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Anti-allodynic effects of loperamide and fentanyl against topical capsaicin-induced allodynia in un-anesthetized primates[1]

Authors:

Eduardo R. Butelman, Todd J. Harris and Mary Jeanne Kreek

The Rockefeller University

1230 York Ave (Box 171)

New York NY 10024

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Corresponding Author:

Dr. E. Butelman

Rockefeller University (Box 171)

1230 York Ave

New York NY 10024

Telephone: (212)327-8247

FAX: (212)327-8574

e-mail: butelme@mail.rockefeller.edu

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Abbreviations: BL: baseline; **CAP:** capsaicin; **LOP:** loperamide; **FEN:** fentanyl; **MNTX:** methylnaltrexone; **NTX:** naltrexone; **OLDA:** N-oleoyldopamine; **Veh:** vehicle.

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Abstract:

Capsaicin produces thermal allodynia in animals and humans, by acting as an agonist at vanilloid VR1 (also known as TRPV1) receptors. VR1 receptors are widely distributed in the periphery (e.g., on primary afferent neurons). These studies examined the ability of loperamide (0.1-1 mg/kg, s.c.; a μ-opioid agonist which is peripherally selective after systemic administration), in preventing and reversing thermal allodynia caused by topical capsaicin (0.004 M) in rhesus monkeys, within a tail withdrawal assay (n=4; 38^{o} and 42^{o}C; normally non-noxious thermal stimuli). The effects of loperamide were compared to those of the centrally-penetrating μ-agonist, fentanyl (0.0032-0.032 mg/kg, s.c.). We also characterized the allodynic effects of the endogenous VR1 agonist ("endovanilloid"), N-oleoyldopamine (OLDA; 0.0013-0.004 M). In this model, loperamide and fentanyl produced dose-dependent prevention of capsaicin-induced allodynia, whereas only fentanyl produced robust reversal of ongoing allodynia. Antagonism experiments with naltrexone (0.1 mg/kg, s.c.) or its analog, methylnaltrexone (0.32 mg/kg, s.c.), which does not readily cross the blood-brain barrier, suggest that the anti-allodynic effects of loperamide and fentanyl were predominantly mediated by peripherally and centrally located μ-receptors, respectively. Loperamide and fentanyl (1 mg/kg and 0.032 mg/kg, respectively) also prevented OLDA (0.004 M)-induced allodynia. Up to the largest dose studied, loperamide was devoid of thermal antinociceptive effects in 48^{o}C (a noxious thermal stimulus, in the absence of capsaicin). By contrast, fentanyl (0.01-0.032 mg/kg) caused dose-dependent antinociception in this sensitive thermal antinociceptive assay (a presumed centrally-mediated effect). These studies show that loperamide, acting as a peripherally selective μ-agonist after systemic administration, can prevent capsaicin-induced thermal allodynia in primates *in vivo*, in the absence of thermal antinociceptive effects.

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Capsaicin is the main pungent component of “hot” chili peppers; this compound can produce thermal allodynia in experimental animals and humans, by acting as an agonist at vanilloid VR1 receptors (also known as TRPV1 receptors) (Caterina and Julius, 2001). VR1 receptors are widely distributed in the periphery and in the CNS (Mezey et al., 2000). With respect to cutaneous tissues, as relevant to these studies, VR1 receptors are located in several structures, including primary afferent neurons and keratinocytes (Caterina and Julius, 2001; Southall et al., 2003). Vanilloid VR1 receptors are sensitized and/or upregulated during conditions associated with tissue damage, including noxious heat, low pH, inflammation or neuropathic insults (Caterina and Julius, 2001; Hudson et al., 2001; Ji et al., 2002). As such, capsaicin-induced allodynia and its modulation are prominent models in the study of pain mechanisms *in vivo* and their pharmacological blockade.

Studies in rodents have indicated that μ-opioid receptors are located in the periphery (e.g., on primary afferents), where they may modulate ascending painful stimuli (Andreev et al., 1994). It has also been reported that μ-opioid receptor populations may increase in density in local neuronal sites following inflammatory or neuropathic insults, in rodents (Zollner et al., 2003; Truong et al., 2003). Thus, “peripherally-selective” μ-opioid agonists or locally administered μ-opioid agonists may be investigated for their potential to block painful conditions, in the relative absence of the undesirable effects of systemically administered, centrally-penetrating μ-agonists (e.g., respiratory depression, pruritus, cognitive effects) (Twillman et al., 1999; DeHaven-Hudkins et al., 1999; O'Mahony et al., 2001; Ko et al., 2004).

In these studies, we therefore compared loperamide (which acts as a peripherally selective μ-agonist after parenteral administration), with fentanyl, a centrally penetrating μ-agonist, in a recently developed model of topical capsaicin-induced thermal allodynia in unanesthetized rhesus monkeys (Butelman et al., 2003). Loperamide’s peripheral selectivity after parenteral administration is likely due its property as a substrate for a P-glycoprotein (multi-drug resistance) transporter, located in the blood-brain barrier (Schinkel et al., 1996; Wandel et al., 2002). Therefore, although loperamide is a lipophilic molecule of moderate molecular weight (MW=513.5), it is removed from potential accumulation at central sites by this transporter. Both loperamide and fentanyl have binding selectivity for μ- over κ or

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δ- receptors *in vitro*, and both are also highly efficacious μ-agonists (Toll et al., 1997; DeHaven-Hudkins et al., 1999). For example, loperamide's binding affinity at cloned human μ-receptors exhibits a K_i of 3.3 nM (DeHaven-Hudkins et al., 1999). Loperamide also has lower affinity at calcium channels labeled by (-)-[^{3}H]desmethoxyverapamil *in vitro*, with IC_{50} values in the 100-200 nM range; a loperamide-induced blockade of calcium-channel currents has also been observed *in vitro* (Reynolds et al., 1986; Church et al., 1994).

Prior allodynia/hyperalgesia studies in unanesthetized primates have focused on injected, rather than topical capsaicin or other agents (Ko et al., 1998a; Brandt et al., 2001). Interestingly, injected and topical capsaicin may produce somewhat different effects on sensory neurons *in vivo* (LaMotte et al., 1992). For example, a greater degree of within-session desensitization of primary afferent function was reported after injected vs. topical capsaicin in humans (LaMotte et al., 1992). Both injected and topical modes of capsaicin administration have been used as experimental pain stimuli in humans (LaMotte et al., 1992; Anderson et al., 2002). In this study, we examined the relative ability of loperamide and fentanyl to modulate thermal allodynia. We also compared the effects of capsaicin in this model with those of the recently discovered "endovanilloid" (endogenous VR1 agonist) OLDA (Chu et al., 2003). These are the first studies, to our knowledge, that directly compare the anti-allodynic effects of systemically administered peripherally selective and centrally penetrating non-peptidic μ–agonists in primates. These studies also provide, to our knowledge, the first evaluation in primates of the allodynic effects of the recently discovered endovanilloid, OLDA.

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Methods:

Subjects: Adult, gonadally intact rhesus monkey females (*Macaca mulatta*, age range: 8-11 years approximately) were used as subjects. They were singly housed in a room maintained at 20-22^{o}C with controlled humidity, and a 12:12 hour light: dark cycle (lights on at 0700). Experiments took place between the hours of 1000 and 1400. Monkeys were fed approximately 11 jumbo primate chow biscuits (PMI Feeds, Brentwood, MO) daily, supplemented by fruit and multivitamins. Water was freely available in home cages, via an automatic waterspout. Unless otherwise stated, experiments were carried out with an n=4. Before these studies, monkeys had been exposed several times to the experimental situation and were previously chair-trained with the standard "pole and collar" system. Monkeys had never received chronic administration of opioid compounds as part of their prior history.

Procedures:

Topical capsaicin-induced thermal allodynia: The present assay is a recent modification of the warm water tail withdrawal assay, adapted to study topical capsaicin-induced allodynia, based on previous models in humans and primates (Culp et al., 1989; Kupers et al., 1997; Ko et al., 1998a).

As recently reported (Butelman et al., 2003), monkeys sit in primate chairs, within a temperature-controlled room (20^{o}-22^{o}C). Their tails are shaved with standard clippers. Tail withdrawal latencies were timed manually in 0.1 sec increments, up to a maximum (cutoff) latency of 20 sec. Baseline latencies are determined in 38^{o} and 42^{o}C water stimuli (thermal stimuli are used within ±0.3^{o}C of the specified temperature). If a monkey did not remove its tail from the water by 20 sec, the experimenter removed the water, and a 20 sec value was assigned (38^{o} and 42^{o}C thermal stimuli are normally non-noxious in human or non-human primates) (Culp et al., 1989). Following baseline determination, the tail is gently dried and then degreased with an isopropyl alcohol pad. The topical capsaicin patch is applied (as described below) for 15 min. At the end of the topical capsaicin exposure, the patch is removed and testing in the above thermal stimuli occurs at standard intervals (5, 15, 30, 60 and 90 min after capsaicin removal). At each time point, the two thermal stimuli are tested in non-systematic order, with tests in the two stimuli separated from each other by 2 min approximately.

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Topical capsaicin / endovanilloid administration: A 1 cm^2 patch of 2-ply gauze (Johnson&Johnson; Arlington, TX) is attached on waterproof adhesive backing (23 mm diameter; Active Strips, 3M Health Care, St. Paul, MN) (Culp et al., 1989; Kupers et al., 1997). This backing is in turn attached onto elastic adhesive tape (5 cm wide, Elastikon, Johnson&Johnson, Arlington, TX). Capsaicin (typically 0.004 M) is dissolved in a vehicle composed of 70% ethanol and 30% sterile water by volume, approximately 30 min before use. Capsaicin (0.3 ml of the above solution) is slowly injected onto the gauze patch, saturating the patch, and minimizing overflow. Within 30 sec of the capsaicin solution being added, the patch is fastened onto the tail skin by means of the surrounding tape (e.g., 2-6 cm from the distal end). The patch is removed after 15 min exposure, and this is followed by tail withdrawal testing as described above. Allodynia is detected as a decrease in tail withdrawal latency from normally non-noxious thermal stimuli (i.e., 38^o and 42^{o}C). Consecutive sessions with topical capsaicin in the same subject were typically separated by at least 7 days. In selected experiments, the recently discovered endovanilloid, OLDA (0.0013-0.004 M) (Chu et al., 2003), is used topically instead of capsaicin (all other conditions are as described above; the 0.004 M OLDA concentration is near the solubility limit under these conditions).

Anti-allodynia assay: In **allodynia prevention** studies, a single dose of a compound (loperamide or fentanyl) was administered subcutaneously to prevent allodynia caused by topical capsaicin or OLDA (0.004 M), administered as described above. In **allodynia reversal** studies, a single dose of a compound (loperamide or fentanyl) was administered subcutaneously after capsaicin-induced allodynia was ongoing (i.e., approximately 15 min after the removal of topical capsaicin). This post-treatment was followed by testing at the remaining timepoints (30, 60 and 90 min), as above.

Assay of thermal antinociception: In separate studies, the thermal antinociceptive effects of loperamide (0.32-1 mg/kg) or fentanyl (0.01-0.032 mg/kg) were studied for comparison. The main purpose of these probe studies was to determine whether anti-allodynic (above) and antinociceptive effects of these compounds are encountered at the same doses and times. The assay is identical to that above, except that monkeys are tested in the absence of capsaicin treatment, and are tested in 42^{o}C

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(non-noxious) and 48^{o}C (noxious) thermal stimuli. In pilot studies, 48^{o}C was determined the to be the least intense thermal stimulus that would elicit rapid tail withdrawal responses from all the present subjects (not shown). After baseline determination, subjects were injected with s.c. vehicle (0.1 ml/kg) or a single s.c. dose of loperamide or fentanyl in the scapular region. This was followed by measurement of tail withdrawal latencies at standard time points, up to 90 min after injection.

Design

Allodynia Studies:

Studies were carried out in a single determination (n=4), unless otherwise stated. **Capsaicin or OLDA-induced allodynia:** The effects of topical vehicle, and topical capsaicin (0.004 M) applied on the tail were studied in separate experiments. Topical capsaicin (0.004 M) was studied in two baseline determinations, separated from each other by approximately two months (with other intervening experiments occurring during this period, at approximately one-week intervals). In one of these baseline determinations, topical capsaicin was studied alone, and in the other determination, capsaicin was studied after systemic s.c. vehicle pretreatment. The allodynic effects of OLDA (0.0013 and 0.004 M), were studied after topical administration on the tail, under identical conditions (Chu et al., 2003). The lower OLDA concentration (0.0013 M) was studied in one determination, and the higher OLDA concentration (0.004 M) was studied in two determinations, as described above.

Effects of loperamide and fentanyl: The effectiveness of loperamide (0.1-1 mg/kg; s.c.) and fentanyl (0.01-0.032 mg/kg, s.c.) in **preventing** capsaicin-induced allodynia was studied. Loperamide was administered 30 min before the removal of the topical capsaicin patch, whereas fentanyl was administered 15 min before the removal of the topical capsaicin patch. These dose ranges and time were based on prior available studies with parenterally administered loperamide and fentanyl in this species (Yanagita et al., 1979; Negus and Mello, 1999; Ko et al., 2002). In separate antagonism studies, the anti-allodynic effect of the largest loperamide and fentanyl dose were studied 30 min after pretreatment with

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either naltrexone (0.1 mg/kg) or methylnaltrexone (0.32 mg/kg), followed by testing as above. The selection of naltrexone and methylnaltrexone doses was based on available prior studies on the effects of these antagonists in this species, and in humans (Ko et al., 1998b; Yuan et al., 2002; Butelman et al., 2004). The timing of these sessions is further illustrated on Table 1.

In separate studies, the effectiveness of the largest dose of loperamide and fentanyl (1 mg/kg and 0.032 mg/kg, respectively) in **reversing** capsaicin-induced allodynia was studied (compared to vehicle). In these studies, capsaicin (0.004 M) was administered as above, with tests at 5 and 15 min after removal of the capsaicin patch. Immediately **after** the 15 min test, the subjects were injected with loperamide or fentanyl, and this was followed by standard testing at the remaining timepoints, (30, 60 and 90 min after capsaicin removal).

Antinociception Studies:

The thermal antinociceptive effects of loperamide (0.32-1 mg/kg) and fentanyl (0.01-0.032 mg/kg) were studied against a 48^{o}C stimulus, in the absence of capsaicin. In one subject, a smaller fentanyl dose (0.0032 mg/kg) was also studied, in order to obtain data sufficient to calculate an $ED_{10"}$ potency value (see Data analysis section, below). Prior studies show that fentanyl causes thermal antinociception in this species over the present dose range (Negus and Mello, 1999).

Data analysis: Tail withdrawal latency was the dependent variable in these studies. This was obtained from 38^{o} and 42^{o}C in the presence of capsaicin or OLDA (anti-allodynia), or from 48^{o}C in the absence of capsaicin (antinociception). Data are presented graphically as mean $\pm$ SEM. Data are analyzed in either one or two-way repeated measures ANOVAs, followed by Dunnett's or Newman-Keuls tests (SPSS-Sigmastat and Graphpad Prism). The level of significance (α) was set at the 0.05 level throughout. In selected cases, anti-allodynic or antinociceptive potency is defined as $ED_{10"}$ values (i.e., the agonist dose that would result in a 10 sec latency; similar to ED_{50}, given the present 20 sec cutoff). Such $ED_{10"}$ values were calculated by linear regression of individual log dose points above and below the 10 sec level of effect (95% confidence limits were calculated as t[SEM] (Kenakin, 1993).

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Test compounds: Naltrexone HCl (kindly supplied by the NIH-NIDA Drug Supply System, Baltimore MD), naltrexone methobromide (methylnaltrexone; kindly supplied by Dr. C.S. Yuan, Dept. of Anesthesiology, University of Chicago), and fentanyl citrate (Sigma, St.Louis, MO) were dissolved in sterile water. Loperamide HCl (Sigma, St.Louis, MO) was dissolved in ethanol (10%) / Tween 80 (10%) / sterile water (80%). Capsaicin (98% pure; Sigma, St. Louis MO) and N-oleoyldopamine (OLDA; Tocris Cookson, Ellisville MO), were prepared in ethanol (70%) / sterile water (30%) vehicle, approximately 30 min before topical use. The pH of all topically applied solutions was approximately 7. Doses of all compounds are in the forms described above.

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Results

Effects of topically administered vehicle, capsaicin or OLDA: Baseline tail withdrawal latencies in 38^{o} and 42^{o}C uniformly reached cutoff (20 sec) in the present subjects. Tail administration of topical vehicle (70% ethanol) under the conditions described above, did not affect tail withdrawal latencies over a standard 90 min test (Fig. 1). By contrast, topical administration of capsaicin on the tail (0.004 M; one determination alone and one determination after systemic s.c. vehicle pretreatment) caused robust allodynia (similarly to previously reported determinations) (Butelman et al., 2003). In a pilot session, identical administration of topical capsaicin at a remote site (the calf area of the leg) did not result in any allodynia (not shown). These two determinations of capsaicin on the tail were made approximately 2 months apart (with other intervening capsaicin tests, at approximately 1 week intervals). In all graphs and analyses, the two above determinations with topical capsaicin (0.004 M) on the tail were averaged. One-way repeated measures ANOVAs for time after capsaicin (and pre-capsaicin baseline) yielded a significant effect of time in 38^{o}C ($F[5,15]=51.36$) and 42^{o}C ($F[5,15]=269.9$).

The apparent onset of peak allodynia caused by capsaicin (0.004 M) was 15-30 min after the removal of the topical patch, as demonstrated by the presence of near-maximal allodynia in 42^{o}C, starting from the 15 min time point. In a single probe determination, capsaicin (0.004 M)- induced allodynia was found to dissipate completely by 24 h after topical administration, in both 38^{o} and 42^{o}C (not shown).

The endovanilloid OLDA (0.0013-0.004 M) caused concentration-dependent allodynia, in 90 min tests. The larger OLDA concentration was studied twice (one determination alone and one determination after systemic s.c. vehicle pretreatment); these two determinations were averaged for graphical and statistical purposes. A two-way (time X OLDA concentration) repeated measures ANOVA in 38^{o}C was significant for time ($F[4,12]=9.55$), treatment ($F[2,6]=55.25$) and their interaction ($F[8,24]=8.76$). A similar ANOVA in 42^{o}C also yielded significant effects of time ($F[4,12]=3.35$), treatment ($F[2,6]=19.21$) and their interaction ($F[8,24]=3.46$). However, a maximal degree of allodynia was observed with the largest OLDA concentration only at the more intense thermal stimulus (42^{o}C; Fig. 1). A larger topical concentration of

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OLDA could not be studied under the present conditions, due to solubility limits. In a single probe determination, OLDA (0.004 M)- induced allodynia was found to dissipate completely by 24 h after topical administration, in both 38^{o} and 42^{o}C (not shown).

Effects of loperamide and fentanyl in preventing capsaicin-induced allodynia: Based on the present determinations (above) and prior concentration-effect studies (Butelman et al., 2003), the 0.004 M capsaicin topical treatment was selected as a standard allodynia-inducing stimulus.

Loperamide (0.1-1 mg/kg; s.c.) was administered 30 min before the removal of the topical capsaicin patch, under the aforementioned conditions. Loperamide caused a robust dose-dependent prevention of capsaicin-induced allodynia. In 38^{o}C, a two-way (time X loperamide dose) repeated measures ANOVA was significant for time ($F[4,12]=8.57$) and for loperamide dose ($F[3,9]=5.02$). In 42^{o}C, similar findings were obtained (time: $F[4,12]=10.59$; loperamide dose: $F[3,9]=9.39$). This dose-dependent effect could be clearly observed at a time of peak capsaicin allodynia (e.g., 30 min after the removal of the topical capsaicin patch; Fig. 2; Table 2). No obvious behavioral effects of s.c. loperamide were observable under these conditions, by an experimenter experienced in behavioral rating in this species.

In separate studies, fentanyl (0.0032-0.032 mg/kg; s.c.) was administered 15 min before the removal of the topical capsaicin patch. Fentanyl also caused dose-dependent prevention of allodynia (Fig. 3). In 38^{o}C, a two-way (time X fentanyl dose) repeated measures ANOVA was significant for time ($F[4,12]=5.75$) and for fentanyl dose ($F[3,9]=5.35$). In 42^{o}C, similar findings were obtained (time: $F[4,12]=4.04$; fentanyl dose: $F[3,9]=5.66$; see also Table 2). In one of the four subjects, the largest fentanyl dose (0.032 mg/kg) did not result in latencies ≥10 sec, at a time of peak capsaicin allodynia (30 min after the removal of the capsaicin patch). A larger fentanyl dose (e.g., 0.056 mg/kg) could not be studied in this subject, due to occurrence of respiratory depression observed in a pilot study.

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Antagonism of the ability of loperamide and fentanyl to prevent allodynia:

In four separate experiments, the largest doses of loperamide and fentanyl used above (1 mg/kg and 0.032 mg/kg, respectively) were studied after 30 min pretreatment with naltrexone (0.1 mg/kg; s.c.) or its analog, methylnaltrexone (0.32 mg/kg; s.c.). The findings are summarized graphically at a time of peak capsaicin-induced allodynia (i.e., 30 min after removal of the topical capsaicin patch, in 42^{o}C) (Fig. 4). A 2-way (treatment X time) repeated measures ANOVA was completed for the effects of loperamide (1 mg/kg) alone or after naltrexone or methylnaltrexone pretreatment, in 42^{o}C. A significant effect of treatment was found ($F[2,6]=11.32$), and a Dunnett's test revealed that both naltrexone and methylnaltrexone pretreatment conditions were different from loperamide alone. Effects on 38^{o}C were not analyzed in these probe antagonism studies (the 1 mg/kg loperamide dose is supra-maximal for two of four subjects with the 38^{o} stimulus; Fig. 2). An analogous 2-way repeated measures ANOVA was completed for fentanyl (0.032 mg/kg) alone or after naltrexone or methylnaltrexone pretreatment, in 42^{o}C. A significant effect of time was found ($F[4,12]=4.41$). In a follow-up one-way repeated measures ANOVA at a standard time of peak allodynia (30 min after topical capsaicin removal), a significant effect of treatment was found ($F[2,6]=5.22$); Dunnett's tests revealed that the naltrexone (0.1 mg/kg) pretreatment condition was different from fentanyl (0.032 mg/kg) alone. By contrast, the methylnaltrexone (0.32 mg/kg) pretreatment condition was not different from fentanyl alone.

Effects of loperamide and fentanyl in reversing ongoing capsaicin-induced allodynia: In separate studies, the largest loperamide and fentanyl doses used above (1 mg/kg and 0.032 mg/kg; respectively) were administered immediately after the onset of robust allodynia, under the present conditions. That is, loperamide, fentanyl (or vehicle) were administered immediately after the 15 min test after the removal of topical capsaicin (see Figs. 1, 5). In 38^{o}C, a 2-way repeated measures ANOVA (time X treatment) narrowly missed significance ($F[2,6]=4.54$; p 0.06), whereas a Dunnett's test revealed that fentanyl (but not loperamide) was significantly different from vehicle. In 42^{o}C, a similar 2-way ANOVA was significant for treatment ($F[2,6]=9.45$), and a Dunett's test confirmed that fentanyl (but not loperamide) was significantly different from vehicle.

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Effects of loperamide and fentanyl in preventing endovanilloid (OLDA)-induced allodynia: The largest dose of loperamide and fentanyl (1 mg and 0.032 mg/kg, respectively) were also administered before topical OLDA (0.004 M), under identical conditions to those described above (Fig. 6). Given the lack of robust OLDA – induced allodynia in 38^{o}C (see Fig. 1), only data in 42^{o}C were analyzed. A 2-way repeated measures ANOVA (time X treatment) revealed a significant effect of time ($F[4,12]=8.77$) and treatment ($F[2,6]=7.90$). Dunnett's tests revealed that both loperamide and fentanyl conditions were significantly different from OLDA alone.

Antinociceptive effects of loperamide and fentanyl: In these subjects, 48^{o}C (in the absence of topical capsaicin) was the lowest temperature at which rapid (e.g., <3 sec) and consistent tail withdrawal latencies were observed (pilot studies). Therefore, the antinociceptive effects of s.c. vehicle, loperamide and fentanyl were studied against the 48^{o}C thermal stimulus, in order to sensitively detect antinociceptive effects. In a 90-min vehicle test, pre-injection latencies in 48^{o}C had a mean of 1.8 sec (SEM=0.5). Vehicle administration (s.c.; 0.1 ml/kg) was without effect on these latencies (Fig. 7). Latencies in 42^{o}C were at cutoff (20 sec) throughout the vehicle test (not shown). Loperamide, up to the largest dose studied above (1 mg/kg) was also without effect in 48^{o}C, whereas fentanyl (0.01-0.032 mg/kg) produced a dose-dependent antinociceptive effect. A 2-way (time X fentanyl dose) ANOVA revealed a significant effect of time ($F[5,15]=4.24$) and an interaction between time and fentanyl dose ($F[10,30]=2.85$). A Dunnett's test revealed that the larger dose of fentanyl (0.032 mg/kg) was significantly different from vehicle. A one-way repeated measures ANOVA at this fentanyl dose (for time [baseline and post-fentanyl, 0.032 mg/kg]), revealed a significant effect of time ($F[6,18]=5.98$). Newman-Keuls tests revealed that the 30 and 45 min time points post fentanyl were significantly different from baseline. These two time points (30 and 45 min post fentanyl) were not different from each other.

A summary of anti-allodynic and antinociceptive dose-effect curves and potency (ED_{10}" values) for loperamide and fentanyl is presented graphically in Figure 8 and Table 2. Overall, fentanyl was more potent than loperamide as an anti-allodynic agent, either in 38^{o} and 42^{o}C. Fentanyl exhibited similar

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potency across all the present endpoints, as an anti-allodynic or antinociceptive agent. Loperamide appeared to exhibit an intensity-dependent profile as an anti-allodynic agent, in that the dose-effect curve for 38^{o}C was to the left of that for 42^{o}C (although ED_{10}" values were not significantly different). Loperamide was ineffective as an antinociceptive agent, over this dose range.

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Discussion

In the present studies, loperamide (0.1-1 mg/kg; s.c.), acting as a peripherally selective μ-agonist after s.c. administration, produced a prevention of topical capsaicin-induced allodynia. However, up to 1 mg/kg, loperamide was completely devoid of thermal antinociceptive effects in these subjects (a presumed centrally mediated effect of μ-agonists). By comparison, the centrally penetrating μ-agonist, fentanyl, produced both anti-allodynia and antinociception over a similar dose range (fentanyl potency in each of these endpoints was very similar). These findings suggest that systemically administered, peripherally selective μ-agonists may have anti-allodynic effects *in vivo* in primates, in the relative absence of robust centrally-mediated effects, such as antinociception. This is consistent with previous findings in rodents, using systemic loperamide (Takasuna et al., 1994; Reichert et al., 2001). Other studies in rodents also indicate that local (e.g., injected or topical) loperamide, also produces antinociceptive effects in certain pain models (Nozaki-Taguchi and Yaksh, 1999; DeHaven-Hudkins et al., 2002; Menendez et al., 2003).

Loperamide doses larger than 1 mg/kg could not be tested under the present solubility conditions. In another study with loperamide in primates, substantially larger loperamide doses (e.g., 8 mg/kg, s.c.) were associated with acute toxicity, including respiratory failure (Yanagita et al., 1979). At the largest dose used herein, loperamide did not have obvious behavioral effects, and no untoward effects were observed during or after loperamide dosing sessions.

Probe antagonist studies were completed with naltrexone and its analog, methylnaltrexone (which only poorly crosses the blood brain barrier). Loperamide's anti-allodynic effects were sensitive to both naltrexone (0.1 mg/kg) and methylnaltrexone (0.32 mg/kg). However, the anti-allodynic effects of fentanyl were sensitive to naltrexone but not methylnaltrexone. The present naltrexone dose is sufficient to antagonize diverse μ-receptor mediated effects in this species; it is presumed that naltrexone would be able to occupy both central and peripheral μ-opioid receptor pools, following systemic administration (France et al., 1990; Ko et al., 1998b). The presently used s.c. methylnaltrexone dose is sufficient to produce a blockade of the effects of morphine on intestinal transit in humans (an effect presumed to be

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mediated by peripherally located μ-receptors) (Yuan et al., 2002). This methylnaltrexone dose is also sufficient to cause a blockade in a κ-opioid neuroendocrine effect in this species; this neuroendocrine effect (prolactin release) is presumed to be mediated outside the blood-brain barrier (Butelman et al., 2004). *Ex vivo* studies suggest that methylnaltrexone is a more potent antagonist of μ- than κ- receptor mediated effects (Yuan and Foss, 1999). Taken together, the above findings lead to the conclusion that the presently used methylnaltrexone dose is sufficient to cause a blockade of peripheral μ-receptor systems, under the present conditions. Both loperamide and fentanyl have binding selectivity for μ- over κ or δ- receptors (Toll et al., 1997; DeHaven-Hudkins et al., 1999). Overall, the present antagonism studies are therefore consistent with the conclusion that loperamide and fentanyl produced their anti-allodynic effects by acting predominantly at peripherally and centrally located μ-receptors, respectively.

The effects of the opioid antagonists alone were not studied herein. Under specific experimental conditions, there have been reports of opioid antagonists (e.g., naloxone) enhancing capsaicin-induced pain ratings in humans (Anderson et al., 2002). Such a potential enhancement would not have been easily detectable under the present conditions, due to the presence of maximal "floor" allodynia at several of the present timepoints after topical capsaicin.

Loperamide appeared to be less effective than fentanyl in reversing, rather than preventing topical capsaicin-induced allodynia. These are the first studies in primates in which μ-agonists' ability in preventing and reversing capsaicin-induced allodynia have been directly investigated, to our knowledge. The present probe experiments suggest that the triggering of allodynia (in this model) may be sensitive to peripheral μ-opioid effects, whereas the maintenance of ongoing allodynia may be relatively less sensitive to such effects (as evidenced by the limited ability of loperamide relative to fentanyl). Interestingly, in mice, i.p. morphine (acting through a proposed peripherally mediated opioid mechanism) was more potent in the acetic-acid induced writhing assay when administered as a pretreatment than when administered after the onset of the writhing (Reichert et al., 2001). As mentioned above, both peripheral VR1 and μ-opioid receptor populations (e.g., in primary afferents) exhibit a substantial degree of plasticity, in animal models of neuropathic or inflammatory insults (Ji et al., 2002; Zollner et al., 2003; Truong et al., 2003). Therefore, loperamide's present lack of effectiveness in reversing ongoing allodynia

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in this model does not necessarily indicate that loperamide would be ineffective in experimental pain models (or in clinical situations) with ongoing neuropathic or inflammatory pain, in which such plasticity may have occurred.

As a further caveat, loperamide (and presumably other peripherally selective μ-agonists) may have potentially undesirable effects mediated by peripheral opioid receptors. Such potential undesirable effects could include constipation, immune and neuroendocrine effects. The "therapeutic window" for systemically administered loperamide in causing anti-allodynia versus causing the aforementioned peripherally-mediated undesirable effects has not been directly studied, to our knowledge.

The recently discovered endovanilloid OLDA (Chu et al., 2003) also produced concentration-dependent thermal allodynia, after topical administration in primates. OLDA displayed a similar profile of maximum allodynia in 42^0C as capsaicin, and maximal allodynia was observed at a similar topical concentration (0.004 M) (present study; Butelman et al., 2003). *In vitro*, OLDA was also approximately equipotent and equieffective to capsaicin, as an agonist at cloned VR1 receptors (Chu et al., 2003). Unlike other endovanilloids (e.g., anandamide, N-arachidonyldopamine), OLDA has a relatively high degree of selectivity for VR1 receptors over CB1 cannabinoid receptors (Chu et al., 2003). Thus, OLDA may be an especially valuable tool to study the *in vivo* effects of endovanilloids. Locally administered OLDA caused thermal hyperalgesia in mice (Chu et al., 2003). The presently reported studies are the first data with OLDA in non-human primates, to our knowledge, and are consistent with a role for this endovanilloid in the process of thermal allodynia. In probe studies, loperamide and fentanyl both prevented OLDA-induced allodynia (similarly to their effect on capsaicin-induced allodynia). These are, to our knowledge, the first studies on opioid blockade of OLDA-induced allodynia in any species. Overall, these initial studies suggest that endovanilloid-induced allodynia may be sensitive to the same types of opioid analgesics, as capsaicin-induced allodynia (a compound widely used to model endogenous allodynic states, which may potentially depend in part on endovanilloid release).

Prior *in vitro* and *in vivo* studies with experimental animals have identified complex inter-species differences in VR1 receptor pharmacology (e.g., rat vs. human) (Szallasi and Blumberg, 1993; Walker et al., 2003). Radioligand binding studies indicate that the *in vitro* pharmacology of native macaque VR1

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receptor (e.g., from dorsal root ganglia) may be only modestly different than that of human VR1 receptors (we are not aware of published sources of information on cloned non-human primate VR1 receptors) (Szabo et al., 2002). Therefore, available data at this time suggest that macaques may be valuable model species, due to the similarity in the *in vitro* VR1 receptor pharmacology with that of humans, as well as the similarity in the *in vivo* allodynic effects of topical capsaicin (Culp et al., 1989; Kupers et al., 1997).

Overall, the present studies show that loperamide, which acts as a peripherally selective μ-agonist after systemic administration, can prevent capsaicin-induced thermal allodynia in primates, in the absence of centrally mediated thermal antinociceptive effects. This suggests that systemically administered peripherally selective μ-agonists could potentially produce anti-allodynia in the relative absence of undesirable centrally-mediated effects (e.g., cognitive side effects and respiratory depression) (O'Mahony et al., 2001). Given the reported relevance of peripheral μ-opioid analgesia in a variety of rodent pain models including visceral pain, burn pain and bone pain (Takasuna et al., 1994; Houghton et al., 1998; Nozaki-Taguchi and Yaksh, 1999; Junger et al., 2002; Menendez et al., 2003), the present studies support the hypothesis that peripherally selective μ-opioids are valuable pharmacotherapeutic targets for these painful conditions, and may be a useful adjunct to current therapeutic approaches.

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Footnotes

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Reprint requests should be sent to E. Butelman, Rockefeller University (Box 171), 1230 York Ave, New York NY 10021.

[1]The present studies were reviewed by the Rockefeller University IACUC, and are in accordance with the Guide for the Care and Use of Laboratory Animals, as promulgated by the U.S. National Institutes of Health.

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Figure Legends

Figure 1. Time course and concentration-dependence of the thermal allodynic effect of topical capsaicin (0.004 M; left panels) or OLDA (0.0013-0.004 M; right panels) in 38^{o} and 42^{o}C water (upper and lower panels, respectively). All data are mean ±SEM (n=4) of one determination, other than the capsaicin and OLDA 0.004 M data, which are the mean of 2 determinations each. Abscissae: Time in minutes from the removal of the topical patch; BL indicates baseline latencies. Ordinates: Tail withdrawal latencies in sec (20 sec maximum allowed latency).

Figure 2. Prevention of topical capsaicin (0.004 M) –induced thermal allodynia by s.c. loperamide. Loperamide doses are in mg/kg; all other details as in Fig. 1.

Figure 3. Prevention of topical capsaicin (0.004 M) –induced thermal allodynia by s.c. fentanyl. Fentanyl doses are in mg/kg; all other details as in Fig. 1 and 2.

Figure 4. Summary of antagonism experiments with naltrexone (NTX, 0.1 mg/kg, s.c.) or methylnaltrexone (MNTX, 0.32 mg/kg, s.c.), to investigate sensitivity of loperamide or fentanyl (1 mg/kg and 0.032 mg/kg, respectively) -induced prevention of capsaicin-induced allodynia. Data are summarized for the 42^{o}C thermal stimulus, at a time of peak capsaicin-induced allodynia (30 min after removal of the topical capsaicin patch; all n=4). Mean of 1-2 determinations (statistical analyses are in text).

Figure 5. Reversal of topical capsaicin (0.004 M) –induced allodynia by s.c. vehicle or s.c. loperamide or fentanyl (1 mg/kg and 0.032 mg/kg, respectively). In these experiments, vehicle, loperamide or fentanyl were injected immediately **following** the test occurring 15 min after topical capsaicin removal.

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Figure 6. Prevention of topical OLDA (0.004 M) –induced thermal allodynia by s.c. loperamide or fentanyl (1 mg/kg and 0.032 mg/kg, respectively). Mean of 1-2 determinations. All other details as in Fig. 1.

Figure 7. Antinociceptive effects of s.c. vehicle, s.c. loperamide (0.32-1 mg/kg) or s.c. fentanyl (0.01-0.032 mg/kg) in 48^{o}C water (a normally noxious stimulus), in the absence of capsaicin. Abscissa: Time in minutes from s.c. administration; BL indicates baseline latencies. Ordinate: Tail withdrawal latencies in sec (20 sec maximum allowed latency).

Figure 8. Anti-allodynic (38^{o} and 42^{o}C; with capsaicin) and antinociceptive (48^{o}C; in the absence of capsaicin) dose-effect curves for loperamide and fentanyl. Anti-allodynic data are presented for the time point 30 min after the removal of topical capsaicin. Antinociception data are presented for an equivalent time point after the administration of loperamide or fentanyl (i.e., 60 and 45 min, respectively). All data are n=4. Abscissa: Dose in mg/kg; ordinate: Tail withdrawal latencies in sec (20 sec maximum allowed latency).

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Table 1. Time line for allodynia prevention experiments

(after baseline latency determination in 38^{o} and 42^{o}C)

	loperamide experiments	**fentanyl experiments**
Time (min)		
-30	s.c. loperamide	NA
-15	NA	s.c. fentanyl
-15 to 0	topical capsaicin	topical capsaicin
+5	test latencies	test latencies
+15	test latencies	test latencies
+30	test latencies	test latencies
+60	test latencies	test latencies
+90	test latencies	test latencies

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Table 2. Anti-allodynic and antinociceptive potency of loperamide and fentanyl (n=4; unless otherwise stated)

Agonist	**Anti-allodynia $ED_{10"}$ [95%CL][1]**		**Antinociception $ED_{10"}$ [95%CL][2]**
	With topical capsaicin		**Without topical capsaicin**
	38°C	**42°C**	**48°C**
Loperamide	0.18 [0.05-0.6]	0.59 [0.25-1.4]	Inactive to 1 mg/kg
Fentanyl[3]	0.0078 [0.0014-0.042]	0.012 [0.0022-0.063]	0.014 [0.0017-0.11]

[1]$ED_{10"}$ values are in mg/kg, measured at a time of peak capsaicin-induced allodynia (i.e., 30 min after topical capsaicin removal).

[2]Determined, for each opioid agonist, at an equivalent time to that in the allodynia studies (i.e., 60 min and 45 post loperamide and fentanyl injection, respectively).

[3]All fentanyl $ED_{10"}$ values based on n=3. The fourth subject did not attain the 10" level of effect in any fentanyl condition, up to the largest fentanyl dose studied. Larger fentanyl doses could not be evaluated in this subject, due to the occurrence of respiratory depression.

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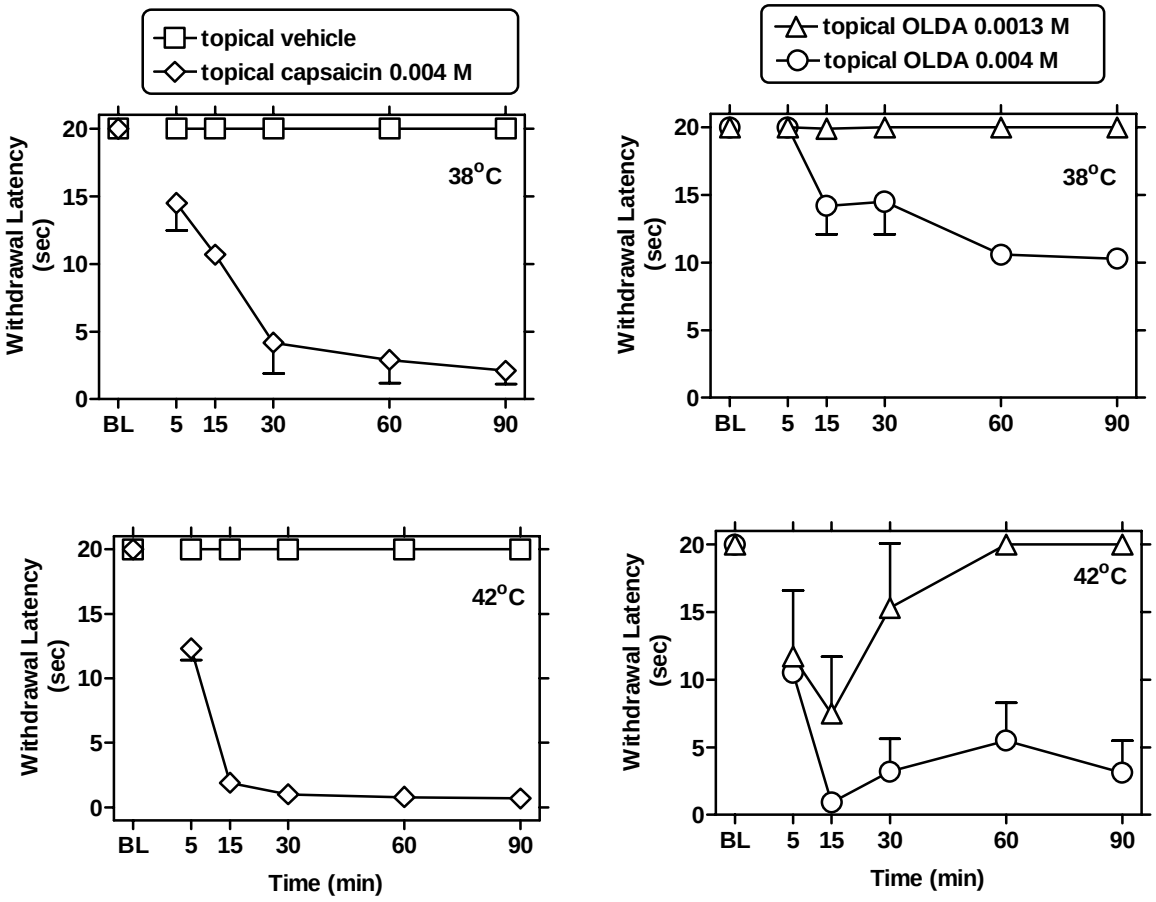

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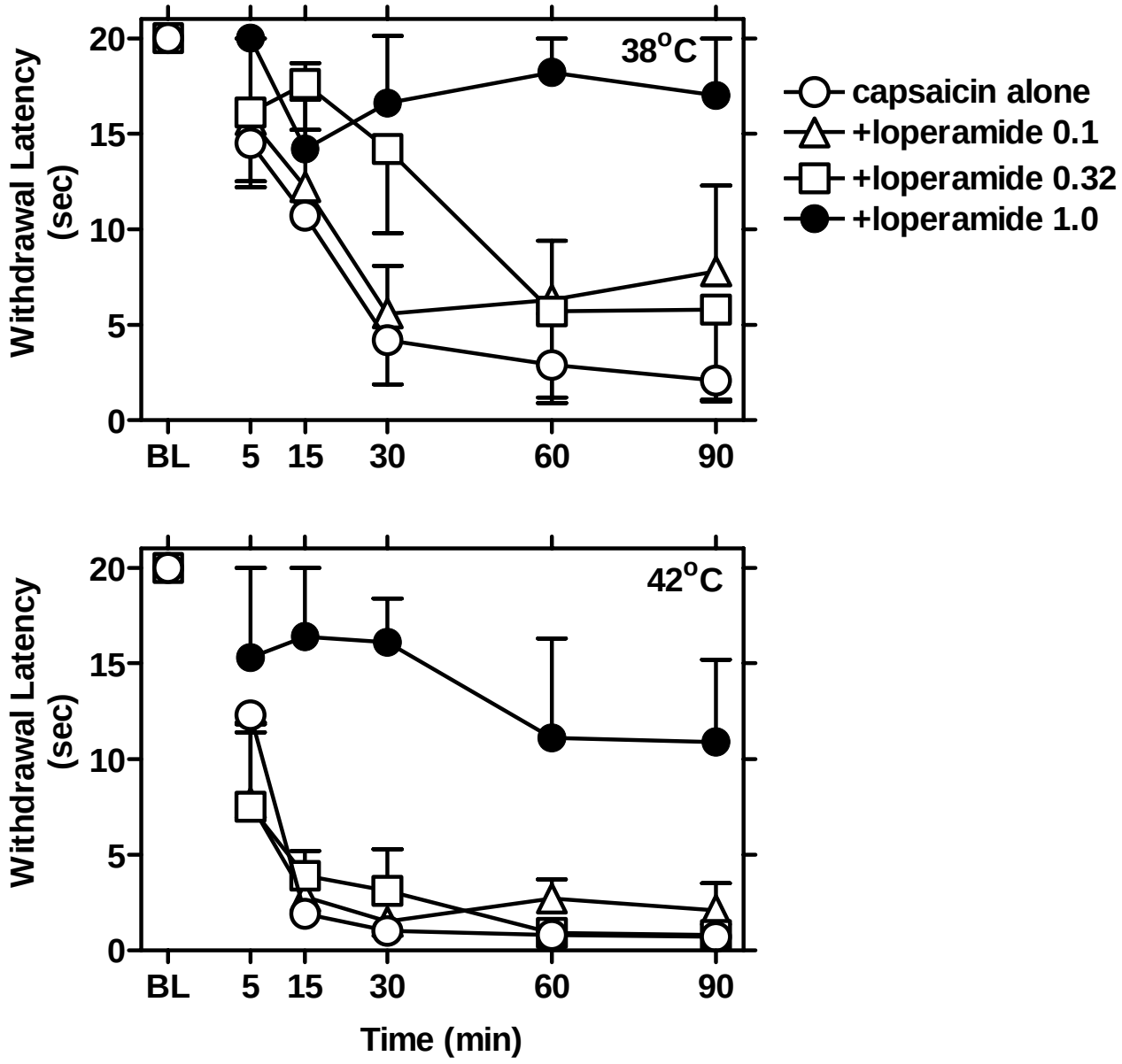

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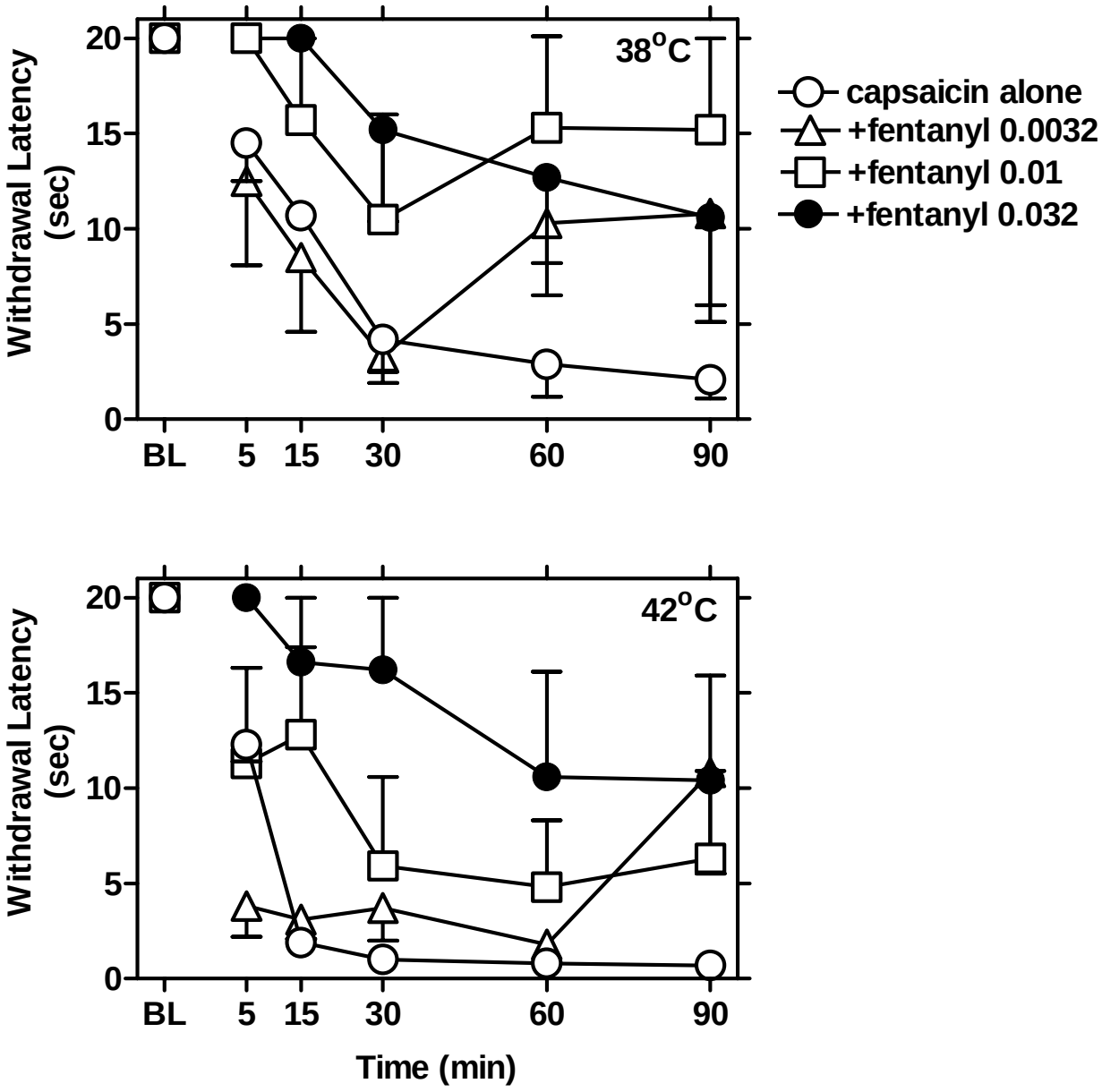

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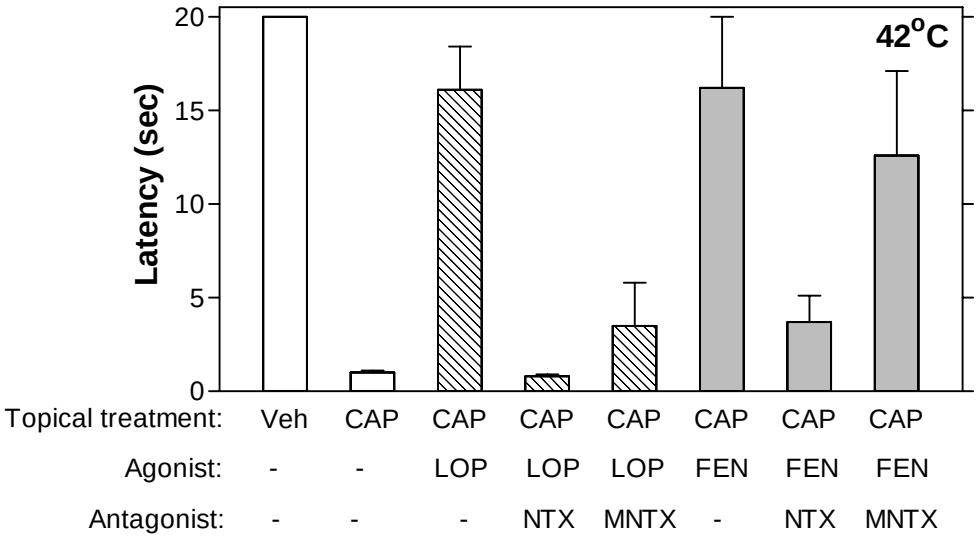

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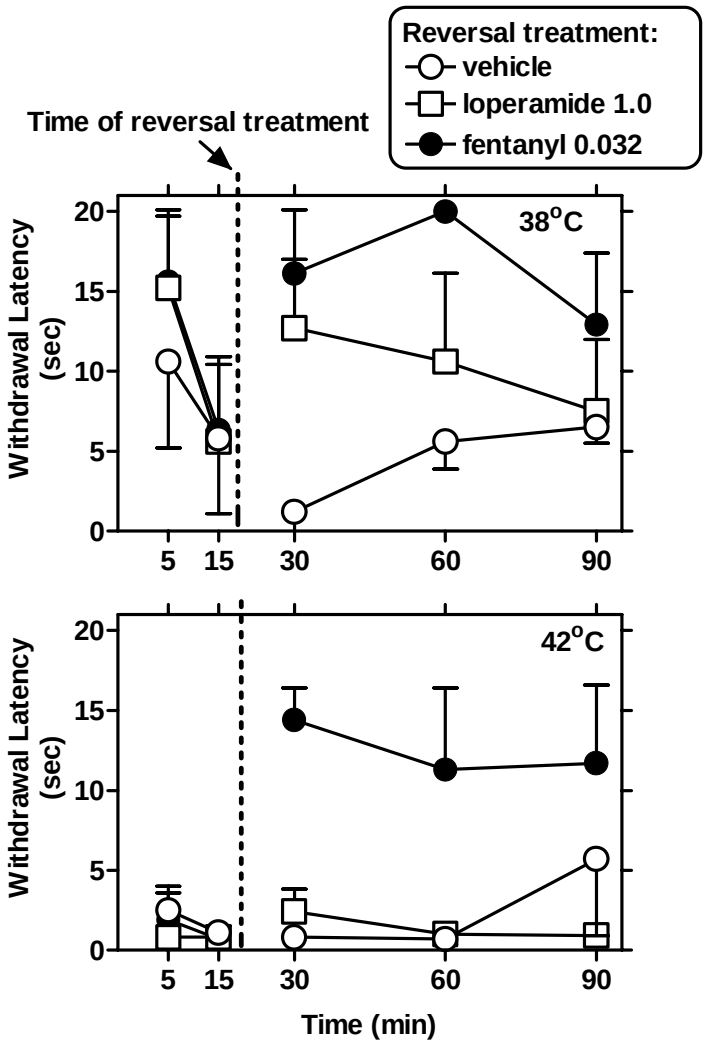

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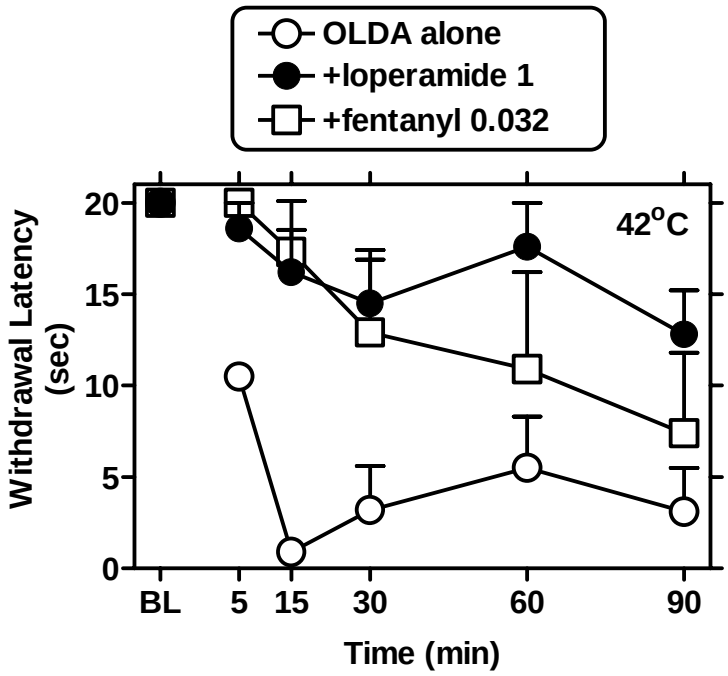

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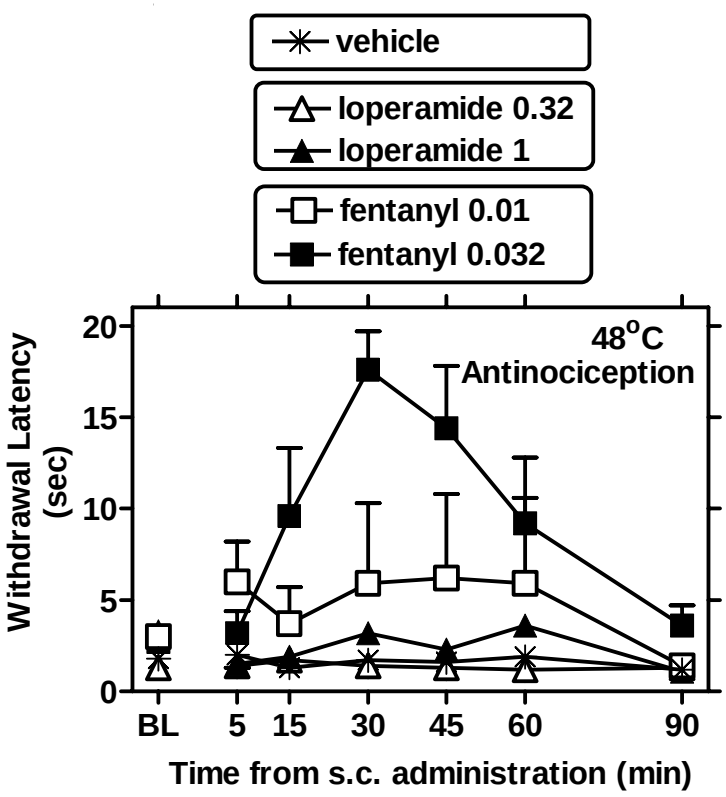

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