Inflammatory pain in the rabbit: A new, efficient method for measuring mechanical hyperalgesia in the hind paw

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Abstract

The discovery of novel analgesic compounds that target some receptors can be challenging due to species differences in ligand pharmacology. If a putative analgesic compound has markedly lower affinity for rodent versus other mammalian orthologs of a receptor, the evaluation of antinociceptive efficacy in non-rodent species becomes necessary. Here, we describe a new, efficient method for measuring inflammation-associated nociception in conscious rabbits. An electronic von Frey device is used, consisting of a rigid plastic tip connected to a force transducer in a hand-held probe. The plastic tip is applied to the plantar surface of a hind paw with increasing force until a withdrawal response is observed. The maximum force (g) tolerated by the rabbit (i.e., withdrawal threshold) is recorded. In young, conscious rabbits (500–700 g), baseline hind paw withdrawal thresholds typically fell within the 60–80 g range. Three hours after injection of the inflammatory agent carrageenan (3%, 200 L, intra-plantar), withdrawal thresholds dropped by ∼30–40 g, indicating the presence of punctate mechanical hyperalgesia. The development of hyperalgesia was dose dependently prevented by the NSAID indomethacin (ED50 = 2.56 mg/kg, p.o.) or the bradykinin B2 receptor peptide antagonist HOE 140 (intrapaw administration). An established hyperalgesia was dose dependently reversed by morphine sulfate (ED50 = 0.096 mg/kg, s.c.) or the bradykinin B1 receptor peptide antagonist [des-Arg10,Leu9]-kallidin (ED50 = 0.45 mg/kg, s.c.). Rabbits treated with the novel B1 receptor small molecule antagonist compound A also showed dose-dependent reversal of hyperalgesia (ED50 = 0.20 mg/kg, s.c.) and analysis of plasma samples taken from these rabbits showed that, unlike other rabbit pain models, the current method permits the evaluation of pharmacokinetic–pharmacodynamic (PK–PD) relationships (compound A plasma EC50 = 402.6 nM). We conclude that the Electrovonfrey® method can be used in rabbits with inflammatory pain to generate reliable dose- and plasma concentration-effect curves for different classes of analgesics.

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The discovery of novel analgesic compounds that interact with some molecular targets can be a challenging process at the preclinical stage due to species differences in ligand pharmacology. For many targets, their associated ligands interact differently with different species orthologs of the target (e.g., high binding affinity for the human ortholog, low binding affinity for rodent orthologs). This can be due to species differences relating to one or more factors, such as amino acid (aa) sequence identity within the target itself (e.g., within an orthosteric or allosteric ligand binding pocket/crevice) or aa sequence identity within accessory proteins that modulate the target’s function. As such, species differences in in vivo and/or in vitro pharmacology have been described for targets in multiple classes. These include G protein-coupled receptors (GPCRs; classes 1 and 2), monoamine transporters, ion channels and kinases.

In the GPCR superfamily alone, a large number of classes 1 and 2 receptors have shown marked species differences in ligand pharmacology. Class 1 GPCRs in this category include the bradykinin B1 receptor (Ransom et al., 2004) and bradykinin B2 receptor (Marceau et al., 2003), cannabinoid CB2 receptor (Mukherjee et al., 2004), endothelin ETB (Reynolds et al., 1995), muscarinic cholinergic receptors M2 and M4 (Van Den Beukel et al., 1997) and neurokinin receptor NK1 (Aramori et al., 1994; Pradier et al., 1995), neurokinin receptor NK2 (Meini et al., 2004) and neurokinin receptor NK3 (Chung et al., 1995). Class 2 GPCRs in this category include the CGRP1...
(Hershey et al., 2005; Mallee et al., 2002), glucagon-like peptide 1 (GLP1) receptor (Tibaduiza et al., 2001) and VIP/PACAP receptor VPAC1 (Couvaineau et al., 1996). The vanilloid receptor TRPV1 (Valenzano and Sun, 2004), serotonin transporter SERT (Barker et al., 1994; Roman et al., 2004) and the kinase porphobilinogen synthase (Kervinen et al., 2001) are examples of ion channels, transporters and kinases, respectively, that show species differences in pharmacology.

Several of the targets described above have been implicated in pain transmission/sensitization/modulation. In several notable cases, novel ligands with potential analgesic properties were discovered that displayed markedly lower affinity for rodent versus human orthologs of the receptor. This precluded the use of standard rodent pain models for the preclinical assessment of analgesic efficacy in these cases. For example, the relatively low affinity of novel ligands for rat versus human orthologs of B1 (Kuduk et al., 2004), CGRP1 (Doods et al., 2000; Hershey et al., 2005), and NK1 (Rupniak et al., 1996) receptors resulted in investigators turning to pain models developed in rabbits, monkeys and gerbils, respectively, for pharmacodynamic readouts.

Rabbits share with humans neuroanatomical pathways relating to pain processing and pain modulation. Accordingly, rabbits represent a potential alternative to the high-cost, low-throughput analgesic drug discovery studies that are sometimes performed in non-human primates. However, while pain models described for monkey and gerbil have some degree of reliability, the rabbit pain models described in the literature to date are lacking in the areas of validity, reliability, throughput and truly quantifiable endpoints (see Section 3). Here, we describe a novel method for quantifying inflammatory nociception in the rabbit involving an electronic von Frey (Electrovonfrey) apparatus that addresses many of these deficiencies.

1. Methods

In the methodology presented herein, the inflammatory agent carrageenan is injected into a hind paw to induce local inflammation, in a manner similar to inflammatory pain models described for rat (Kayser and Guilbaud, 1987) and mouse (Jones et al., 2006). The Electrovonfrey apparatus then is used to quantify the degree of mechanical hyperalgesia present in the hind paw. We begin by describing the establishment of the end point methodology, followed by our evaluation of analgesics from different classes that have been shown to be effective in preventing or reversing inflammatory noceception in rodents.

1.1. Subjects

All experiments were performed at Amgen Inc. All experimental protocols were evaluated by and approved by Amgen’s Institutional Animal Care and Use Committee. Accordingly, experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Experimental subjects were male New Zealand White rabbits (Western Oregon Rabbit Company, Philomath, OR) weighing 500–700 g at the time of the experiment. Rabbits were housed three per cage on a 12 h/12 h light–dark schedule with food and water freely available. The period between arrival at our housing facility and behavioral testing was 5–7 days.

1.2. General procedure

1.2.1. Measuring baseline mechanical thresholds (prior to inflammation)

For each rabbit, the experimental procedure was performed within a single day and each rabbit was sacrificed immediately following completion of the experiment. On each experiment day, rabbits were retrieved from the housing facility and brought to a procedure room. Each rabbit was weighed and the plantar surface of its right hind paw was shaved. A small dot was placed near the center of the plantar surface using a marker. Each rabbit then was placed into an individual Plexiglas enclosure with a mesh floor and allowed to acclimate to the enclosure for 30 min.

After the 30 min acclimation period, baseline punctate withdrawal thresholds were determined for each animal. An automated von Frey apparatus was used (Electrovonfrey; IITC Life Sciences, Woodland Hills, CA), consisting of a hand-held probe unit connected to a main unit (see the IITC web site at http://www.iitcinc.com for a complete description of the apparatus). A “rigid tip” version of the apparatus was used, in which a rigid plastic tip (punctate in nature—analogue to a standard von Frey monofilament) was connected to a force transducer in the probe. Using the hand-held probe, the experimenter applied the plastic tip to the marker dot on the plantar surface in an upward motion with increasing force until a withdrawal response was observed. The withdrawal threshold (i.e., maximum tolerated punctate pressure; gram units, g) then was read from the main unit.

Three withdrawal readings were taken from the right hind paw of each rabbit, and the mean of the three readings was used as the rabbit’s baseline withdrawal threshold. A minimum interval of 2 min was used between each of the three withdrawal trials.

After completion of baseline mechanical withdrawal readings, each rabbit was removed from its Plexiglas enclosure and assigned to a treatment group. Within each treatment group, an effort was made to ensure that a normal distribution of baseline withdrawal latencies was represented (i.e., each treatment group contained rabbits with relatively low, medium and high baseline withdrawal thresholds).

1.2.2. Induction of inflammation-associated hyperalgesia

The next phase of experimentation involved induction of inflammation in a hind paw (and drug treatment, described below). Each rabbit received an injection of lambda carrageenan (1–3% in physiological saline, 100–300 μL injection volume) or its saline vehicle s.c. under the plantar surface of the right hind paw. The injection was performed such that the point of entry of the needle was remote from the marker dot but the bolus was centered under the dot. Immediately after the carrageenan injection, the rabbit was placed in a holding cage. For most of the experiments described herein, determination of punctate mechanical withdrawal threshold after inflammation induction
occurred at the 3 h time point post-carrageenan injection. As such, 2.5 h after carrageenan injection each rabbit was returned to its Plexiglas® enclosure. At 3 h post-carrageenan injection, three more mechanical withdrawal readings were obtained from the rabbit. Each reading was separated by 2 min, and the mean of the three readings was used as the rabbit’s post-carrageenan punctate mechanical withdrawal threshold (g).

For one experiment (see Fig. 4), a time-course study was carried out in which the magnitude of mechanical hyperalgesia was ascertained hourly for 8 h following injection of 3% carrageenan into a hind paw. Again, the mean of three mechanical withdrawal readings obtained at each hour served as the rabbit’s punctate mechanical withdrawal threshold at that time point.

1.2.3. Drug treatments

Within the basic testing paradigm described above, drug treatments were given in an attempt to either (1) prevent the development of hyperalgesia, or (2) reverse an already established hyperalgesia. The time point of administration of a particular drug relative to induction of inflammation depended on (1) the goal of the experiment (i.e., preventing vs. reversing hyperalgesia) and (2) pharmacokinetic factors relating to the drug in question. The exact time points of administration of the different drugs tested are addressed in the next section.

In all cases, the experimenter operating the Electrovonfrey® device (i.e., taking the withdrawal threshold readings) was blind with respect to the treatment conditions of individual rabbits.

1.3. Drug administration

Indomethacin (a non-steroidal anti-inflammatory drug or NSAID) and HOE 140 (peptide antagonist for the bradykinin B2 receptor) were administered at the same time as carrageenan in an attempt to prevent the development of hyperalgesia. As such, indomethacin was administered orally immediately prior to carrageenan injection. HOE 140 was administered s.c. under the plantar surface of the hind paw simultaneously with carrageenan.

Morphine sulfate (the prototypical opioid analgesic), [des-Arg10,Leu7]-kallidin (DALK; peptide antagonist for the bradykinin B1 receptor) and compound A (small molecule antagonist of the bradykinin B1 receptor) were administered after the carrageenan injection (i.e., post-carrageenan) in an attempt to reverse an established hyperalgesia. As such, morphine sulfate and DALK were administered subcutaneously (s.c.) 2.5 h post-carrageenan (i.e., 30 min prior to the 3 h post-carrageenan mechanical withdrawal readings) and compound A was administered s.c. 2 h post-carrageenan.

Additional information relating to drugs tested (vehicles, injection volumes, etc.) is located below in Section 1.6.

1.4. Blood sample collection

In the compound A experiment, blood samples also were collected from all rabbits tested. After taking the final behavioral reading, a butterfly catheter (23 gauge) was inserted into the marginal vein of one ear. Approximately 1 mL of blood was collected into a heparinized tube. Approximately 200 μL of plasma was obtained by immediate centrifugation of 400 μL whole blood at 14,000 × g for 10 min at 4°C. The plasma samples were submitted for analytical determination of the plasma concentration of compound A (see below).

1.5. Analysis of plasma samples (compound A experiment)

Rabbit plasma samples (60 μL) were extracted using protein precipitation to isolate the analyte and internal standard (IS). Extracted samples were separated by reverse-phase liquid chromatography on a Phenomenex C18 2.0 mm × 50 mm (5 μm) column. The reagents were water (mobile phase A) and methanol (mobile phase B). A gradient going from 10% to 70% of mobile phase B at a flow rate of 0.7 mL/min was utilized, with a total run time of 3.0 min.

Compound A concentrations were determined by LC–MS/MS using atmospheric pressure chemical ionization (APCI) with multiple reaction monitoring (MRM) in the positive ion mode. Compound A concentrations were determined by a weighted (1/√x2) linear regression of peak area ratios (peak area of compound A/IS peak area) versus the theoretical concentrations of the calibration standards.

1.6. Materials

Lambda carrageenan (Sigma–Aldrich Co.) was dissolved in 0.9% physiological saline. Morphine sulfate (Baxter Healthcare Corp.) and DALK (Midwest Bio-Tech Inc., Indianapolis, IN) were dissolved in phosphate buffered saline (PBS) and injected s.c. at a volume of 1 mL/kg. Indomethacin (Sigma–Aldrich Co.) was prepared as a suspension in 1% methylcellulose and injected p.o. at a volume of 5 mL/kg. Compound A is a novel small molecule antagonist of the bradykinin B1 receptor (synthesized by Amgen chemists) that selectively inhibits the B1 receptor in vitro with a functional IC50 of approximately 11 nM at the rabbit ortholog. It is devoid of activity at the bradykinin B2 receptor. Compound A was dissolved in 20% Captisol® (CyDex, Lenexa, KS) in water and injected s.c. at a volume of 1 mL/kg. HOE 140 (American Peptide Co., Sunnyvale, CA) was dissolved in distilled water at various concentrations and mixed with a 4% carrageenan solution at a ratio of 1:3 (final carrageenan concentration = 3%). In this way, HOE 140 could be administered locally into the paw simultaneously with carrageenan.

Calculations of doses for morphine sulfate were based on the free base equivalent. Doses of HOE 140 and DALK reported are based on the peptide content of the solid material.

1.7. Statistical analyses

The mean pre-carrageenan withdrawal threshold was subtracted from the mean post-carrageenan withdrawal threshold to provide a threshold “difference score” for each rabbit. For a particular experiment, the difference scores for rabbits in each treatment group (e.g., vehicle, doses 1–3, etc.) were averaged and the means and standard errors of the mean (S.E.M.s) plotted. Data generated from the experiments in which different carrageenan percentages or carrageenan volumes were compared...
were analyzed by a one-factor independent groups analysis of variance (ANOVA). If a significant \( F \)-ratio was observed, the ANOVA was followed by Tukey’s HSD post hoc multiple comparisons tests to compare individual groups with every other group in the experiment.

In experiments in which analgesic compounds were evaluated, dose-effect data for each compound were analyzed in two major ways. Firstly, a one-factor independent groups analysis of variance (ANOVA) was used. If a significant \( F \)-ratio was observed, the ANOVA was followed by Dunnett’s post hoc multiple comparisons tests to compare individual dosage groups with the vehicle control group. Secondly, log dose-effect relationships were analyzed using nonlinear least-squares regression (curve fitting for sigmoidal dose-response; GraphPad Prism statistical analysis and graphing program). The “bottom” of the dose-effect curve for a particular compound (on or around the mean of the vehicle control group) was unconstrained while the “top” of the dose-effect curve was constrained to a value of zero (a post-carrageenan minus pre-carrageenan “difference score” of zero represents maximal inhibition of hyperalgesia). The ED\(_{50}\) value (i.e., the dose resulting in 50% of the maximum possible anti-hyperalgesic effect) and 95% confidence intervals (CI\(_{95}\)) were calculated. A maximum effect (i.e., top of the curve) also was calculated if complete inhibition of hyperalgesia was not observed at the highest dose tested.

For compound A, additional statistical analyses were performed on data retrieved from the plasma samples. Log plasma concentrations and behavioral “difference scores” for individual animals from all dosing groups were correlated by nonlinear least-squares regression (curve fitting for sigmoidal dose-response) to generate a plasma EC\(_{50}\) (plasma concentration resulting in 50% of the maximum possible anti-hyperalgesia effect) and 95% confidence intervals (CI\(_{95}\)).

Finally, Student’s \( t \)-tests for independent groups were used to compare treatment groups in which physiological saline, not carrageenan, was administered into the paw.

2. Results

2.1. Carrageenan-induced hyperalgesia—determination of optimal carrageenan concentration

For our first experiment, we chose to compare three different percentages of carrageenan (1, 2 or 3%) and vehicle (physiological saline) with respect to magnitude of punctate mechanical hyperalgesia generated. Injection volume was kept constant at 200 \( \mu \)L. Post-carrageenan withdrawal thresholds were measured at 3 h in all cases. A one-factor ANOVA performed on mechanical threshold “difference scores” revealed a significant main effect of carrageenan percentage (\( F(3, 32) = 8.17; \ P < 0.01 \)). Post hoc Tukey’s HSD tests revealed that the difference scores associated with the carrageenan concentrations of 1, 2 and 3% were significantly different from those associated with vehicle treatment (\( P < 0.05, <0.01 \) and <0.001, respectively; see Fig. 1). Although the mean difference scores increased progressively with increasing carrageenan concentration (−22.6, −23.68 and −30.09, respectively), none of the carrageenan treatment groups was significantly different from either of the other two carrageenan treatment groups (Tukey’s HSD tests, \( P > 0.05 \); Fig. 1).

Since the mean difference score was largest in the 3% carrageenan treatment group, this concentration was judged to be the most favorable of the three. Additional analyses of the raw data in this treatment group versus the vehicle treatment group were revealing with respect to the magnitude of the hyperalgesia “window”. When punctate mechanical withdrawal thresholds were measured 3 h after hind paw injection of vehicle, thresholds were not different from those observed at baseline (68.84 ± 7.22 g vs. 63.73 ± 5.77 g, respectively, \( P > 0.05 \); Fig. 2). In contrast, when punctate mechanical withdrawal thresholds were measured 3 h after hind paw injection of 3% carrageenan, robust hyperalgesia was observed when compared...
with baseline (67.64 ± 4.97 g vs. 37.54 ± 4.25 g, respectively, 
P < 0.01; Fig. 2).

2.2. Carrageenan-induced hyperalgesia—determination of optimal carrageenan injection volume

For the next experiment, we evaluated the effect of injection volume on the magnitude of punctate mechanical hyperalgesia. Holding the concentration of carrageenan constant at 3%, we tested the magnitude of hyperalgesia injection associated with injection volumes of 100, 200 and 300 μL. A one-factor ANOVA performed on mechanical threshold “difference scores” revealed no significant main effect of injection volume (F(2, 24) = 0.087; 
P > 0.05; Fig. 3), indicating that there was no significant difference between any of the injection volumes tested. Although there were no statistically significant differences detected between the injection volumes, the injection volume of 200 μL was judged to be the most favorable of the three because its mean difference score was slightly higher than the other two (100 μL: mean = −34.6; 200 μL: mean = −35.4; 300 μL: mean = −32.85) and its standard error of the mean was slightly lower than the other two (100 μL: S.E.M. = 3.88; 200 μL: S.E.M. = 3.58; 300 μL: S.E.M. = 3.76).

2.3. Carrageenan-induced hyperalgesia—determination of the time-course of hyperalgesia

Based on the results described above, we chose 3% carrageenan at an injection volume of 200 μL to explore the time-course of punctate mechanical hyperalgesia. Either carrageenan or vehicle (physiological saline, also injected at 200 μL) was injected into the right hind paw and punctate mechanical thresholds were evaluated every hour for the next 8 h. A two-factor ANOVA performed on mechanical threshold “difference scores” revealed a significant interaction between hind paw treatment and time (F(7, 84) = 3.28; 
P < 0.01; Fig. 4). Post hoc Tukey’s HSD tests revealed that carrageenan produced significant hyperalgesia at the 3 h (P < 0.01), 4 h (P < 0.01) and 5 h (P < 0.05) time points, as compared with vehicle. The magnitude of hyperalgesia appeared to peak at the 3 and 4 h time points, with identical mean difference scores of −27.4 observed at both time points. The S.E.M. observed at the 3 h time point was slightly less than that observed at the 4 h time point (4.34 vs. 4.57, respectively). Regardless, the degrees of hyperalgesia observed at the 3, 4 and 5 h time points were not significantly different from each other (Tukey’s HSD tests, 
P > 0.05; Fig. 4). Although a small non-significant trend toward hyperalgesia remained at the 7 and 8 h time points, the magnitude was relatively small (Fig. 4).

On the basis of the results described above, the following carrageenan parameters were used for all experiments in which analgesic compounds were tested (see next sections): (1) concentration = 3%; (2) injection volume = 200 μL; (3) post-carrageenan time point for measurement of mechanical withdrawal threshold = 3 h.

2.4. Prevention of hyperalgesia

2.4.1. Indomethacin

A one-factor ANOVA performed on mechanical threshold “difference scores” revealed a significant main effect of indomethacin dose (doses tested: 0, 3, 10, 30 mg/kg; F(3, 27) = 5.903; 
P < 0.05). Post hoc Dunnett’s tests revealed that the difference scores associated with the doses of 10 and 30 mg/kg, p.o. were significantly different from those associated with vehicle treatment (P < 0.05 and <0.01, respectively) (see Fig. 5).

Nonlinear regression analysis performed on the dose-effect data showed that p.o. indomethacin dose dependently prevented the development of carrageenan-induced mechanical hyperalgesia with an ED50 of 1.62 mg/kg (CI95: 0.23–11.18). Indomethacin completely prevented the development of hyperalgesia at the highest dose tested (30 mg/kg).

Fig. 4. Time-course of hyperalgesia associated with hind paw injection of carrageenan (3%, 200 μL). The magnitude of hyperalgesia was greatest at the 3 and 4 h time points. ∗P < 0.05, ∗∗P < 0.01, one-factor ANOVA followed by Tukey’s HSD post hoc multiple comparison test, as compared with saline at the same time point.
Fig. 5. Dose-dependent reversal of carrageenan-induced punctate mechanical hyperalgesia by p.o. indomethacin. The vehicle for indomethacin was 1% methylcellulose. *P < 0.05, **P < 0.01, one-factor ANOVA followed by Dunnett’s post hoc multiple comparison test, as compared with the Vehicle (p.o.) + Carr (paw) group.

In rabbits injected with physiological saline in the hind paw, the highest dose of indomethacin tested did not increase punctate mechanical withdrawal thresholds above baseline levels (P > 0.05, Student’s t-test; Fig. 5).

2.4.2. HOE 140

A one-factor ANOVA performed on mechanical threshold “difference scores” revealed a significant main effect of local HOE 140 dose (local doses tested: 4.13, 12.38, 41.25 μg; F(3, 27) = 5.019; P < 0.05). Post hoc Dunnett’s tests revealed that the difference scores associated with the local (hind paw) doses of 12.36 and 41.2 μg were significantly different from those associated with vehicle treatment (P < 0.05) (see Fig. 6).

Nonlinear regression analysis to calculate ED$_{50}$ was not performed on the HOE 140 data. The highest dose of HOE 140 tested resulted in sub-maximal prevention of hyperalgesia (Fig. 6). Since it was not possible to test higher doses of HOE 140 due to solubility issues, it was concluded that nonlinear regression would not yield a meaningful ED$_{50}$ value.

In rabbits injected with physiological saline in the hind paw, the highest dose of HOE 140 tested did not increase punctate mechanical withdrawal thresholds above baseline levels (P > 0.05, Student’s t-test; Fig. 6).

2.5. Reversal of hyperalgesia

2.5.1. Morphine sulfate

A one-factor ANOVA performed on mechanical threshold “difference scores” revealed a significant main effect of morphine dose (doses tested: 0.03, 0.1, 0.3, 1 mg/kg; F(3, 19) = 10.5; P < 0.05). Post hoc Dunnett’s tests revealed that the difference scores associated with the dose of 1 mg/kg, s.c. were significantly different from those associated with s.c. vehicle treatment (P < 0.01) (see Fig. 7).

Nonlinear regression analysis performed on the dose-effect data showed that s.c. morphine dose dependently reversed carrageenan-induced mechanical hyperalgesia with an ED$_{50}$ of 0.11 mg/kg (CI$_{95}$: 0.03–0.43). Morphine completely reversed the hyperalgesia at the highest dose tested (1 mg/kg).

Fig. 6. Dose-dependent reversal of carrageenan-induced punctate mechanical hyperalgesia by s.c. HOE 140. The vehicle for HOE 140 was distilled water. *P < 0.05, one-factor ANOVA followed by Dunnett’s post hoc multiple comparison test, as compared with the Vehicle (s.c.) + Carr (paw) group.

Fig. 7. Dose-dependent reversal of carrageenan-induced punctate mechanical hyperalgesia by s.c. morphine sulfate. The vehicle for morphine sulfate was PBS. **P < 0.01, one-factor ANOVA followed by Dunnett’s post hoc multiple comparison test, as compared with the Vehicle (s.c.) + Carr (paw) group.
In rabbits injected with physiological saline in the hind paw, the highest dose of morphine tested did not increase punctate mechanical withdrawal thresholds above baseline levels ($P>0.05$, Student’s $t$-test; Fig. 7).

### 2.5.2. \{des-Arg$^{10}$,Leu$^{9}$\}-kallidin (DALK)

A one-factor ANOVA performed on mechanical threshold “difference scores” revealed a significant main effect of DALK dose (doses tested: 0.1, 0.3, 1, 3, 10, 30 mg/kg; $F(5,46)=4.95; P<0.05$). Post hoc Dunnett’s tests revealed that the difference scores associated with the doses of 0.3, 1, 3, 10, and 30 mg/kg, s.c. were significantly different from those associated with vehicle treatment ($P<0.05$ and $<0.01$, respectively) (see Fig. 8).

Nonlinear regression analysis performed on the dose-effect data showed that s.c. DALK dose dependently reversed carrageenan-induced mechanical hyperalgesia with an $ED_{50}$ of 0.45 mg/kg (CI$_{95}$: 0.17–1.12). Despite being highly efficacious, DALK did not appear to completely reverse hyperalgesia in all animals at the highest dose tested (30 mg/kg). When difference scores were converted to %MPE values (% of maximum possible effect; 100% efficacy was defined as a “difference score” of zero), the maximal anti-hyperalgesic effect of DALK calculated by a regression analysis was 83% (CI$_{95}$: 63–100%).

In rabbits injected with physiological saline in the hind paw, the highest dose of DALK tested did not increase punctate mechanical withdrawal thresholds above baseline levels ($P>0.05$, Student’s $t$-test; Fig. 8).

### 2.5.3. Compound A

The structure of compound A is shown in Fig. 9. A one-factor ANOVA performed on mechanical threshold “difference scores” revealed a significant main effect of compound A dose (doses tested: 10, 15, 30, 100 mg/kg; $F(4,31)=3.103; P<0.05$). Post hoc Dunnett’s tests revealed that the difference scores associated with 30 and 100 mg/kg, s.c. were significantly different from those associated with vehicle treatment ($P<0.05$ and $<0.01$, respectively) (see Fig. 10a).

Nonlinear regression analysis performed on the dose-effect data showed that s.c. compound A dose dependently reversed carrageenan-induced mechanical hyperalgesia with an $ED_{50}$ of 20.19 mg/kg (CI$_{95}$: 4.24–96.13). Compound A completely inhibited the hyperalgesia at the highest dose tested (100 mg/kg, s.c.; Fig. 10a). The pharmacokinetic data for compound A revealed dose-dependent increases in plasma concentration (Fig. 10b) and a plasma EC$_{50}$, with respect to efficacy, of 402.6 nM (CI$_{95}$: 50.1–3235.0).

### 2.6. Power analyses

For our final statistical analyses, we performed formal power analyses on a subset of the vehicle data sets presented herein to ascertain optimal group sizes under various conditions. In these analyses, vehicle data were taken from four experiments (the experiments in which analgesics were tested) and combined to create a pooled overall mean “difference score” and pooled standard deviation (S.D.) associated with the difference scores. The overall mean “difference score” was $-31.2$ and the associated S.D. was 16.9. The goal was to find the group size needed to show a 60%, 70%, 80% or 90% change from vehicle with power fixed at 80% or 90% when comparing 1, 2, 3 or 4 dose groups to the vehicle control group (e.g., ANOVA followed by Dunnett’s post hoc test). The S.D. was taken to be 16.9, the control value was rounded to $-30$, and the 60%, 70%, 80%, and 90% change values were taken as $-12$, $-9$, $-6$ and $-3$, respectively. All calculations were done using SAS V9.1.3 on a Windows Professional operating system. SAS “proc glm” was used to find the overall mean and pooled S.D., and SAS “proc power” was used to find the sample sizes. The results are presented in Table 1.

The results of the power analysis suggest, for example, that if one designs a series of experiments in which three doses of compound are evaluated against a vehicle control group and, each
Fig. 10. Dose-dependent reversal of carrageenan-induced punctate mechanical hyperalgesia by s.c. compound A. The vehicle for compound A was 20% Captisol® in water. *P < 0.05, **P < 0.01, one-factor ANOVA followed by Dunnett’s post hoc multiple comparison test, as compared with the Vehicle (s.c.) + Carr (paw) group.

Table 1

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</tbody>
</table>

Information on dose group contains 10 rabbits, then a reduction of inflammation-associated hyperalgesia by 90% in any one of the dose groups (i.e., increasing the mean “difference score” from $-30 \pm S.E.M.$ to $-3 \pm S.E.M.$) will be statistically significant 80% of the time (see Table 1).

3. Discussion

The present data show that the Electrovonfrey® device (IITC Life Sciences) can be used to quantify punctate mechanical hyperalgesia in the inflamed hind paw of the conscious rabbit. The data generated by the device are pressure values (in gram units) and, as such, qualify as ratio-scale data. Accord-ingly, parametric statistical analyses (e.g., standard ANOVA, regression analyses) can be performed on the data. The results of our experiments illustrated in Figs. 1−4 suggested that the following carrageenan parameters resulted in optimal hyperalgesia for drug testing purposes: (1) concentration = 3%; (2) injection volume = 200 μL; (3) post-carrageenan time point for measurement of mechanical withdrawal threshold = 3 h. As can be seen from all experiments presented herein, the magnitude of hyperalgesia associated with these carrageenan parameters generally falls within the 30−40 g range in rabbits that are not treated with analgesic agents (Figs. 1, 3−8 and 10).

Also, it is clear from the present results that the variability of the behavioral data associated with the Electrovonfrey® methodology is low enough to permit the generation of reliable dose-effect functions in the rabbit across multiple classes of analgesic agents and multiple routes of administration. When administered at the same time as hind paw carrageenan, systemically administered indomethacin (Fig. 5) or local HOE 140 (Fig. 6) prevented the development of carrageenan-induced hyperalgesia in a manner similar to effects of these compounds on inflammatory nociception in the rat (Belichard et al., 2000; Buritova et al., 1997; Francischi et al., 2002). Additionally, when administered 2−2.5 h after carrageenan administration, systemically administered morphine sulfate (Fig. 7), DALK (Fig. 8) or compound A (Fig. 10) reversed established carrageenan-induced mechanical hyperalgesia. Like indomethacin and HOE 140, the present effects of morphine and DALK mirror the effects of these compounds on inflammatory nociception in the rat (Belichard et al., 2000; Whiteside et al., 2005). In summary, the ED$_{50}$ values
for the reference analgesics reported here are similar to those reported in rat models of inflammatory hyperalgesia in which classical methods of measuring mechanical hyperalgesia (e.g., the Randall–Selitto method) are employed. A similar comparison between rabbit and rat cannot be made for compound A because this compound belongs to a class of molecules that shows very low affinity for rodent orthologs of the bradykinin B1 receptor. Thus, evaluation of compound A’s efficacy in rodent pain models was deemed impractical.

In addition to proving reliable for the generation of dose-effect functions for analgesic compounds in rabbits, the Electrovonfrey® methodology appears to be sensitive enough to allow determination of plasma concentration-effect functions (i.e., pharmacokinetic–pharmacodynamic (PK–PD) relationships) (Fig. 10). In addition to compound A, we have tested other, related small molecules and seen similar PK–PD relationships (data not shown). Our review of the literature suggests that this is the first rabbit pain model with sufficient end point resolution to allow this sort of analysis.

The model described herein adds to a relatively small collection of reports describing other pain models developed in the rabbit. These models are summarized in Table 2 and fall into the broad categories of inflammatory pain, visceral pain and acute pain. To date, there is no published report indicating the presence or absence of “gross, purposeful movement”. The MAC served as the quantifiable end point that permitted measurement of an analgesic drug effect. If the presence of a drug decreased the MAC of halothane seen at the time the behavioral response occurred, the authors concluded that the drug was producing analgesia (presumably the analgesic effects of the drug were compensating for the lower concentration of halothane).

Another rabbit inflammatory pain model has been described in which decerebrate rabbits receive an injection of complete Freund’s adjuvant (CFA) into a hind paw (Kuduk et al., 2004; Su et al., 2003). Like the Antognini (1993) model, nociceptive stimulation in this model is mechanical in nature and is described as “noxious pinch”. The investigators employ a quantifiable nociception-related end point that is electrophysiological, not behavioral, in nature. Briefly, the investigators measure single, motor unit responses in the biceps femoris/semitendinous muscle upon pinch of the inflamed hind paw. The investigators label an increase in motor unit action potentials a “nociceptive reflex response”, suggesting that the increase in motor unit activity is a consequence of nociceptive transmission in primary afferent nociceptors. Presumably, noxious pinch of the inflamed hind paw produces a larger number of motor unit action potentials than does noxious pinch of a non-inflamed paw and compounds can be evaluated for anti-hyperalgesic efficacy within this paradigm. Although the investigators indeed may be measuring a response that reflects nociception, nociception per se is not measured and the decerebrate nature of these rabbits makes it possible that normal spinal cord processing of nociceptive reflexes is imbalanced as compared with normal animals. This is due to the absence of pain modulatory circuitry that descends to the spinal dorsal horn from the rostral ventromedial medulla and elsewhere. In the normal animal, descending pain modulatory circuitry profoundly influences the “set point” of neural circuits in the spinal cord that mediate simple nociceptive reflexes as well as the transmission of nociceptive signals to higher brain centers (Mason, 2005). At a minimum, decerebration might result in ED50’s for analgesics that are markedly different from those seen in the awake, conscious rabbit; in support of this notion, interruption of descending pain modulatory circuitry in rats by spinal transaction results in an increase in the potency of clonidine in the tail flick test (Advokat, 2002).

The rabbit inflammatory pain model described herein offers several distinct advantages relative to the models described above. First, our rabbits are conscious and unrestrained (as opposed to anesthetized or decerebrated), making it likely that nociceptive processing is closer to normal in these rabbits as compared with rabbits in the other models. Second, a quantifiable behavioral end point is measured that, in the presence of inflammation, directly reflects a sensitization of nociceptive processing and indicates the presence of robust punctate mechanical hyperalgesia. This end point shows acceptable variability within treatment groups and allows the generation of reliable dose-effect and plasma concentration-effect curves for analgesics of multiple classes. Third, the throughput/efficiency of the present model is much higher than other rabbit pain models. While the nature of the models described above permits a throughput of, at most, 2–3 animals per day per investigator, the present model has a throughput of 12+ animals per day per investigator.

The other rabbit pain models described in the literature fall under the categories of visceral and acute pain (see Table 2). A visceral pain model has been described that involves electrical stimulation of the splanchnic nerve (Cai et al., 1994; Hu et al., 1994). The investigators determine the current necessary to observe an “obvious behavioral response” and then measure how much the current requirement increases in the presence of an analgesic. The acute pain models summarized in Table 2 involve a range of nociceptive stimulation parameters that fall under the categories of thermal, mechanical, chemical and electrical. The regions of the body stimulated include the hind paw, fore paw, ear, tooth pulp and cheekbone. The behavioral end points employed include some that are described in somewhat vague terms and are difficult to quantify (e.g., “signs of discomfort”, “gross, purposeful movement”, “escape movement response”, “vigorous attempt to remove the paw”) and more specific, quantifiable responses (“lick/chew response”, “ear flick”). All of the models summarized in Table 2 suffer in one respect or another.
<table>
<thead>
<tr>
<th>Report(s)</th>
<th>Inflammation present?</th>
<th>Nociceptive Stimulus</th>
<th>Region stimulated</th>
<th>Anesthesia or decerebration used?</th>
<th>Restraint employed (conscious rabbits)?</th>
<th>Behavioral end point(s) measured</th>
<th>End point quantified</th>
<th>Throughput</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory pain: mechanical</strong></td>
<td></td>
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</tr>
<tr>
<td>Dong et al. (2004)</td>
<td>Yes: carrageenan in hind paw</td>
<td>Mechanical: electrovonfrey</td>
<td>Hind paw</td>
<td>No</td>
<td>No</td>
<td>Paw withdrawal</td>
<td>Force (g) required to elicit paw withdrawal</td>
<td>High</td>
</tr>
<tr>
<td>Antognini (1993)</td>
<td>Yes: carrageenan injected in tail</td>
<td>Mechanical clamp (hemostat)</td>
<td>Tail</td>
<td>Yes: halothane</td>
<td>n/a</td>
<td>“Gross, purposeful movement”</td>
<td>Minimum halothane concentration required</td>
<td>Low</td>
</tr>
<tr>
<td>Su et al. (2003), Kaduk et al. (2004)</td>
<td>Yes: complete Freund’s adjuvant (CFA) injected into hind paw</td>
<td>Mechanical: pinch</td>
<td>Hind paw</td>
<td>Yes: decerebration</td>
<td>n/a</td>
<td>Motor unit responses in biceps femoris/semitendinous muscle</td>
<td>Number of action potentials during stimulus</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Inflammatory pain: chemical</strong></td>
<td></td>
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<tr>
<td>Lombeck and Juan (1974), Schweizer et al. (1984), Schweizer and Brom (1985)</td>
<td>Strictly, no, but intra-arterial injection of inflammatory mediators</td>
<td>Chemical: prostaglandins, bradykinin, acetylcholine</td>
<td>Perivascular nociceptors in ear</td>
<td>Yes: sodium pento-barbitone</td>
<td>Yes</td>
<td>“Head flick” response of facial musculature</td>
<td>Quantal: response occurred or not</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Visceral pain</strong></td>
<td></td>
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<tr>
<td>Cai et al. (1994), Hu et al. (1994)</td>
<td>No</td>
<td>Electrical stimulation</td>
<td>Splanchnic nerve</td>
<td>No</td>
<td>Yes</td>
<td>“Obvious behavioral response”</td>
<td>Current required to elicit behavioral response</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Acute pain: thermal</strong></td>
<td></td>
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<tr>
<td>Williams et al. (1986)</td>
<td>No</td>
<td>Thermal: heat lamp</td>
<td>Ear</td>
<td>No</td>
<td>Yes</td>
<td>Removal of ear from heat source</td>
<td>Latency to remove ear</td>
<td>Medium</td>
</tr>
<tr>
<td>Hughes et al. (1993), Doherty et al. (1995)</td>
<td>No</td>
<td>Mechanical clamp (hemostat)</td>
<td>Hind paw</td>
<td>No</td>
<td>Yes</td>
<td>“Signs of discomfort”</td>
<td>Latency to the onset of “signs of discomfort”</td>
<td>Low</td>
</tr>
<tr>
<td>Hu et al. (1996)</td>
<td>No</td>
<td>Mechanical: pressure algometer</td>
<td>Hind paw</td>
<td>No</td>
<td>Yes</td>
<td>“Vigorous attempt to remove the paw”</td>
<td>Pressure (kPa) at which the response is elicited</td>
<td>Medium</td>
</tr>
<tr>
<td>Mason et al. (2002)</td>
<td>No</td>
<td>Mechanical: paw pinch</td>
<td>Hind paw</td>
<td>Yes: decerebration</td>
<td>n/a</td>
<td>Motor unit responses in biceps femoris/semitendinous muscle</td>
<td>Number of action potentials during stimulus</td>
<td>Low</td>
</tr>
<tr>
<td>Hayashida et al. (2003a,b)</td>
<td>No</td>
<td>Mechanical clamp (hemostat)</td>
<td>Fore paw</td>
<td>No</td>
<td>Yes</td>
<td>“Gross purposeful movement”</td>
<td>Number of rabbits showing “gross purposeful movement”</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Acute pain: electrical stimulation</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Wang and Xu (1993)</td>
<td>No</td>
<td>Electrical stimulation</td>
<td>Ear</td>
<td>No</td>
<td></td>
<td>“Ear flick” or rubbing ear with fore paw</td>
<td>Current required to elicit response(s)</td>
<td>Medium</td>
</tr>
<tr>
<td>Shepheard et al. (1999)</td>
<td>No</td>
<td>Electrical stimulation</td>
<td>Hind paw</td>
<td>Yes: decerebration</td>
<td>n/a</td>
<td>Motor unit responses in biceps femoris/semitendinous muscle</td>
<td>Number of action potentials during stimulus</td>
<td>Low</td>
</tr>
<tr>
<td>Hayashida et al. (2003a,b)</td>
<td>No</td>
<td>Electrical stimulation</td>
<td>Fore paw</td>
<td>No</td>
<td>Yes</td>
<td>(1) “Head lift” response; (2) “escape movement” response</td>
<td>Voltage required to evoke behavioral responses</td>
<td>Low</td>
</tr>
</tbody>
</table>
from technical complexity (e.g., complicated surgery), low efficiency/throughput and low end point resolution (i.e., difficulty in reliably generating dose-effect functions for analogesics). Furthermore, the use of restraint in all of the models in which conscious rabbits are used introduces the potential confounding factors of stress-induced hypoalgesia or stress-induced hyperalgesia (Manning, 2004). These factors can interfere with the detection of, or confound the interpretation of, apparent analgesic effects of drugs.

In summary, we have developed a novel inflammatory pain model in the rabbit that involves hind paw injection of carrageenan and use of an electronic von Frey device to quantify inflammation-associated mechanical hyperalgesia. The model is reliable, efficient, has a relatively high throughput capacity, does not involve the use of general anesthetics or decerebration and does not involve the use of prolonged restraint. The variability and resolution associated with the behavioral end point are such that generation of dose- and plasma concentration-effect functions is possible for analgesic compounds in a manner unprecedented for a rabbit pain model. This model may be useful in future analgesic drug discovery efforts in which species-specific pharmacology is a factor.

Acknowledgments

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