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DiPOA ([8-(3,3-diphenyl-propyl)-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl]-acetic acid), a novel, systemically-available and peripherally-restricted mu opioid agonist with anti-hyperalgesic activity: 2. In vivo pharmacological characterization in the rat.

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Running title: In vivo characterization of DiPOA

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Abbreviations:

CCI, chronic constriction injury; CNS, central nervous system; DAMGO, [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin; DiPOA, [8-(3,3-Diphenyl-propyl)-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl]-acetic acid; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglia; FCA, Freud's complete adjuvant; i.p., intraperitoneal; ORL-1, opioid receptor-like 1; p.o, *per os* (by mouth); PNS, peripheral nervous system; PSN, partial sciatic nerve ligation; PWT, paw withdrawal threshold; s.c., subcutaneously; SEM, standard error of the mean; SNL, spinal nerve ligation

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ABSTRACT

Mu opioid receptors are expressed throughout the central and peripheral nervous systems. Peripheral inflammation leads to an increase in mu receptor present on the peripheral terminals of primary sensory neurons. Activation of peripheral mu receptors produces potent anti-hyperalgesic effects in both humans and animals. Here we describe the in vivo pharmacological properties of the structurally novel, highly potent, systemically–available yet peripherally-restricted mu opioid agonist, DiPOA. DiPOA administered intra-peritonealy (i.p.) produced naltrexone-sensitive, dose-dependent reversal of Freud's complete adjuvant (FCA) induced inflammatory mechanical hyperalgesia (1-10 mg/kg). Maximum percent reversal (67%) was seen one-hour post-administration at 10 mg/kg (the highest dose studied). DiPOA also proved anti-hyperalgesic in a model of post-surgical pain with a maximum percent reversal of 85% one-hour post-administration at 30 mg/kg i.p. (the highest dose studied). DiPOA administered i.p. had no effect in the tail flick assay of acute pain (0.1-10 mg/kg), produced no ataxia as measured by latency to fall from an accelerating rotarod (3-30 mg/kg) and was not anti-hyperalgesic in the Seltzer model of neuropathic pain (1-10 mg/kg). This is the first report of a peripherally-restricted, small molecule mu opioid agonist that is non-sedating, anti-hyperalgesic and effective against inflammatory and post-surgical pain when administered systemically.

To date, four members of the opioid receptor family have been cloned and characterized, and include the mu, kappa, delta and opioid receptor-like 1 (ORL-1) receptors (Pleuvry, 2003). All are G-protein coupled receptors and mediate inhibition of adenylate cyclase through activation of GTP-binding proteins. In addition, opioid receptor agonism results in the opening of receptor-operated potassium channels and suppression of voltage-gated calcium currents (Duggan and North, 1983). Several classes of endogenous peptidic ligands have been identified for the opioid receptors, including the enkephalins, dynorphins, endorphins and nociceptin (Terenius, 2000). These ligands are distributed throughout the central and peripheral nervous systems (CNS and PNS) as well as in peripheral tissues.

Opioid receptors are also expressed throughout the CNS and their activation results in potent analgesia via inhibition of ascending excitatory nociceptive transmissions and activation of descending inhibitory systems (Yaksh 1999; Fields and Basbaum, 1999). Activation of CNS opioid receptors also results in diminished responsiveness of the brainstem respiratory centers to carbon dioxide (Gutstein and Akil, 2001) and stimulation of dopaminergic pathways, particularly the nucleus accumbens (Kieffer and Gaveriaus-Ruff, 2002); these effects lead to respiratory depression and dependence, respectively. It is important to note that for exogenous compounds to elicit these effects penetration into the CNS is required. In situ hybridization and immunohistochemistry has localized opioid receptor mRNA and protein to the PNS; specifically to the cell bodies of primary afferent sensory neurons, located in the dorsal root ganglia (DRG) (Wang and Wessendorf, 2001). Furthermore, the presence of functional opioid receptors has been demonstrated on both the peripheral terminals, located in the skin and end organs, and the presynaptic terminals, located in the spinal cord (Stein, 1993; Wenk et al., 2003).

Inflammation can play multiple roles in modulating peripheral opioid receptor function. Inflammatory conditions may sensitize peripheral opioid receptors. It has been demonstrated

that increased cAMP and reduced pH, two events that occur at the site of inflammation, can increase the efficacy of opioid agonists (Selley et al., 1993) and enhance opioid-induced attenuation of neuronal excitability (Ingram and Williams, 1994). Immune cells, such as macrophages, that express opioid receptors and contain enkephalin and endorphin are commonly found at sites of inflammation (Mousa, 2003). Importantly, inflammatory mediators, such as interleukin-1, can stimulate the release of opioid peptides from macrophages, the result of which may be an endogenous attempt to attenuate pain and inflammation. Furthermore, inflammatory conditions increase the axonal transport of mu opioid receptors, resulting in increased receptor density at peripheral sites (Zöllner et al., 2003). Finally, inflammation can disrupt the perineurium, which results in a more efficient binding of opioids to opioid receptors (Antonijevic et al., 1995).

Indeed, local administration of exogenous opioids at the site of inflammation has shown therapeutic utility in animals and humans (for review, see Stein et al., 2003). In humans, intra-articular administration of morphine reverses the hyperalgesia associated with osteoarthritis (Stein et al., 1999) or resulting from arthroscopic knee surgery (Kalso et al., 1997). Pre-clinically the inflammation and hyperalgesia caused by acute chemical injury to the rat cornea were reversed by direct application of morphine to the surface of the eye (Wenk et al., 2003). Additionally, local administration of the mu agonists loperamide, morphine or fentanyl is anti-hyperalgesic in rodent models of inflammatory pain (DeHaven-Hudkins et al. 2002; Stein et al., 1988; Zhou et al., 1998). This data suggests that an exogenous, peripherally-restricted mu opioid agonist when systemically administered would provide effective pain relief against hyperalgesia associated with inflammation without eliciting centrally-mediated adverse effects.

In the accompanying manuscript, (Valenzano et al.) DiPOA ([8-(3,3-diphenyl-propyl)-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl]-acetic acid) is described as a high affinity, high efficacy

and systemically-available mu receptor agonist (systemically-available is defined as significant concentrations of compound in the plasma following systemic administration). Therefore, DiPOA provides us with the opportunity of testing the hypothesis that a systemically administered, peripherally-restricted mu opioid agonist would be anti-hyperalgesic in rat pain models of inflammatory hyperalgeisia without eliciting centrally-mediated side effects. As such we would expect DiPOA to be anti-hyperalgesic in the FCA model of inflammatory pain without demonstrating analgesia in the rat tail flick assay of nociception or causing sedation as measured by the rotarod assay. Here we describe the effects of DiPOA in models of acute, inflammatory, neuropathic and incision-induced pain. We have compared the effects of DiPOA with those of the classic and clinically-relevant opioid, morphine, in the acute and inflammatory models. Additionally we have assessed the effects of the compound on motor function and investigated the ability of an opioid receptor antagonist to reverse the effects on inflammatory pain.

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MATERIALS AND METHODS

Compounds and Administration Procedures. The structure for DiPOA is disclosed in the accompanying manuscript (Valenzano et al.), the synthetic route for DiPOA (free base) has been recently disclosed in patent application WO 2003101953 (2nd June, 2003). DiPOA was used in all experiments as it free base (molecular weight 483.6, K_i at mu = 0.76 $\pm$ 0.15 nM, solubility in 100 mM K_2HPO_4 at pH=7.4 > 50 uM). DiPOA was administered intraperitoneally (i.p.) in 25% β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) dissolved in distilled water in a dose volume of 2 ml/kg (the dosage formulation were sonicated for 2.5 h prior to administration). The opioid agonist morphine (Sigma-Aldrich) was administered subcutaneously (s.c.) or i.p. in 0.9% saline in a dose volume of 2 ml/kg. The anticonvulsant gabapentin (Kemprotec, Middlesborough, UK) was administered i.p. in 0.9% saline in a dose volume of 2 ml/kg. The non-steroidal anti-inflammatory drug indomethacin (Sigma-Aldrich) and the cycloxogenase-2 inhibitor celecoxib (Toronto Research Chemicals, Toronto, Canada) were administered orally (p.o.) in 0.5% methyl-cellulose (Sigma-Aldrich) dissolved in distilled water. The mu antagonist naltrexone (Sigma-Aldrich) was administered i.p. in 0.9% saline in a dose volume of 2 ml/kg.

Animals. The Purdue Institutional Animal Care and Use Committee approved all animal procedures according to the guidelines of the Office of Laboratory Animal Welfare. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 180 to 200 g at the start of acute and inflammatory experiments or 90-110 g at the start of nerve ligation experiments, were used. Animals were group-housed and had free access to food and water at all times, except prior to oral administration of drugs when food was removed 12 hours before dosing. For comparison with compound-treated groups, animals treated with appropriate drug vehicle were included in each experiment. The volume of administration and all other experimental procedures and conditions for vehicle and compound-treated rats, were identical.

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Acute Analgesia. The effect of DiPOA on acute analgesia was investigated using the tail flick assay (D'Amour and Smith, 1941). Animals were placed on the apparatus (Ugo Basile, Varese, Italy) and an infrared beam was focused onto the tail, 5 cm from the tip. The latency to tail flick was assessed. Cut-off was set at 30 seconds and the intensity was set to 35%. Latency was determined once for each rat at each time point. Baseline latency was determined and 1 hour later the rats received a single dose of 0.1, 0.3, 1, 3 or 10 mg/kg DiPOA, 10 mg/kg morphine (the positive control) or vehicle (i.p. volume = 2 ml/kg). Latency to tail flick was again determined 1, 3 and 5 h post drug administration.

Inflammatory Hyperalgesia. The efficacy of DiPOA against hyperalgesia associated with inflammation was investigated using the Freund's complete adjuvant (FCA) model. For this assay, hind paw withdrawal thresholds (PWT) to a noxious mechanical stimulus were determined using an analygesymeter (model 7200; Ugo Basile, Varese, Italy). Cut-off was set at 250 g and the endpoint was taken as complete paw withdrawal. PWT was determined once for each rat at each time point. Baseline PWT was determined, the rats were anaesthetised with isofluorane (2% in oxygen) and received an intraplantar injection of 50% FCA (50 µl, diluted in saline) to the left hind paw. Twenty-four hours following FCA injection, pre-drug PWT were measured and the rats received a single dose of 1, 3 or 10 mg/kg DiPOA, 30 mg/kg morphine (the positive control) or vehicle (i.p. volume = 2 ml/kg). (In addition the effect of DiPOA (10 mg/kg, i.p.) was assessed on PWT of animals that did not receive an intraplantar injection of FCA). PWT was again determined 1, 3, 5 and 24 h post drug administration. For naltrexone inhibition, rats received a single dose of 10 mg/kg naltrexone 10 minutes prior to 10 mg/kg DiPOA. Percent reversal of hyperalgesia for each rat was calculated according to the following equation:

$$\%reversal = \frac{postdose\,threshold - predose\,threshold}{baseline\,threshold - predose\,threshold} \quad (1)$$

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Neuropathic Hyperalgesia. The partial sciatic nerve ligation model (PSN) was used as a model of nerve injury-related pain in rats, as described previously by Seltzer et al. (1990). Baseline PWT was determined and partial ligation of the left sciatic nerve was performed under isofluorane (2% in oxygen) inhalation anaesthesia. After induction of anaesthesia, the left thigh was shaved and prepared in a sterile manner. The sciatic nerve was exposed at high thigh level through a small incision and was carefully cleared of surrounding connective tissue at a site near the trocanther just distal to the point at which the posterior biceps semitendinosus nerve branches off the common sciatic nerve. A 7-0 silk suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle and tightly ligated so that the dorsal one-third to one-half of the nerve thickness was held within the ligature and the wound was closed. Sham-operated control rats underwent an identical procedure on the left hind limb, however the sciatic was not manipulated or ligated. After surgery, animals were weighed and allowed to recover before being returned to their home cages. Twenty-one days following nerve ligation, pre-drug PWT were measured and the rats received a single dose of 1, 3 or 10 mg/kg DiPOA, 100 mg/kg gabapentin (the positive control) or vehicle (i.p. volume = 2 ml/kg). PWT was again determined 1, 3, 5 and 24 h post drug administration.

Post-Surgical Hyperalgesia. The effect of DiPOA on post-surgical pain was assessed using an incisonal pain model, as described previously by Brennan et al. (1996). Baseline PWT was determined and the plantar surface of the left hind paw prepared in a sterile manner. Under isofluorane (2% in oxygen) inhalation anaesthesia, a 1 cm longitudinal incision was made with a number 10 scalpel, through skin and fascia of the plantar aspect of the paw starting 0.5 cm from the proximal edge of the heel and extending toward the toes. The plantaris muscle was elevated and incised longitudinally. Following haemostasis with gentle pressure the skin was opposed with 2 single interrupted sutures using 5-0 nylon. The wound site was covered with povidone-

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iodine anti-biotic powder (PRN Pharmacal, Pensacola, FL) and the animals allowed to recover in their home cages. Unoperated rats served as controls. Twenty-four hours following plantar incision, predrug PWT were measured and the rats received a single dose of 3, 10 or 30 mg/kg DiPOA, vehicle (i.p. volume = 2 ml/kg) or 30 mg/kg indomethacin (the positive control) orally (p.o. volume = 10 ml/kg). PWT was again determined 1, 3, 5 and 24 h post drug administration. Percent reversal of hyperalgesia for each rat was calculated according to the following equation:

$$\%reversal = \frac{postdose\,threshold - predose\,threshold}{baseline\,threshold - predose\,threshold} \quad (2)$$

Ataxia/Motor Coordination. To examine the potential effects of DiPOA on motor performance, rats were tested using the rotarod assay. The rotarod speed was set to accelerate from 4 to 40 rpm over 300 seconds, with the maximum time spent on the rotarod set at 300 seconds. Rats (n = 8-10/group) received two training trials on the first day, were fasted overnight and then received a single dose of either 3, 10 or 30 mg/kg DiPOA or 10 mg/kg morphine (s.c.) as a positive control. Rats were tested on the rotarod 0.5, 1 and 2 hours following drug administration. Data is presented as percent of baseline latency.

Statistical Analysis. Untransformed data was analysed using a one-way analysis of variance. In instances were a main effect was detected, planned comparisons were made using Fisher's PLSD test. The level of significance was set at $p < 0.05$. Data are shown as mean ± the standard error of the mean (SEM).

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RESULTS

DiPOA does not affect Acute Nociception. Opioids that penetrate the blood-brain barrier, such as morphine, are analgesic in models of acute nociception. We tested the effects of DiPOA on acute nociception using the tail flick assay (D'Amour and Smith, 1941). DiPOA did not affect latency to tail flick at 1, 3 or 5 hours following administration of doses up to 10 mg/kg, whereas morphine (10 mg/kg, s.c.) produced a significant increase in latency to tail flick 1 hour post administration ($p < 0.05$; Fig. 1).

DiPOA Reduces Mechanical Hyperalgesia Associated with Inflammation. Intraplantar injection of 50 μl of FCA into the hind paw resulted in the development of mechanical hyperalgesia as indicated by a decreased PWT to a noxious mechanical stimulus (Fig. 2). Intraperitoneal administration of DiPOA produced a dose-dependent reduction in mechanical hyperalgesia 1 hour post-administration ($F_{(5,49)} = 20.621$; $p < 0.0001$; Fig. 2). DiPOA (1, 3 and 10 mg/kg i.p.) produced statistically significant anti-hyperalgesia 1 hour following administration ($p < 0.05$). A dose-dependent trend was observed 3 and 5 hours following administration, however statistical significance was not reached. The maximum percent reversal, (67%), was achieved 1 hour following the 10 mg/kg dose. Sedation was not noted at any dose of DiPOA at any of the time points tested. Intraperitoneal administration of morphine (30 mg/kg) also produced a statistically significant reversal or hyperalgesia 1 and 3 hours post-administration (Fig. 2), however sedation was noted in all animals treated with this dose. (When administered to animals that did not receive an intraplantar injection of FCA, DiPOA [10 mg/kg, i.p.] did not produce a statistically significant change in PWT 1, 3, 5 or 24 hours following administration, data not shown).

Naltrexone Inhibits the Anti-Hyperalgesic Effect of DiPOA against Inflammatory Pain. As described above intraplantar injection of 50 μl of FCA resulted in the development of mechanical

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hyperalgesia as indicated by a decreased PWT to a noxious mechanical stimulus. Intraperitoneal administration of DiPOA (10 mg/kg) produced a statistically significant reduction in mechanical hyperalgesia 1 hour post-administration ($p < 0.05$; Fig. 3), which was completely inhibited by intraperitoneal pre-treatment with naltrexone (10 mg/kg); no statistical significance was observed as compared to vehicle treated controls ($p > 0.15$; Fig. 3). Oral administration of celecoxib (30 mg/kg) also produced a statistically significant reversal of hyperalgesia 1 hour post-administration (Fig. 3). When administered alone in this model, naltrexone (10 mg/kg) has no effect on PWT (data not shown).

DiPOA does not Reduce Mechanical Hyperalgesia Associated with Nerve Injury. Partial ligation of the sciatic nerve resulted in the development of mechanical hyperalgesia within 3 weeks of surgery. Intraperitoneal administration of DiPOA 21 days after partial ligation of the sciatic nerve did not result in statistically significant reversal of mechanical hyperalgesia (data not shown). Intraperitoneal administration of gabapentin (100 mg/kg) produced a statistically significant reversal of hyperalgesia 1 and 3 hours post-administration.

DiPOA Reduces Mechanical Hyperalgesia Associated with Surgical Incision. Incision of the plantar surface of the hind paw resulted in the development of mechanical hyperalgesia as indicated by a decreased PWT to a noxious mechanical stimulus 24 hours post-incision (Fig. 4). Intraperitoneal administration of DiPOA produced a dose-dependent reduction in mechanical hyperalgesia 1 ($F_{(5,54)} = 23.131$; $p < 0.0001$), 3 ($F_{(5,54)} = 14.907$; $p < 0.0001$) and 5 ($F_{(5,54)} = 31.926$; $p < 0.0001$) hours post-administration. DiPOA (3, 10 and 30 mg/kg i.p.) produced statistically significant anti-hyperalgesia 1 and 3 hour following administration ($p < 0.05$; Fig. 4). Thirty mg/kg produced significant anti-hyperalgesia 5 hours post dosing ($p < 0.05$; Fig.4). The maximum percent reversal, (85%), was achieved 1 hour following 30 mg/kg. Oral administration

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of indomethacin (30 mg/kg) also produced a statistically significant reversal of hyperalgesia 1 and 3 hours post-administration.

DiPOA does not cause Ataxia or a Loss of Motor Coordination. A common centrally-mediated side effect of opioids is ataxia, which can confound the interpretation of behavioral assays. We tested rats for motor function using the rotarod assay. DiPOA did not affect rotarod performance at 0.5, 1 or 2 hours following administration of doses up to 30 mg/kg, whereas morphine (10 mg/kg, s.c.) produced a significant decrease in rotarod performance 0.5 and 1 hour post administration ($p < 0.05$; Fig. 5).

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DISCUSSION

A link between opioid receptors and pain is well established. The mu opioid receptor is present on neurons of both the CNS and PNS, in addition to inflammatory cells such as macrophages (for review, see Mousa, 2003). Systemic administration of mu opioid agonists that penetrate the blood-brain barrier, such as morphine, produces analgesia (Fields and Basbaum, 1999), however this also causes unwanted and dose-limiting side effects such as sedation, respiratory depression and dependence (Kieffer and Gaveriaus-Ruff, 2002). Activation of opioid receptors in the PNS, by local administration of morphine for example, has shown efficacy in both animals and humans following an inflammatory insult (for review see Stein et al., 2003). We hypothesized that an exogenous, systemically-available and peripherally-restricted opioid agonist would provide effective pain relief in an inflammatory setting without centrally-mediated adverse effects; the accompanying paper (Valenzano et al.) describes such a molecule. Here, we report that systemic administration of DiPOA produced significant and naltrexone-sensitive reversal of mechanical hyperalgesia associated with FCA-induced inflammation. In addition, DiPOA was also effective against hyperalgesia induced by plantar incision, yet had no effect in the partial ligation model of neuropathic pain at the doses tested. These effects were observed in the absence of ataxia or effects on acute nociception, two outcomes commonly associated with opioid analgesics.

The results presented here regarding reversal of inflammatory hyperalgesia are in line with those of DeHaven-Hudkins et al. (2002) who demonstrated that local administration of the potent mu agonist loperamide is anti-hyperalgesic in the FCA model. Local administration of alternative mu agonists such as fentanyl (Stein et al., 1988) and the peptide [D-Ala2,MePhe4,Gly(ol)5]enkephalin (DAMGO) (Zhou et al., 1998; Stein et al., 1989) have also proven anti-hyperalgesic following intraplantar injection of FCA.

In the present study intraperitoneal administration of DiPOA has no effect on acute nociception as measured by the tail flick assay. This result is in direct contrast to those obtained with morphine, which leads to profound effects on acute nociception. We believe this difference is due to the extremely low levels of DiPOA that penetrate the blood-brain barrier (Valenzano et al.). Therefore this data provides strong evidence that effects on acute nociception are mediated by central opioid receptors. The literature regarding the involvement of peripheral mu receptors in acute nociception is controversial. Clinical studies indicate that local application of opioid agonists along uninjured nerve axons does not reliably produce analgesic effects (Pickard et al., 1997; Likar et al., 2001). This result may indicate that peripheral opioid analgesia is not achievable in the absence of an injury or inflammation. Alternatively, it may indicate that binding of opioids to opioid receptors does not occur on nerve axons. Pre-clinically, peri-neural application of either morphine or fentanyl is also not analgesic (Grant et al., 2001). Intraplantar injection of DAMGO (Stein et al., 1989) or loperamide (Dehaven-Hudkins et al., 1999) into non-inflamed rat paws or loperamide administered intravenously (Jurwitz et al., 1994) has no effect on acute nociception. These studies argue that peripheral mu receptors are not involved in acute nociception and are therefore in agreement with the conclusions of the current study. This is in disagreement with the results of Kolesnikov and Pasternak (1999) who demonstrated that when the tail of a mouse is immersed in a dimethyl sulfoxide (DMSO) solution containing either morphine or DAMGO, potent dose-dependent analgesia results only in the treated area. These seemingly opposing results may be explained by differences in administration procedures; the use of DMSO (Kolesnikov and Pasternak, 1999), may disrupt the perineurium causing an enhancement of opioid coupling, analogous to the situation following inflammation. Indeed following intraplantar injection of mannitol, a compound that increases perineurial permeability without causing inflammation, local administration of DAMGO or fentanyl inhibits acute nociception with a similar potency to that observed after local administration to FCA treated animals (Antonijevic et al., 1995).

Although at least 3 studies have demonstrated anti-hyperalgesic efficacy of locally administered mu opioid agonists in either the chronic constriction injury (CCI) or spinal nerve ligation (SNL) rat models of neuropathic pain (Truong et al., 2003; Martinez et al., 2002; Pertovaara and Wei, 2001), we did not find that DiPOA was effective in the PSN model of neuropathic pain at doses that were effective against inflammatory pain. There are three potential contributing factors that may explain this difference. Firstly, the action of morphine in the CCI model of neuropathic pain has been attributed, at least partially, to action at the kappa opioid receptor (Catheline et al., 1996), this may also hold true for the effect of morphine in the SNL model. As DiPOA has a higher selectivity for mu over kappa, as compared to morphine, this may contribute to the lack of efficacy in the PSN model. Secondly, both the CCI and SNL models involve a more substantial inflammatory component as compared to the PSN model (Lindenlaub and Sommer, 2000; Eaton, 2003), which may account for the efficacy of peripherally applied opioid agonists observed using these models. Finally, the time point in these studies was 14 days post surgery or less; at this time there may be a significant inflammatory component due to the surgery. In the present study a 21 day time point post-surgery was chosen to minimize any the effect of surgery induced-inflammation. In this study, DiPOA was in-effective over the same range of doses that produced anti-hyperalgesia in the rat FCA model of inflammatory hyperalgesia.

In addition to reversing inflammation-induced pain, systemically administered DiPOA also reversed hyperalgesia induced by plantar incision. To our knowledge this is the first demonstration that activation of peripheral mu receptors can inhibit pain elicited by an incision in the rat and suggests that this model of post-operation pain has an inflammatory component.

Our demonstration of a complete inhibition of the anti-hyperalgesic effect of DiPOA against inflammatory hyperalgesia by pre-administration of the opioid receptor antagonist, naltrexone,

demonstrates that DiPOA is acting through opioid receptors in vivo. Furthermore we believe that the mechanism of action of DiPOA is predominantly through mu opioid receptors. Firstly, as compared to morphine, DiPOA has higher affinity, potency and efficacy at the mu receptor in vitro; it also demonstrates selectivity for mu over kappa, ORL-1 and delta receptors (Valenzano et al.). Secondly, if the mechanism of action of DiPOA is predominantly through the stimulation of kappa or ORL-1 receptors then we would expect to see effects on acute nociception (Machelska et al., 1999a) and/or on neuropathic pain (Catheline et al., 1996; Abdulla and Smith, 1998).

As previously discussed both peripheral mu and peripheral kappa receptors have been implicated in pain modulation. Our data suggests that development of a peripheralised, orally available, small molecule mu agonist would have clinically utility in the treatment of inflammatory and post-surgical pain. Clinical proof-of-concept exists for peripheral mu mediated anti-hyperalgesia in studies where morphine was applied locally. We anticipate that the major side effect of a peripheralised, orally available, small molecule mu agonist, to be constipation. In contrast a peripheral kappa agonist, asimadoline, has been shown to be analgesic (Machelska et al., 1999b) and may therefore have a wider therapeutic utility, including neuropathic pain. However, in the same pre-clinical study a low dose of asimadoline was hyperalgesic. Furthermore, administration of asimadoline to patients following knee surgery resulted in no beneficial effect (Machelska et al., 1999b). This lack of efficacy may have been compound specific and data from further clinical trials with alternative peripheral kappa agonists is needed before the clinical validity of peripheral kappa receptors can be concluded.

The in vivo characterization of DiPOA in acute and chronic pain models presented here, together with the in vitro and pharmacokinetic profile presented in the accompanying manuscript, suggest a pharmacological profile unique from other opioids commonly used to

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study peripheral mechanisms of pain modulation. Our findings provide strong support for a role of peripheral mu opioid receptors in the pathology of pain associated with inflammation and argue against the involvement of these receptors in acute nociception, neuropathic pain and motor coordination. In conclusion, DiPOA represents the first description of a peripherally-restricted, small molecule mu opioid agonist that is non-sedating, anti-hyperalgesic and effective against inflammatory and post-surgical pain when administered systemically.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. DiPOA has no effect on acute analgesia as measured using the tail flick assay. The latency to tail flick was measured before and 1, 3 and 5 hours following administration of vehicle, DiPOA or morphine (i.p.) Asterisks denote significance ($p < 0.05$) from vehicle-treated group according to Fisher's PLSD post-hoc test. B = Baseline latency. Data shown are mean ± SEM (n = 8-10 rats/group).

Figure 2. DiPOA dose-dependently reverses inflammatory mechanical hyperalgesia. Rats received an intraplantar injection of 50 µl of saline (open squares) or 50% FCA (all other groups) into the hind paw, followed by intraperitoneal administration of DiPOA, morphine or vehicle 24 h later. Asterisks denote significance ($p < 0.05$) from FCA/vehicle-treated group according to Fisher's PLSD post-hoc test. B = Baseline latency, Pre = predrug reading. Data shown are mean ± SEM; error bars represent SEM (n = 8-10 rats/group).

Figure 3. Naltrexone completely inhibits the anti-hyperalgesic effect of DiPOA. Rats received an intraplantar injection of 50 µl of saline (open column) or 50% FCA (all other groups), followed 24 h later by administration of naltrexone or vehicle (i.p.). DiPOA, celecoxib or vehicle was administered (i.p.), 10 minutes after naltrexone or vehicle, and the animals were tested 1 h later. Asterisks denote significance ($p < 0.05$) from FCA/vehicle/vehicle-treated group according to Fisher's PLSD post-hoc test. Data shown are mean ± SEM; error bars represent SEM (n = 8-10 rats/group).

Figure 4. DiPOA dose-dependently reverses post-surgical mechanical hyperalgesia. Control rats were not operated on (open squares), all other groups received a 1 cm incision to the plantar surface of the left hind paw, followed 24 h later by administration of DiPOA, vehicle (i.p.)

or indomethacin (p.o.). Asterisks denote significance ($p < 0.05$) from incised/vehicle-treated group according to Fisher's PLSD post-hoc test. B = Baseline latency, Pre = predrug reading Data shown are mean ± SEM; error bars represent SEM (n = 8-10 rats/group).

Figure 5. DiPOA has no effect of motor performance as measured using the rotarod assay. Rats were placed on a rotarod set to accelerate from 4 to 40 rpm over 300 seconds and the latency to fall off the rotarod was recorded 30mins, 1 and 2 h post-administration of vehicle, DiPOA (i.p.) or morphine (s.c.). Asterisks denote significance ($p < 0.05$) from vehicle-treated group according to Fisher's PLSD post-hoc test. Data shown are mean percent of baseline value ± SEM; error bars represent SEM (n = 8-10 rats/group).

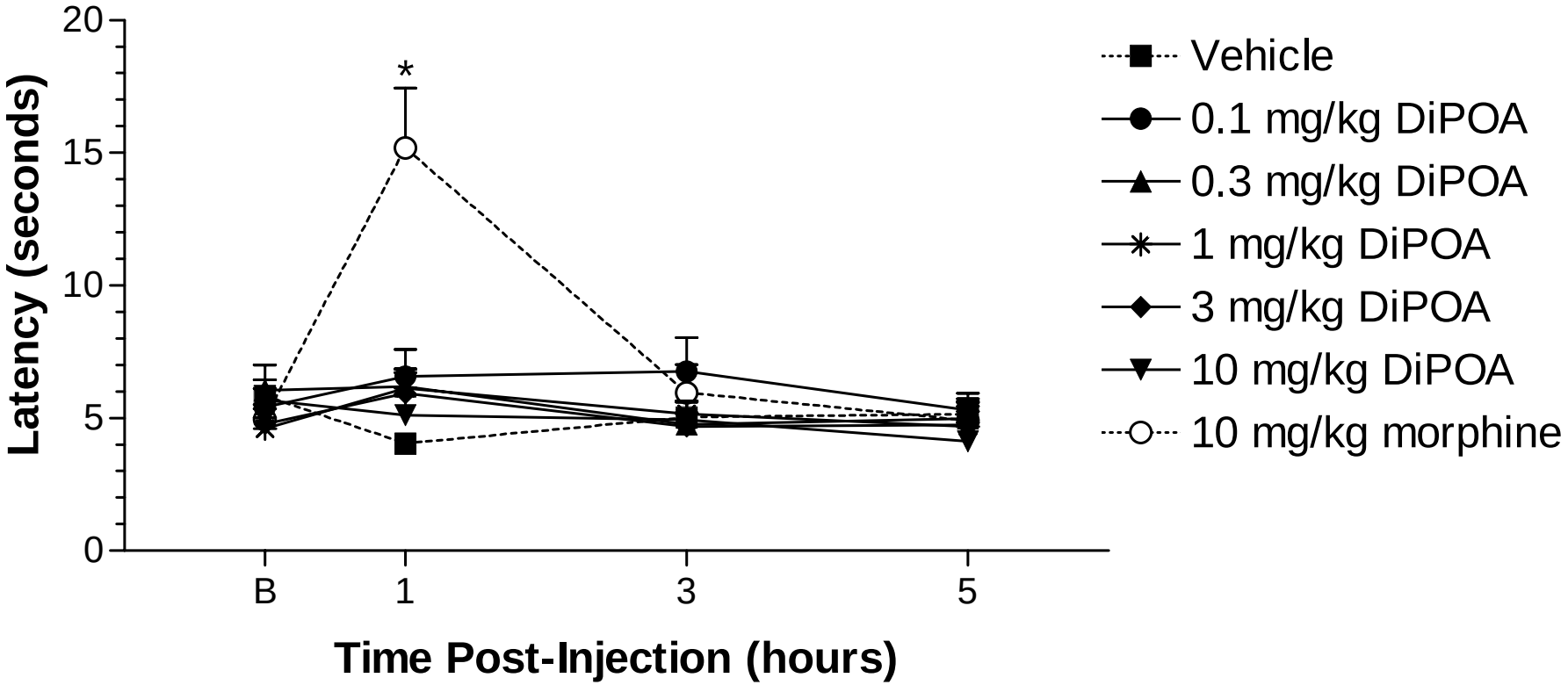

Figure 1

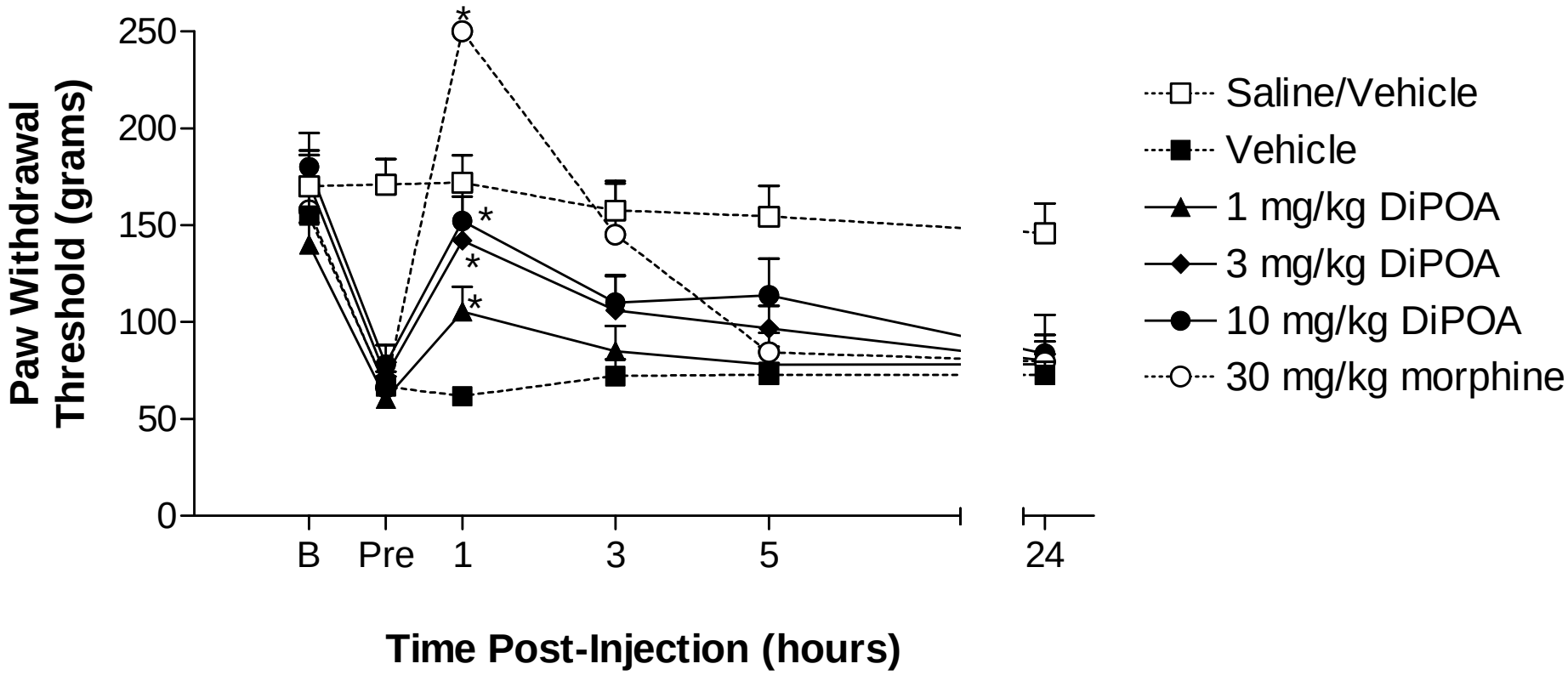

Figure 2

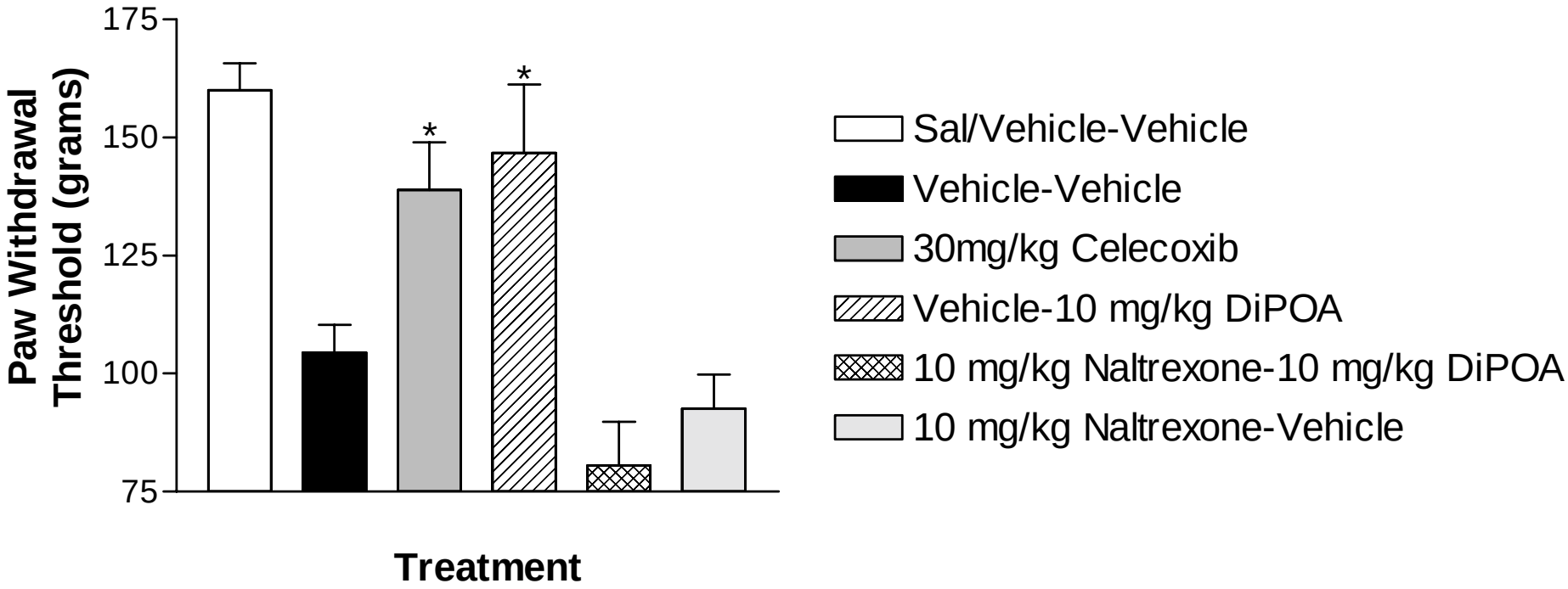

Figure 3

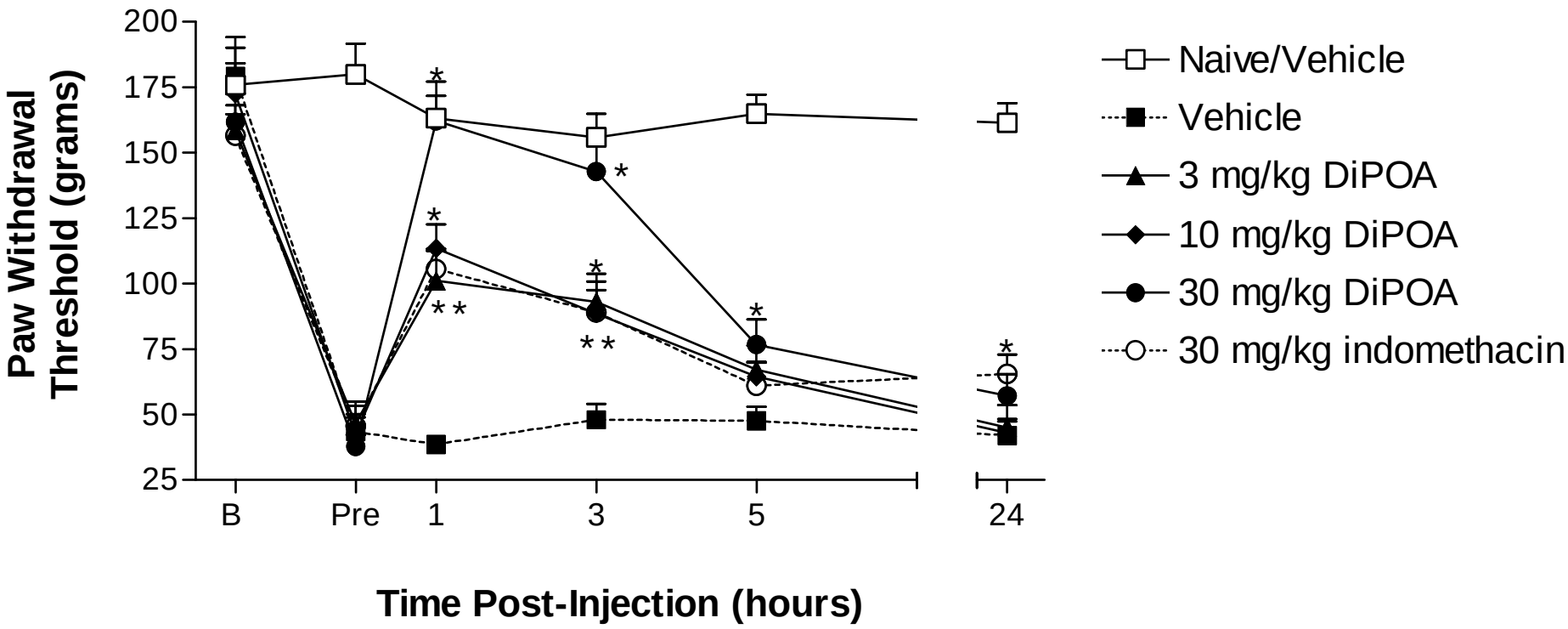

Figure 4

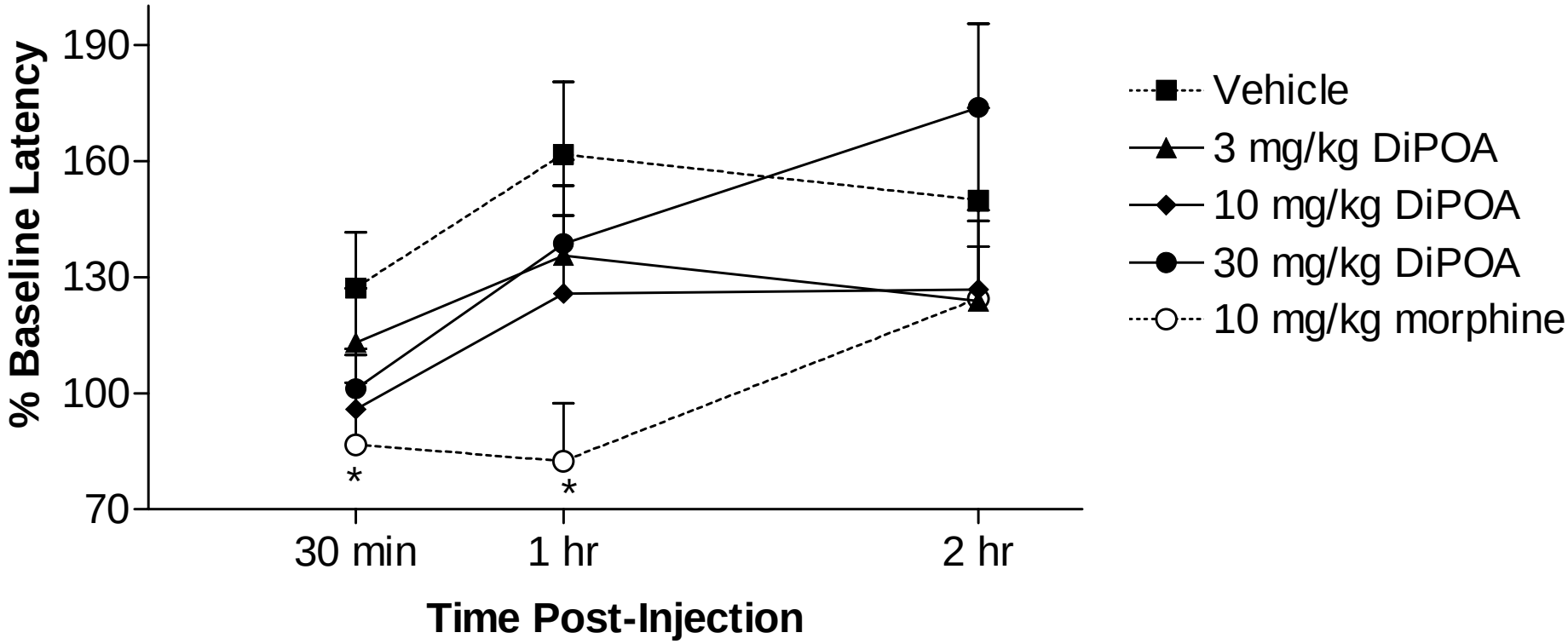

Figure 5