■ LABORATORY INVESTIGATIONS

Touch-evoked Agitation Produced by Spinally Administered Phospholipid Emulsion and Liposomes in Rats

Structure-Activity Relation

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Background: Phospholipid-based liposomes can alter the kinetics of spinally administered agents. We have observed that spinal delivery of these preparations results in an unexpected touch-evoked agitation, a state in which light touch evokes vocalization by the rat. In the current study we characterized this agitated state induced by various liposome preparations.

Methods: Rats prepared with lumbar intrathecal catheters received a variety of phospholipids delivered spinally as emulsions or as liposomes. Before and after injection, the animal's hot-plate latency (52.5°C surface), spontaneous mobility, and spontaneous and evoked pain behavior were assessed.

Results: Spinal delivery of L-α-phosphatidylcholine of egg yolk (L-EPC) and the phospholipase hydrolysis product (lyso-L-α-phosphatidylcholine [lyso-L-EPC]) produced dose-dependent touch-evoked agitation; the order of potency and rapidity of onset was lyso-L-EPC > L-EPC, with no difference in activity whether administered as an emulsion or as a liposome preparation. Examination of the activity of a series of pure phospholipids revealed the ordering of touch-evoked agitation potency (where PC = phosphatidylcholine) to be L-monopalmitoyl-PC (lyso product of L-dipalmitoyl-PC) > L-dipalmitoyl-PC > L-distearoyl-PC, L-dioleoyl-PC ≫ L-dilauroyl-PC > L-dimyristoyl-PC; D-dipalmitoyl-PC = 0. The effect of unsaturation on touch-evoked agitation cannot be predicted because dioleoyl-PC and dioleoyl phosphatidylglycerol produced touch-evoked agitation but dipalmitoleoyl-PC did not. Substitution of glycerol for choline as the head group had no influence on touch-evoked agitation. Spinal treatment with an inhibitor of phospholipase (mepacrine) or a cyclooxygenase (ketorolac) blocked the touch-evoked agitation of L-EPC but not that of lyso-L-EPC.

Conclusions: These results emphasize that certain L-isomeric phospholipids with their gel-transition temperatures near body temperature can produce prominent touch-evoked agitation after spinal delivery, an effect likely mediated by a phospholipase hydrolysis product. This touch-evoked agitation, which is consistent with neurotoxicity reported in the early literature on lysophospholipids, suggests that the choice of lipids for the formulation of liposomes intended for spinal drug delivery should be carefully considered. (Key words: Anesthetic techniques, spinal: liposomes; phospholipids. Pain.)

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SEVERAL reports have indicated that intrathecal or epidural administration of liposome-encapsulated opioids,[1] local anesthetics,[2] or chemotherapeutic agents[3] can increase the duration of drug action and minimize systemic or supraspinal side effects by slowing or reducing drug redistribution to extraspinal sites. Liposomes are lipid membrane vesicles that can serve as a drug carrier or a controlled drug delivery system. Water-soluble drugs are held within the aqueous interior of the liposomes, whereas water-insoluble drugs dissolve in or become incorporated into the lipid bilayer.[4] The release rate of an encapsulated drug can be controlled by manipulating the lipid composition and the size and structure of the liposomes. Therefore, liposomes potentially can be used to attenuate peak drug concentration and to provide sustained drug release, thereby improving the therapeutic index of the drug.

Traditionally, natural phospholipids such as L-α-phosphatidylcholines (L-α-PCs) with neutral net charge in physiologic conditions are used to construct liposomes that closely resemble biologic membranes. Liposomes made of naturally occurring phospholipids are generally considered safe for parenteral use. Little

YANEZ *ET AL.*

or no toxicity is observed with doses as large as 1 g of intravenously administered liposomes containing phosphatidylcholine (PC) or phosphatidylglycerol.[5,6] A similar lack of toxicity has been reported for other synthetic L-α-PCs, such as L-α-dipalmitoyl and L-α-dimyristoyl-PC. In contrast, investigations into the toxicity of liposomes after administration into the central nervous system have yielded conflicting results. Adams *et al.*[7] reported that intracerebral administration of 5–10 mg positively charged liposomes containing a mixture of phosphatidyl choline, stearylamine, and cholesterol (5:1:5) produced epileptic seizures and respiratory failure in mice. However, in a study by Kimelberg,[8] similar liposome preparations injected into the lateral cerebral ventricles of monkeys and rats, to 24 mg/kg, did not produce obvious toxicity for 60 days. More recently, Firth *et al.*[9] reported that liposomes containing phosphatidyl choline and cholesterol (without stearylamine) did not produce toxicity when administered intracerebrally to rats.

We have reported the use of neutral liposomes containing L-α-dipalmitoyl-PC as a carrier to modify the redistribution kinetics of intraspinally delivered alfentanil, in an attempt to improve the therapeutic index of the opioid analgesic agents.[1] Intracerebral administration of 10 mg L-α-dipalmitoyl-PC had previously been reported not to produce any toxicity in mice.[10] However, in the course of studying the spinal delivery of L-α-dipalmitoyl-PC liposome-encapsulated alfentanil in rats, we observed unusual behavior in the control animals receiving non-drug-containing liposomes. This effect elicited by blank liposomes appeared as touch-evoked agitation that began 30–60 min after intrathecal injection.[11] Lyso derivatives result from the degradation of the liposomes and are potentially neurotoxic. The previously mentioned toxicity studies[5–9] have focused on the liposomes and not their parent compound. Lyso compounds deserve to be evaluated along with the parent compounds for possible toxicity.

In this study, we characterized the agitation evoked by phospholipids in the form of emulsions or liposomes. A series of phospholipids with various acyl substitutes were examined. The results suggest that touch-evoked agitation may be associated with the production of lipid metabolites by the action of phospholipases in the intrathecal space. Moreover, we showed that this abnormal behavior can be circumvented by using phospholipids that are less susceptible to breakdown by lipases.

Materials and Methods

These studies were carried out according to protocols approved by the Institutional Animal Care Committee at the University of California, San Diego, California, and the Fred Hutchinson Cancer Research Center, Seattle, Washington.

Animal Preparation

Male Sprague-Dawley rats (275–300 g) were housed individually and maintained on a 12-h day/night cycle with food and water *ad libitum*. After 3–5 days, the rats were prepared with chronic intrathecal catheters. In brief, during halothane anesthesia, the rat was placed in a stereotaxic head holder and the cisternal membrane was exposed by a midline incision. A PE-10 catheter was then inserted through the cisterna and passed to the level of the lumbar enlargement (9 cm).[12] The wound was closed. After 5 days, rats were used in the experiments to be described below.

Drugs and Injection

Drugs were prepared in sterile physiologic saline such that the dose was delivered in a final volume of 10 μl. For injection, this volume was delivered into the spinal catheter of the nonanesthetized rat by a gear-driven microinjection syringe. After the injection of the drug, the catheter was flushed with a further volume of 10 μl saline to clear the catheter.

The chemical structures and abbreviations of the phospholipids and the commercial sources and the forms in which the lipids were delivered are presented in table 1. All chemicals were reagent grade or better. The rationale for choosing these lipids was to determine (1) the role of phase transition properties (determined by chain length) and (2) the role of the phospholipid head group charge on the observed behavior.

Agents were delivered as an emulsion or as a liposome preparation. The emulsion was prepared by mixing the appropriate mass of lipid with sterile preservative-free saline. In preparing the liposome, 100 mg of the appropriate lipid in chloroform/methanol (1:1, volume in volume) were dried under a gentle stream of N_2 gas in a sterile glass tube. The dried lipids were desiccated for 1 h to remove the residual organic solvents. To each preparation, 1 ml of phosphate-buffered saline was added and incubated at 55°C water bath for 30 min. The suspensions were vortexed immediately and subsequently sonicated at 55°C in a bath-type sonicator until translucent liposome suspensions were obtained.

Table 1. Summary of Lipid Molecules Delivered Spinally, Their Physical Characteristics, and Injection Form

| | | Fatty Acyl Chain | | |
Phospholipid and Derivatives (isomer and name)	Abbreviation	Length	Degree of Unsaturation	$T_c(°C)^*$
L-α-Dilauroyl phosphatidylcholine	L-DLPC	12	0	−2
L-α-Dimyristoyl phosphatidylcholine	L-DMPC	14	0	23
L-α-Dipalmitoyl phosphatidylcholine	L-DPPC	16	0	41.5
L-α-Dipalmitoleoyl phosphatidylcholine	L-DPoPC	16	1	−36
L-α-Dipalmitoyl phosphatidylglycerol	L-DPPG	16	0	41
L-α-Dioleoyl phosphatidylcholine	L-DOPC	18	1	−20
L-α-Dioleoyl phosphatidylglycerol	L-DOPG	18	1	−18
L-α-Disteroyl phosphatidylcholine	L-DSPC	18	0	55
L-α-Diarachidoyl phosphatidylcholine	L-DAPC	20	0	66
L-α-Egg phosphatidylcholine	EPC	Mix	Mix	—
D-α-Dipalmitoyl phosphatidylcholine	D-DPPC	16	0	41
Lyso-derivative of EPC	Lyso-L-EPC			
Lyso-derivative of L-DPPC-(1-palmitoyl-2 hydroxy-sn-glycero-3-phosphocholine)	Lyso-L-DPPC			

* T_c: gel-to-liquid transition temperature.[31]

These liposome suspensions were flash frozen with methanol dry ice, then thawed at room temperature, and warmed to 55°C. This freeze-and-thaw procedure was repeated three times, and the final incubation at 55°C was for 30 min. The suspensions were then placed in a 100-ml beaker containing 50 ml 55°C water and equilibrated to 20°C (room temperature). These liposomes were diluted with pharmaceutical-grade saline immediately before injection into rats. Peroxidation was checked before liposome preparation, and a fraction of the liposomes was saved and checked for lipid degradation after animal administration by using thin-layer chromatography. In addition, liposomes were kept at pH 7.4 throughout the preparation. They were used within 48 h.

Other agents that were injected included the phospholipase inhibitor mepacrine (Sigma, St. Louis, MO) and the cyclooxygenase inhibitor ketorolac (Syntex, Palo Alto, CA).

Testing

Five to eight rats were used in each group and all rats were used only once. Before and after injection, the animal's hot-plate response latency, spontaneous mobility and touch-evoked agitation were assessed. Hot-plate response latency was examined by placing the animal on a 52.5°C surface. The time between placing on the surface and the licking of the hind paw was taken as the response measure. No response within 60 s was taken as a failure to respond and that value was assigned as the score. Spontaneous mobility was assessed by noting the time required for the animal to move once it had been positioned with its forepaws placed on a horizontal bar 4 cm from the floor. Failure to move within 20 s was cause to terminate the test and assign that score. Touch-evoked agitation was scored as follows: 0 = normal quiescent behavior; 1 = occasional squeaking, mild agitation induced by light touch; 2 = significant spontaneous agitation, spontaneous squeaking, exaggerated agitation behavior induced by light touch to the flank and thorax. Tests were carried out before and at 5, 60, 120, and 180 min after injection. The overall effect was expressed by a cumulative touch-evoked agitation score, equal to the sum of the scores at the four time points with a maximum of 8. Behavioral examination was also carried out 24 h after injection.

Statistics

Hot-plate and spontaneous mobility results are expressed as percentages of the maximum possible effects. Touch-evoked agitation was expressed as the number of animals that displayed agitation at each time point. In addition, for comparison of touch-evoked agitation activity, the serial touch-evoked agitation scores (0–2) recorded over the interval 0–3 h were summed. All data is expressed with the standard error of the mean. All comparisons were made by one-way analysis of variance. If the analysis of variance indicated significant results, intergroup comparisons were carried

YANEZ *ET AL.*

by Fisher's test for least significant difference. *P* values less than 0.05 were considered significant.

Results

Intrathecal Lipid Emulsions

The intrathecal injection of saline did not cause touch-evoked agitation in any of the animals nor did it effect their reaction on the hot-plate or mobility test over the ensuing 3-h interval. In comparison, 10 μl of a phospholipid emulsion of PC of egg yolk (L-EPC) or its principle constituent, L-dipalmitoyl-PC (L-DPPC) had no acute effect on behavior for at least 5 min but showed a reliable onset of touch-evoked agitation by 60 min (fig. 1). The touch-evoked agitation consisted of vocalization induced by gently stroking the back of the animal and a clearly enhanced irritability in response to simple handling. Examination of the distribution of sensitivity revealed that tactile stimulation of the upper body had little effect; however, stroking of lower dermatomes, particularly along the lower back and flank revealed a prominent enhancement of the vocalization and the signs of irritation.

Concurrent with the touch-evoked agitation, there was a progressive increase in the hot-plate latency (fig. 1). Despite the failure to display licking of the hind paw, these animals displayed increasing agitation while on the hot plate.

When placed on the test bar, the treated animals were alert, sniffed the air, and oriented toward sound with the head, while remaining poised for extended periods in front of the horizontal bar. At the doses where touch-evoked agitation and decreased mobility was observed, the animals retained normal motor strength as evidenced by their ability to ambulate and to stand in front of the test bar. It is relevant to note that the increased latency to move from the test bar correlated with the increased latency to respond on the hot plate (fig. 1).

Although they were not systematically examined, all animals (except those receiving L-lyso-EPC; see below) typically showed recovery of spontaneous activity and hot-plate scores along with a loss of touch-evoked agitation by 24 h. The magnitude of the agitation evoked by intrathecal lipids was dose dependent over the range of 100–1,000 mg total dose (fig. 2).

The time to onset of these effects was shortened when the phospholipase A_2 product of PC, L-lyso-EPC, was administered intrathecally. Equal degrees of touch-evoked agitation were produced by 1,000 mg L-EPC as
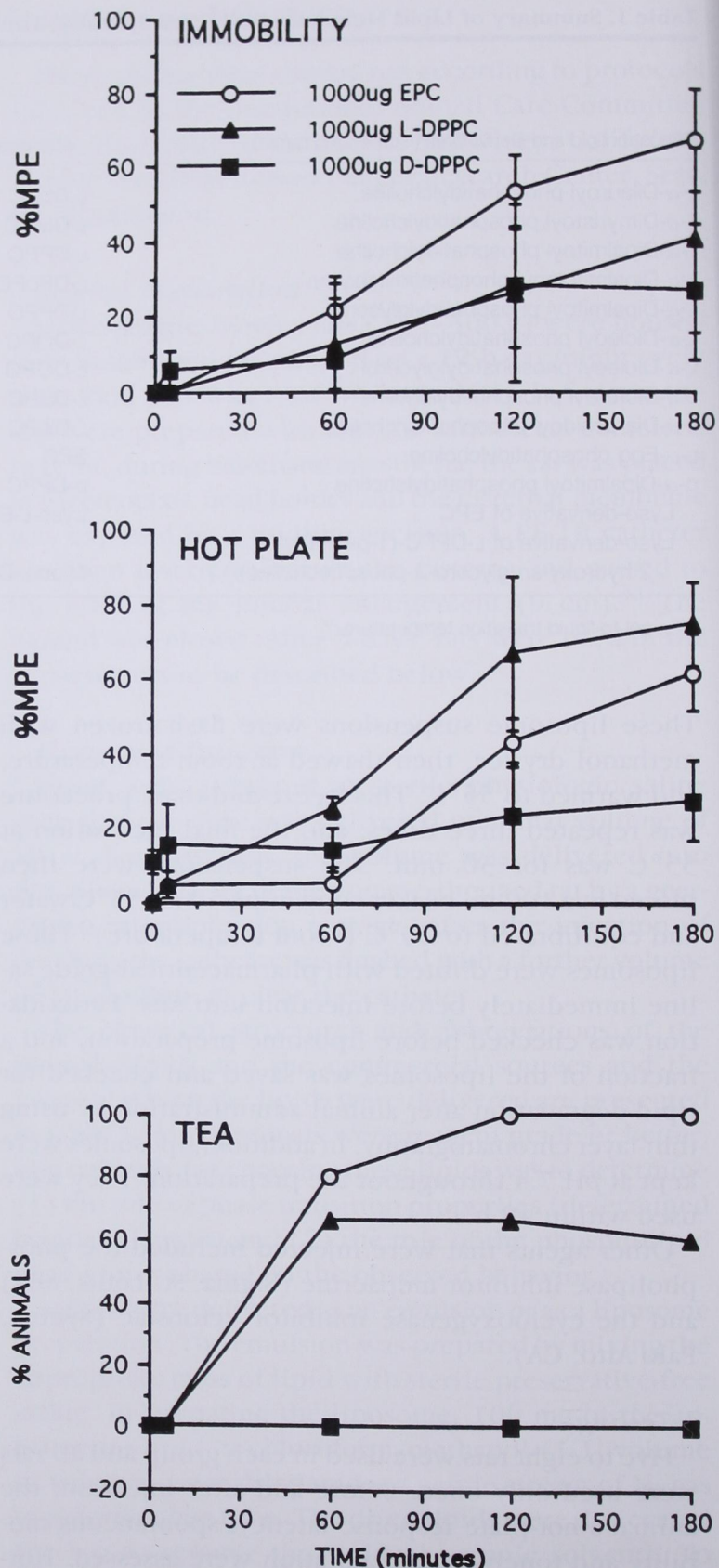

Fig. 1. Time-effect curves for immobility (*top*), hot-plate response latency (*middle*), and percentage of animals displaying touch-evoked agitation (TEA) (*bottom*) after intrathecal delivery of L-α-phosphatidylcholine of egg yolk (L-EPC: 1,000 mg), L-dipalmitidyl phosphatidylcholine (L-DPPC: 1,000 mg), or D-DPPC (1,000 mg).
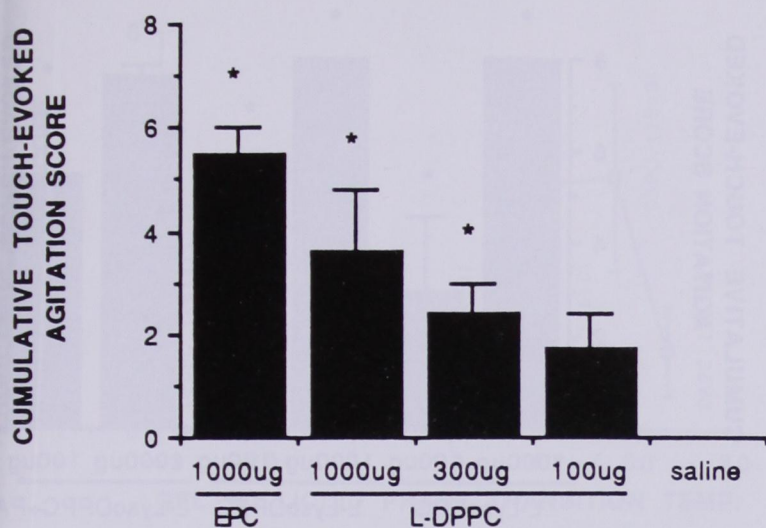
Fig. 2. Degree of touch-evoked agitation produced by spinal delivery of emulsions of L-α-phosphatidylcholine of egg yolk (L-EPC: 1,000 mg), L-dipalmitoyl phosphatidylcholine (L-DPPC: 1,000 mg), or saline. *$P < 0.05$ compared with saline.

compared with 300 mg L-lyso-EPC (fig. 3). Irrespective of dose, L-lyso-EPC (100–1,000 mg) resulted in a clear sign of touch-evoked agitation within 5 min after the injection, in contrast to the 60-min delay typically noted for PC. The time course of touch-evoked agitation for equi-potent intrathecal doses of L-lyso-EPC (300 mg) and L-EPC (1,000 mg) are presented in figure 3. No mortality was noted with L-EPC (1,000 mg), but four of ten rats died within 2 h of the injection of L-lyso-EPC. Deaths were preceded by significant motor weakness.

The lyso derivatives of pure L-DPPC (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, lyso-DPPC, and lyso-DPPC + palmitic acid), were also examined. As with the L-lyso-EPC, the lyso derivatives of L-DPPC produced touch-evoked agitation within 5 min, whereas the L-DPPC emulsion produced about a 60-min delay (fig. 4). All of the lyso derivatives produced a dose-dependent touch-evoked agitation (fig. 5).

Intrathecal Liposomes

The intrathecal injection of liposomes from L-DPPC resulted in similar signs of touch-evoked agitation as those prepared as emulsions, over similar lipid dose ranges (fig. 6). A comparison of the potency of the emulsion with the liposome fraction of L-DPPC delivered spinally, revealed no significant differences.

To investigate the structure-activity relation for the phospholipid-induced touch-evoked agitation, liposomes constructed from a variety of acyl chains were injected at a dose of 1,000 mg. The results of these
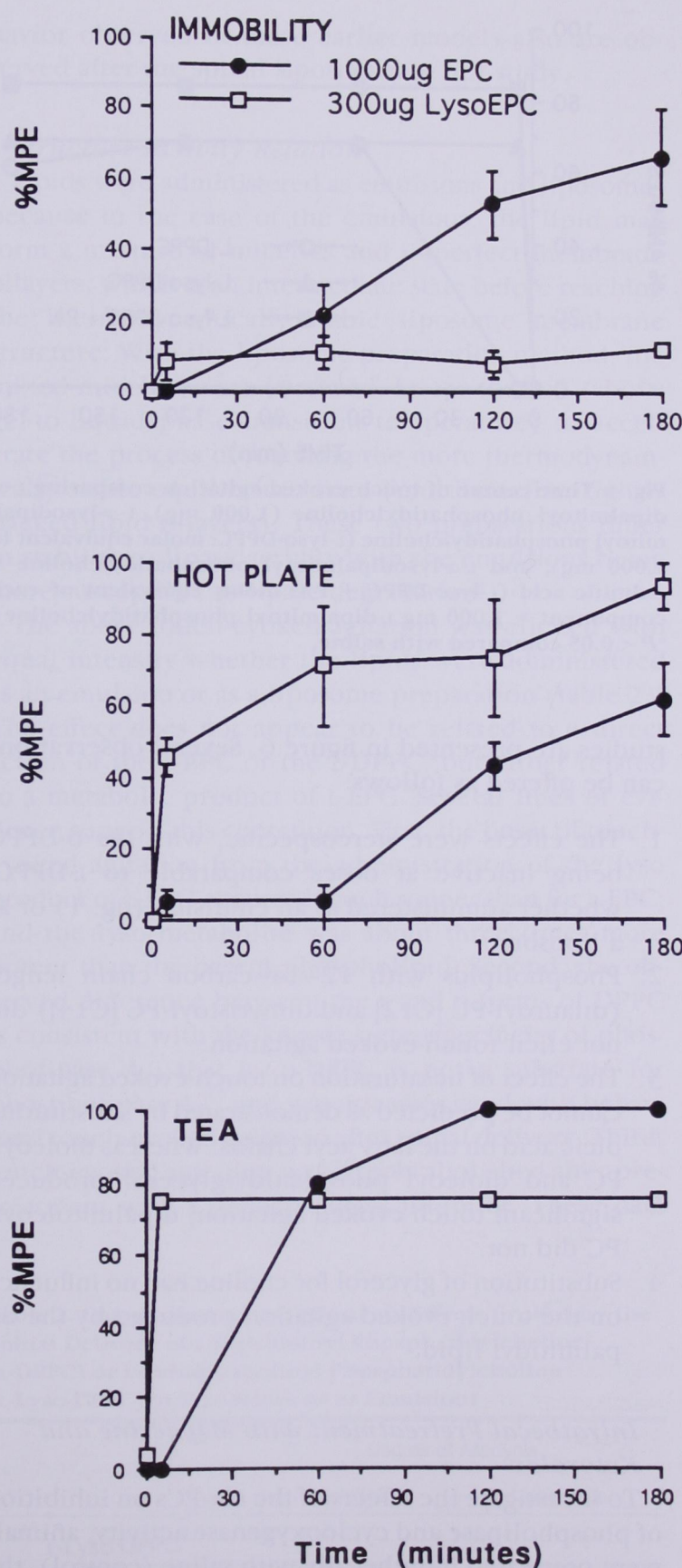

Fig. 3. Time-effect curves for immobility (*top*), hot-plate response latency (*middle*), and percentage of animals displaying touch-evoked agitation (*bottom*) after intrathecal delivery of L-α-phosphatidylcholine (L-α-PC: 1,000 mg) or L-lysophosphotidylcholine (L-lyso-PC: 300 mg).

YANEZ *ET AL.*
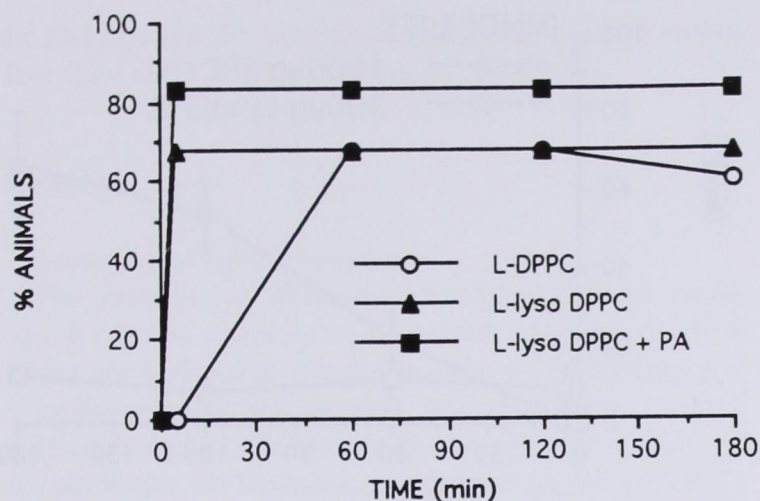

Fig. 4. Time course of touch-evoked agitation comparing L-α-dipalmitoyl phosphatidylcholine (1,000 mg), L-α-lysodipalmitoyl phosphatidylcholine (L-lyso-DPPC: molar equivalent to 1,000 mg), and L-α-lysodipalmitoyl phosphatidylcholine + palmitic acid (L-lyso-DPPC + PA: molar equivalent of each component = 1,000 mg L-dipalmitoyl phosphatidylcholine). *$P < 0.05$ compared with saline.

Fig. 5. Dose-response relation for the lyso metabolites L-α-lysophosphatidylcholine of egg yolk (L-EPC), L-α-lyso-dipalmitoyl phosphatidylcholine (L-lyso-DPPC), and L-α-lysodipalmitoyl phosphatidylcholine + palmitic acid (L-lyso-DPPC + PA). *$P < 0.05$ compared with saline.

studies are presented in figure 6. Several observations can be offered as follows:

1. The effects were stereospecific, with the D-DPPC being inactive at doses comparable to L-DPPC, whether administered as an emulsion (fig. 1) or as a liposome.
2. Phospholipids with 12–14–carbon chain length (dilauroyl-PC [C12] and dimyristoyl-PC [C14]) did not elicit touch-evoked agitation.
3. The effect of unsaturation on touch-evoked agitation cannot be predicted as demonstrated by substituting oleic acid on the fatty acyl chains. Whereas dioleoyl-PC and dioleoyl phosphatidylglycerol produced significant touch-evoked agitation, dipalmitoleoyl-PC did not.
4. Substitution of glycerol for choline had no influence on the touch-evoked agitation produced by the dipalmitidyl lipid.

Intrathecal Pretreatment with Mepacrine and Ketorolac

To investigate the effects of the L-α-PCs on inhibition of phospholipase and cyclooxygenase activity, animals were pretreated intrathecally with saline (control), the phospholipase inhibitor mepacrine (300 mg), or the cyclooxygenase inhibitor ketorolac (30 mg). Each of these injections alone had no effect on motor or sensory behavior. Pretreatment with saline had no effect on the
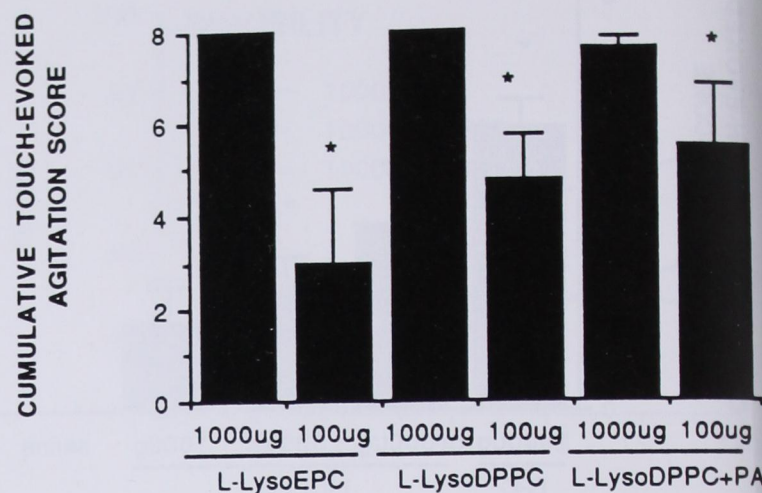
development of touch-evoked agitation after the injection of L-DPPC and L-EPC (fig. 7). In contrast, pretreatment with mepacrine or ketorolac produced a significant reduction in the touch-evoked agitation observed with L-DPPC or L-EPC. Similar pretreatment, however, had no effect on the touch-evoked agitation produced by L-lyso-EPC (fig. 7).

Discussion

Behavioral Effects

In the current work, we found that intrathecal administration of several phospholipids in the form of
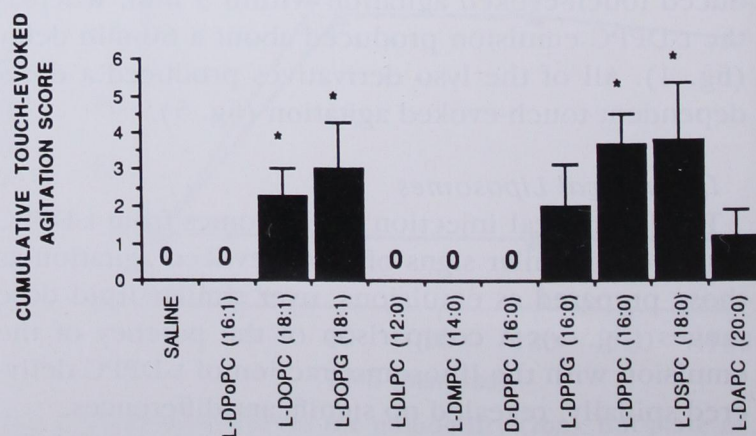

Fig. 6. A comparison of the degree of touch-evoked agitation produced by the spinal delivery of liposomes prepared from various phospholipids (1,000 mg). *$P < 0.05$ compared with saline control.
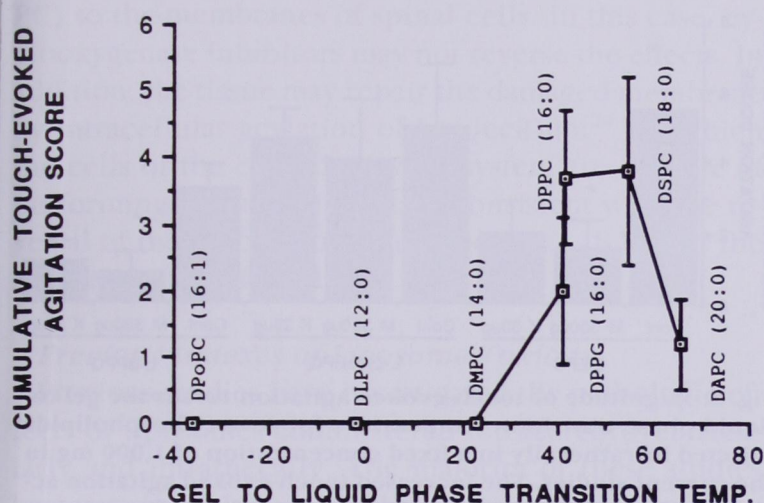
Fig. 7. Degree of touch-evoked agitation measured after the spinal delivery of L-phosphatidylcholine of egg yolk (L-EPC: 1,000 mg), L-lysophosphatidylcholine of egg yolk (L-lyso-EPC: 300 mg), and L-dipalmitoyl phosphatidylcholine (L-DPPC: 1,000 mg) after pretreatment with saline (control), 300 mg mepacrine (M), or 30 mg ketorolac (K). Each bar = mean and SEM of four to six rats. *$P < 0.05$ compared with control.

emulsions or liposomes resulted in the development of pronounced touch-evoked agitation in the rat. Examination of the distribution of sensitivity revealed that tactile stimulation of the upper body had little effect; however, stroking of lower dermatomes, particularly along the lower back and flank revealed a prominent enhancement of the vocalization and signs of irritation. This suggested a dermatomal distribution of the increased sensitivity to touch that corresponds to the lumbar distribution of the injectate. Also the rat failed to display typical spontaneous behavior, as evidenced by the increased intervals of immobility. This lack of movement could be distinguished from that of truncal rigidity (as observed with opiates or major tranquilizers) by virtue of the orienting behaviors of the animal and touch-evoked agitation. Although the hot-plate latencies typically were increased, we do not believe the animals were analgesic because of their pronounced agitated behavior on the heated surface. It was our perception that the failure to move spontaneously and to lick the hind paw on the hot plate reflected avoidance of discomfort evoked by normal limb movements.

It is possible that the observed behavior is allodynia (pain elicited by a nonpainful stimulus). Increasing numbers of reports indicate that in certain animal models, unconditioned low-threshold mechanical stimulation (as in the current studies) yields behavior that appears to correspond to a "pain state" that by definition is allodynia.[13-16] The characteristics of be-

havior observed in these earlier models also are observed after the spinal liposomes in our study.

Structure-Activity Relation

Lipids were administered as emulsions and liposomes because in the case of the emulsions, the lipid may form a mixture of micelles and imperfect membrane bilayers, which is an intermediate state before reaching the thermodynamically stable liposome membrane structure. With the liposome preparation method, the mixed micelles were vortexed, freeze-thawed (above gel-to-liquid phase transition temperature) to accelerate the process of reaching the more thermodynamically stable equilibrium state (multilamellar, tightly packed lipid bilayers). These two preparations differ in stability to lipase activity with the emulsions being more susceptible to lipase degradation.

The above touch-evoked agitation was elicited with equal intensity whether the lipids were administered as an emulsion or as a liposome preparation (table 2). The effect does not appear to be related to a direct action of the L-EPC or the L-DPPC, but rather related to a metabolic product of L-EPC. Several lines of evidence support this contention. First, the onset of touch-evoked agitation from the administration of the lyso product of L-EPC occurred much sooner than for L-EPC, and the lyso metabolite was about three times more potent than the parent phospholipid. Second, the observed difference between the L and D forms of DPPC is consistent with the known stereospecificity of phospholipase A_2; that is, D-DPPC is not a substrate for phospholipase A_2[17] and was not associated with behavioral touch-evoked agitation after spinal delivery. Third, touch-evoked agitation was largely abolished after pretreatment with a phospholipase inhibitor. These data

Table 2. Comparison of the Allodynic Effects Evoked by the Spinal Delivery of L-Dipalmitoyl Phosphatidylcholine (L-DPPC) or L-Lysodipalmitoyl Phosphatidylcholine (L-Lyso-DPPC) as Liposomes or as Emulsions

	Degree of Allodynia	
	Emulsions	Liposomes
L-Lyso DPPC		
1000 μg	8.0 ± 0	—
100 μg	3.0 ± 1.6	—
L-DPPC		
1000 μg	3.6 ± 1.2	3.6 ± 0.8
100 μg	1.7 ± 0.7	—

jointly offer strong support for the finding that the spinal delivery of phosphotidylcholine can evoke a state of touch-evoked agitation that depends on the formation of lyso products of phospholipids.

Analysis of the structure-activity relation revealed that touch-evoked agitation was elicited by some but not all of the phospholipids examined. Importantly, aside from the oleolyl substituted forms, potency of these phospholipids in inducing touch-evoked agitation after intrathecal delivery appeared to be closely related to their phase-transition temperature. A plot of the magnitude of touch-evoked agitation achieved at a fixed dose of phospholipid *versus* the phase transition temperature of the lipid revealed a peak around body temperatures (fig. 8).

The susceptibility of these phospholipids to hydrolysis by phospholipases is markedly facilitated around their respective phase transition temperature.[18] Phospholipases can be found in the central nervous system of humans and rats.[19] These enzymes can hydrolyze phospholipids with the liberation of free fatty acids and the corresponding lyso derivative. In addition, the central nervous system of the rat also has some capacity to acylate the lysolecithin[20] to form lecithin.

Mechanisms of Phospholipid-induced Touch-evoked Agitation

Although the reasoning outlined above suggests that the touch-evoked agitation observed in the current study may be mediated by a phospholipase products, the mechanism underlying the neurobehavioral effects of these products is not known. The current results are consistent with an earlier literature that investigated the effects of several lysophospholipids, most notably the lyso-PC. When lyso-PC was injected in the peripheral nervous system or into the spinal cord, a demyelination[21–24] and subsequent remyelination was produced. Although many of these effects appeared to have a delayed onset, at least one study has reported detectable ultrastructural changes as early as 5 min after injection.[22] Electrophysiologic studies also have indicated that the local delivery of small amounts of lyso-phospholipids into the dorsal part of the spinal column produces significant signs of increased electric activity, axonal cross talk, and mechanical sensitivity of the dorsal columns, presumably reflecting the underlying histopathologic changes. Lyso-PCs that form micelles at a normal temperature are strong detergent. Exposure of cell membranes to detergent would result in loss of the stable lipid-proteins units.[25]
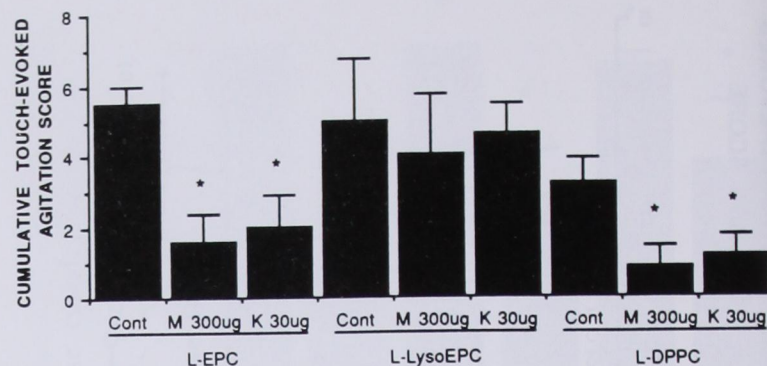

Fig. 8. Magnitude of touch-evoked agitation *versus* the gel-to-liquid phase transition temperature for seven phospholipids injected intrathecally in a fixed concentration of 1,000 mg in the current studies. The increased touch-evoked agitation activity is 22–40°C, indicating that lipids with a gel transition near body temperature are the most likely to induce touch-evoked agitation.

Role of Cyclooxygenase Products

In the current study, ketorolac, a cyclooxygenase inhibitor, was able to attenuate the touch-evoked agitation produced by the L-EPC and the L-DPPC, but not the L-lyso-EPC. These observations were unanticipated. If the interaction of the L-EPC or L-DPPC was uniquely mediated through the formation of the lyso derivative, cyclooxygenase inhibitor should have blocked the effects of the lyso product. There are no data indicating that ketorolac has an inhibitory effect on phospholipase activity. Thus, it is possible that multiple mechanisms mediate the actions of L-EPC or L-DPPC. The first would be the formation of a lyso derivative that has potent membrane effects, and the second is that its interaction may lead to the formation of cyclooxygenase products that induce touch-evoked agitation. Current data strongly emphasizes that cyclooxygenase products in the spinal canal may be released by the presence of phospholipids or its lipolytic products. These products can facilitate release of C-fiber neurotransmitters in the dorsal horn, resulting in pain behavior in a rat model. It has been shown that this cyclooxygenase-induced pain can be suppressed by the spinal administration of cyclooxygenase inhibitors.[26,27] Given the lack of acute pain behavior evoked by the local injection of L-EPC or L-DPPC, it seems unlikely that these agents are in fact inducing a direct activation of afferent input. Moreover, the spinal delivery of several prostanoids, such prostaglandin E_2, prostaglandin F_{2a}, or prostaglandin D_2, results in dose-dependent hyperalgesia or pain behavior[28–30] similar to those observed in these studies. A third mechanism that may mediate the actions of L-EPC or L-DPPC is temporary damage (from lyso-

PC) to the membranes of spinal cells. In this case, cyclooxygenase inhibitors may not reverse the effects. In addition, the tissue may repair the damaged membrane by intracellular acylation of lysolecithin,[20] for which the cells of the central nervous system are capable of performing. In fact, this idea is consistent with the reversal of the touch-evoked agitation 24–48 h after the administration of the lipids.

Previous Indexes of Liposome Toxicity

Previous studies have investigated the pathologic effects of liposomes administered intracerebroventricularly and intrathecally. The majority of these studies have reported the use of a liposome preparation using phosphotidylcholines derived from egg yoke or pure, synthetic L-DPPC. After intracerebroventricular[7,10] or spinal delivery,[1] no behavioral pathologic change has been reported with L-α-PC liposomes in doses similar to those used here. Several reasons may be offered for the discrepant reports of effects on behavior. First, the touch-evoked agitation may have been suppressed by the injected medication. Thus we have observed that opiates,[11] phospholipase inhibitors (current study), and nonsteroidal antiinflammatory agents (current study) can mask the symptoms of touch-evoked agitation. Second, the observed effects occur in about 60 min and then disappeared within 24 h. Accordingly, the failure of previous workers to observe these clear behavioral effects may relate to the acuity of their experiments. Whether the behavioral effects observed in the current studies are correlated with the histopathologic features that have been reported for lysophospholipids remains to be seen. Nevertheless, it seems certain that the direct exposure of spinal cord to surprisingly low concentrations of these phospholipids can induce observable behavioral changes in relatively short intervals (30 min). Histopathologic data have not yet been collected in the current animal model, but these may include, for instance, histopathologic findings in the spinal cord after spinal administration of liposomes.

In summary, it appears likely that a hydrolytic product of an L-isomer phospholipid can evoke significant touch-evoked agitation after spinal delivery. These observations are in accord with the early literature that points to potential tissue toxicity and aberrant neural activity after spinal delivery of lyso metabolites of phospholipids. The observed relation of phospholipase activity and the gel-to-liquid transition temperature of the phospholipids as structural determinants of allo-

dynic effects should guide the future design of liposome-mediated drug delivery systems to the central nervous system for human use. The use of spinal liposome preparations containing phospholipids should be considered cautiously, with due attention to the potential for neurotoxicities. We acknowledge that this is a behavioral study, and that to determine whether a true toxicity exists, histopathologic studies are needed. Therefore, this investigation indicates the need for toxicity studies on the intraspinal administration of liposomes.

References

1. Bernards CM, Luger TJ, Malmberg AB, Hill HH, Yaksh TL: Liposome encapsulation prolongs alfentanil spinal analgesia and alters systemic redistribution in the rat. ANESTHESIOLOGY 77:529–535, 1992

2. Mashimo T, Uchida I, Park M, Shibata A, Nishimura S, Inagaki Y, Yoshiya I: Prolongation of canine epidural anesthesia by liposome encapsulation of lidocaine. Anesth Analg 74:827–834, 1992

3. Kim S, Scheerer S, Geyer MA, Howell SB: Direct cerebrospinal fluid delivery of an antiretroviral agent using multivesicular liposomes. J Infect Dis 162:750–752, 1990

4. Pagano RE, Weinstein JN: Interaction of liposomes with mammalian cells. Annual Review of Biophysics and Bioengineering 7: 435–468, 1978

5. Kimelberg HK, Mayhew EG: Properties and biological effects of liposomes and their use in pharmacology and toxicology. Crit Rev Toxicol 9:25–38, 1978

6. Olson F, Mayhew E, Marlow D, Rustum Y, Szoka F: Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. European Journal of Clinical Oncology 18:167–176, 1982

7. Adams DH, Joyce G, Richardson VJ, Ryman BE, Wisnieuwski HM: Liposome toxicity in the mouse central nervous system. J Neurol Sci 31:173–179, 1977

8. Kimelberg HK: Liposome as carriers for methotrexate, Liposomes in Biological Systems. Edited by Cregoriadis G, Allison AE. New York, John Wiley & Sons, 1980, p 219

9. Firth G, Oliver AS, Mckeran RO: Studies on the intracerebral injection of bleomycin free and entrapped within liposomes in the rat. J Neurol Neurosurg Psychiatry 47:585–590, 1984

10. Storm G, Oussoren C, Peters PAM, Barenholz Y: Tolerability of liposomes in vivo, Liposome Technology. Volume 3. Edited by Gregoriadis G. Boca Raton, CRC Press, 1993, p 345

11. Wallace MS, Yanez AM, Ho RJY, Shen D, Yaksh TL: Antinociception and side effects of liposome-encapsulated alfentanil after spinal delivery in rats. Anesth Analg 79:778–786, 1994

12. Yaksh TL, Rudy TA: Chronic catheterization of the spinal subarachnoid space. Physiol Behav 17:1031–1036, 1976

13. Sherman SE, Loomis CW: Morphine insensitive allodynia is produced by intrathecal strychnine in the lightly anesthetized rat. Pain 56:17–29, 1994

14. Na HS, Leem JW, Chung JM: Abnormalities of mechanoreceptors in a rat model of neuropathic pain: Possible involvement in mediating mechanical allodynia. J Neurophysiol 70:522–528, 1993

15. Yaksh TL: Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: Effects of modulatory receptor systems and excitatory amino acid antagonists. Pain 37:111–123, 1989

16. Yaksh TL, Harty GJ: Pharmacology of the allodynia in rats evoked by high dose intrathecal morphine. J Pharmacol Exp Ther 244:501–507, 1988

17. Grainger DW, Reichert A, Ringsdorf H, Slesse C: Hydrolytic action of phospholipase A_2 in monolayers in the phase transition region: Direct observation of enzyme domain formation using fluorescence microscopy. Biochim Biophys Acta 1023:365–379, 1990

18. Fernandez MS, Mejia R, Zavala E, Pacheco F: Dependence on neutral salt concentration of latency phase in the time course of hydrolysis of dimyristoylphosphatidylcholine liposomes by phospholipase A_2. Biochem Cell Biol 69:715–721, 1990

19. Gallai-Hatchard J, Magee WI, Thompson RHS, Webster GR: The formation of lysophosphatides from di-acyl phosphatides by brain preparations. J Neurochem 9:545–554, 1962

20. Webster GR, Alpern RJ: Studies on the acylation of lysolecithin by rat brain. Biochem J 90:35–42, 1964

21. Blakemore WF, Eames RA, Smith KJ, McDonald WI: Remyelination in the spinal cord of the cat following intraspinal injections of lysolecithin. J Neurol Sci 33:31–34, 1977

22. Hall SM, Gregson NA: The in vivo and ultrastructural effects of injection of lysophosphatidyl choline into myelinated peripheral nerve fibres of the adult mouse. J Cell Sci 9:769–789, 1971

23. Blakemore WF: Invasion of schwann cells into the spinal cord of the rat following local injections of lysolecithin. Neuropathol Appl Neurobiol 2:21–39, 1976

24. Webster GR: Clearing action of lysolecithin on brain homogenates. Nature 180:660–661, 1957

25. Gent WLG, Gregson NA, Gammack DB, Raper JH: The lipid-protein unit in myelin. Nature 204:553–555, 1964

26. Malmberg AB, Yaksh TL: Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. Science 257:1276–1279, 1992

27. Malmberg AB, Yaksh TL: Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J Pharmacol Exp Ther 263:136–146, 1992

28. Taiwo YO, Levine JD: Indomethacin blocks central nociceptive effects of PGF2a. Brain Res 373:81–84, 1986

29. Yaksh TL: Central and peripheral mechanisms for the analgesic action of acetylsalicylic acid, Acetylsalicylic Acid: New Uses for an Old Drug. Edited by Barnet HJM, Hirsh J, Mustard JF. New York, Raven Press, 1982, pp 137–151

30. Uda R, Horiguchi S, Ito SM, Hayaishi O: Nociceptive effects by intrathecal administration of prostaglandin D2, E2 or F2a to conscious mice. Brain Res 510:26–32, 1990

31. Silvius JR: Thermotropic phase transitions of pure lipids in model membranes and their modifications by membrane proteins, Lipid-Protein Interactions. Edited by Silvius JR. New York, John Wiley & Sons, 1992