, MA) was con-
ected, and dialysis tubing was perfused with artificial cerebrospinal
fluid at a rate of 10 μl/min. The artificial cerebrospinal fluid con-
tained 151.1 mM Na+, 2.6 mM K+, 0.9 mM Mg2+, 1.3 mM Ca2+,
122.7 mM Cl−, 21.0 mM HCO3−, 2.5 mM HPO4−, and 3.5 mM dextrose
and was bubbled with 95% O2/5% CO2 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 PGE2 in spinal dialysate was measured by enzyme-
linked immunosorbent assay using a commercially available kit (As-
say Designs 90001; Assay Designs, Ann Arbor, MI). The antibody is
selective for PGE2 with less than 2.0% cross-reactivity to PGF1α,
PGF2α, 6-ketoPGF1α, PGA2, or PGB2 but cross-reacts with PGE1 and
PGE3.

Drug Delivery. Drugs were delivered systemically (i.p.) or spi-
nally (i.t.). Intraperitoneal drugs were delivered uniformly in doses
prepared in volumes of 0.5 ml/kg. Drugs injected i.t. were adminis-
tered 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 5 min at 40°C. Pure enzyme
was added to yield a final volume of 500 μl, and digestion was car-
ried 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(50) calculations.

Inhibition of cyclooxygenase-1 and cyclooxygenase-2 was tested in
vitro using the COX Activity Assay kit (catalog no. 760151F) 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 cannabinoïd 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-aminobutanoate, to a stirred solution of 2-hydroxy-hexadecanoic acid (2.0 mmol) and the ester of 4-aminobutanoate (2.0 mmol) in CH2Cl2 (20 ml), Et3N (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-hydroxybenzotriazole (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, CH, NH2), 2.68 (1H, b, OH), 2.32 (2H, t, J = 7 Hz, CH2COO), 1.80–1.58 (4H, m, CH2CH2COO, CH2), 1.45–1.10 (27H, m, CH2, CH2CH2COO), 0.85 (3H, t, J = 7 Hz, CH3).


**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, CH2CH2), 1.45 (9H, s, C(CH3)3), 1.38–1.15 (24H, m, CH2, CH2CH2COO), 0.89 (3H, t, J = 7 Hz, CH3).


**Results**

**Synthesis and Physical Properties of Test Agent**

Ethyl and tert-butyl 4-aminobutanoate were coupled with 2-hydroxy-hexadecanoic acid using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide as a condensing agent in the presence of 1-hydroxybenzotriazole. The 2-hydroxyamides synthesized were oxidized with NaOCl in the presence of a catalytic amount of 4-acetamido-2,2,6,6-tetramethylpiperi-
dine-1-yloxy free radical to produce compounds AX048 and AX057.

**Characterization of PLA2 Inhibitory Activity: Enzymatic Assay.** The inhibitory effects of AX006, AX010, AX048, and AX057 on pure group IVA PLA2 and group VIA PLA2 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 PLA2 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$_{2\text{a}}$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$_2$. 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.

**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 $\mu$M) had no inhibitory effects upon COX activity (Fig. 4).

![Fig. 2](image1) 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](image2) 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.

![Fig. 4](image3) 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 $\mu$M) but not AX006 (50 $\mu$M), AX010 (50 $\mu$M), AX048 (50 $\mu$M), or AX057 (50 $\mu$M) served to inhibit cyclooxygenase activity at the doses employed.

**TABLE 1**
List of compounds including physical characteristics, in vitro $X_{I(50)}$ values for groups IVA and VIA PLA$_2$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Mol. Wt.</th>
<th>CLogP</th>
<th>Group IVA $X_{I(50)}$ (mole fraction)</th>
<th>Group VIA $X_{I(50)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX006</td>
<td></td>
<td>355.52</td>
<td>6.6</td>
<td>0.024 ± 0.015</td>
<td>N.D.</td>
</tr>
<tr>
<td>AX010</td>
<td></td>
<td>369.54</td>
<td>7.1</td>
<td>N.D.</td>
<td>L.D.</td>
</tr>
<tr>
<td>AX048</td>
<td></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></td>
<td>411.62</td>
<td>8.3</td>
<td>0.031 ± 0.017</td>
<td>0.026 ± 0.014</td>
</tr>
</tbody>
</table>
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 \( \text{ED}_{50} \) was defined as the dose that reduced the hyperalgesia observed in a vehicle-treated animal by 50%. On this basis, the estimated i.p. \( \text{ED}_{50} \) 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-
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). 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 PGE₂ 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 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 (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
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 cPLA₂ and group VIA iPLA₂ revealed that AX010 had at best a weak effect, AX006 was group IVA PLAs preferring, whereas AX048 and AX057 were group IVA cPLA₂ and group VIA iPLA₂ preferring. The profile of activity observed here suggests the importance of both group IVA cPLA₂ and group VIA iPLA₂. We showed that i.t. delivery of methyl arachidonoyl fluorophosphonate and arachidonoyl trifluoromethylketone (AACOCF₃), mixed inhibitors of group IVA cPLA₂ and group VI iPLA₂, produced a dose-dependent inhibition of hyperalgesia induced by intraplantar carrageenan as well as formalin-induced flinching. Moreover, i.t. injection of AACOCF₃ at antihyperalgesic doses decreased PGE₂ release into spinal dialysate evoked by i.t. NMDA (Lucas et al., 2005). In contrast, in those studies, an irreversible group VIA iPLA₂ 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 PGE₂ release. Yeo et al. (2004) reported that i.c.v. injection of AACOCF₃ or 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 PGE₂ release. Yeo et al. (2004) reported that i.c.v. injection of AACOCF₃ 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 cPLA₂ inhibitor, was orally active in inhibiting edema and neutrophil infiltration. 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 reverse transcription-polymerase chain reaction have shown that group IVA cPLA2, group VIA iPLA2, and secretory PLA2 (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 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 PGE2 release, a downstream biomarker believed to be essentially dependent upon PLA2 activity (Svensson and Yaksh, 2002). Although 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 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 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 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 PLA2. 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. 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.](image-url)
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 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 spinal 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 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. 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 postsynaptic facilitation at the spinal level (L’Hirondel et al., 1999; Richards et al., 2003). Arachidonic acid formed by the action of PLA₂β 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 PLA₂. 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). 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 COX. 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 neuron inflammatory processes may also be mediated through their activation of neuraxial PLA₂ isoforms.
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References


Address correspondence to: Dr. Tony L. Yaksh, Department of Anesthesiology University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0818. E-mail: tyaksh@ucsd.edu.