An electronic pressure-meter nociception paw test for rats

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Abstract

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The objective of the present investigation was to compare the sensitivity of an electronic nociceptive mechanical paw test with classical mechanical tests to quantify the intensity variation of inflammatory nociception. The electronic pressure-meter test consists of inducing the hindpaw flexion reflex by poking the plantar region with a polypropylene pipette tip adapted to a hand-held force transducer. This method was compared with the classical von Frey filaments test and with the rat paw constant pressure test, a modification of the Randall and Selitto test developed by our group. When comparing the three methods, the electronic pressure-meter and the rat paw constant pressure test, but not the von Frey filaments test, detected time *vs* treatment interactions in prostaglandin E_2 (PGE_2)-induced hypernociception. Both methods also detected the PGE_2-induced hypernociception in dose- (50-400 ng/paw) and time- (1-4 h) dependent manners, and time *vs* treatment interactions induced by carrageenin (25-400 μg/paw). Furthermore, the electronic pressure-meter test was more sensitive at early times, whereas the constant pressure test was more sensitive at later times. Moreover, the electronic pressure-meter test detected the dose-dependent antinociceptive effect of local indomethacin (30-300 μg/paw) and dipyrone (80-320 μg/paw) on carrageenin- (200 μg/paw) and PGE_2- (100 ng/paw) induced hypernociception, respectively, and also detected the ineffectiveness of indomethacin (300 μg) on the effect of PGE_2. Our results show that the electronic pressure-meter provides a sensitive, objective and quantitative mechanical nociceptive test that could be useful to characterize new nociceptive inflammatory mediators and also to evaluate new peripheral analgesic substances.

Introduction

The objective of the present investigation was to compare the sensitivity of an electronic nociceptive mechanical paw test (electronic pressure-meter) with the classical von Frey filaments test (1) and with our modification of the Randall and Selitto test (2) to quantify variations of inflammatory nociception. In recent years, classical von Frey filaments have become popular among mechanical tests applied to rats (3-5), although

this test presents some disadvantages such as the large number of attempts required to evaluate the nociceptive threshold and the problems with the standardization of the filaments (6). The electronic pressure-meter has been previously used in humans (7) and rats (8). It is an adaptation of the classical von Frey filaments test in which the pressure intensity is recorded automatically after paw removal.

The Randall and Selitto nociceptive test (9) and the writhing test have been used extensively for the development of nonsteroidal anti-inflammatory drugs. The modification of the Randall and Selitto nociceptive test developed by our group uses a small constant pressure and a different behavioral response (freezing reaction) as an end-point (2). This test was instrumental in the discovery of various seminal findings, which were later confirmed by other nociceptive methods such as formalin-induced flinches (10,11), chemically induced writhing (12,13) and the classical Randall and Selitto method (14). In fact, the modified Randall and Selitto test was used in several pioneering studies of inflammatory nociceptor sensitization (hypernociception): the participation of the $cAMP/Ca^{2+}$ pathway in the mechanism of hypernociception (15), the peripheral effect of opiates (16), the cytokine cascade involved in the onset of inflammatory hypernociception (17-24), the peripheral memory of nociceptor sensitization (25,26), and the spinal retrograde sensitization of primary sensory neurons (27).

Compared with chemical tests (acetic acid writhing test and formalin test), mechanical tests have an important practical advantage by allowing the dissociation between the nociceptor sensitization (by an injection of a phlogogen or of a specific inflammatory mediator) and the overt behavioral end-point induced by the mechanical stimulus (28).

In order to evaluate the sensitivity of the electronic pressure-meter test, we sensitized

or induced an inflammatory response in the paws with different doses of prostaglandin E_2 (PGE_2) or with carrageenin, respectively. PGE_2-induced hypernociception was compared to the von Frey filaments test and our modification of the Randall and Selitto test. Carrageenin-induced hypernociception was compared to the modification of the Randall and Selitto test. We also determined if the pressure-meter test was able to detect the qualitative difference previously observed with the constant pressure test in the effect of indomethacin and dipyrone on carrageenin- and PGE_2-induced hypernociception.

Material and Methods

Animals

The experiments were performed on male Wistar rats weighing 180 to 200 g (University of São Paulo, Ribeirão Preto, SP, Brazil) housed in the animal care facility of the School of Medicine of Ribeirão Preto and taken to the testing room at least 1 h before the experiments. Food and water were available *ad libitum*. All behavioral testing was performed between 9:00 am and 4:00 pm and the animals were used only once. Animal care and handling procedures were in accordance with the guidelines of the International Association for the Study of Pain (IASP) on the use of animals in pain research. All efforts were made to minimize the number of animals used and their discomfort.

von Frey filaments and electronic pressure-meter paw tests for rats

Rats were placed in acrylic cages (12 x 20 x 17 cm high) with a wire grid floor (Figure 1, panel A), 15-30 min before the beginning of the tests in a quiet room. During this adaptation period the paws were poked 2-3 times. Before paw stimulation, the animals were quiet, without exploratory defecation

or urination movements and not resting over the paws. In these experiments, either a series of von Frey filaments (Stoelting, Chicago, IL, USA) with logarithmically increasing stiffness (-2.35 to 2.65 log of force, g) or the pressure-meter, which consisted of a hand-held force transducer fitted with a 0.7 mm^2 polypropylene tip (electronic von Frey anesthesiometer, IITC Inc., Life Science Instruments, Woodland Hills, CA, USA), were used. The investigator was trained to apply the filaments or the polypropylene tip perpendicularly to one of the five distal footpads with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal's hindpaw (Figure 1, panel B). The tests consisted of poking the hindpaw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. Each von Frey filament was applied for approximately 3-4 s to induce the endpoint reflex. Testing was initiated with the filament handle marked 5.46, which corresponds to 1.46 log of force (g), which is in the middle of the filament series. The response to this filament defines if a series of a weaker or a stronger filament will be tested. The weakest filament able to elicit a response was considered to be the mechanical threshold (g).

The results are reported as Δ log of force (g) which was calculated by subtracting the value of the measurements (log of force) after treatment from that of the first measurement (before treatment). With the electronic pressure-meter, the intensity of the stimulus was automatically recorded when the paw was withdrawn. The equipment was calibrated to determine the pressure linearly until 80 g. The stimulation of the paw was repeated until the animal presented three similar measurements (the difference between the highest and the lowest measurement should be less than 10 g). The animals were tested before and after the treatments and the results are reported as the Δ withdrawal threshold (g), which was calculated by subtracting the value of the measurements after the

treatments from that of the first measurement (before treatment).

Rat paw constant pressure test
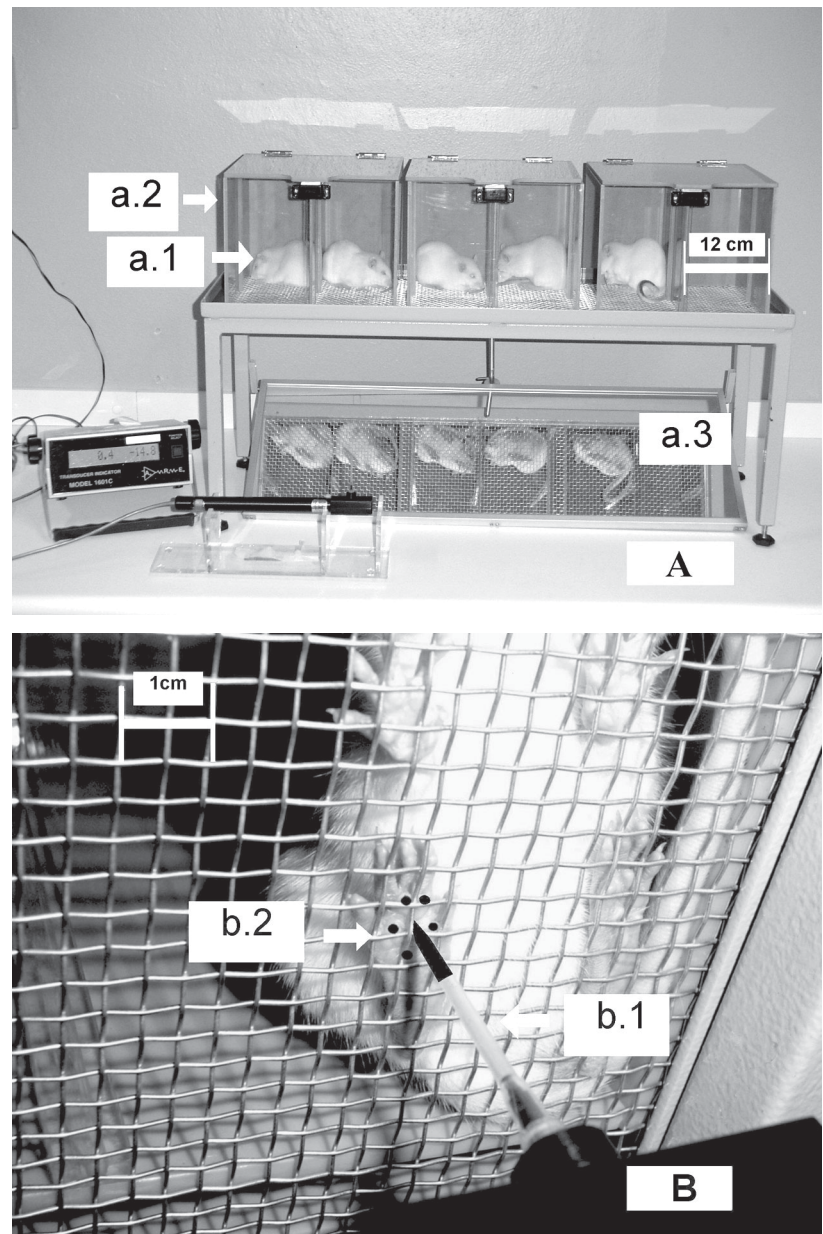
Paw sensitivity was also measured using

Figure 1. Apparatus for the electronic pressure-meter test and the area to which the polypropylene tip should be applied. *Panel A*: Rats (a.1) were placed in acrylic cages (a.2; 12 x 20 x 17 cm high) with a wire grid floor. A tilted mirror (a.3) below the grid provided a clear view of the animal's hindpaw. *Panel B*: A 0.7 mm^2 polypropylene tip (b.1) fitted to a hand-held force transducer was applied perpendicularly among the five distal footpads (b.2; black dots).

the rat paw constant pressure test, which is a modification of the Randall and Selitto test (2). In this method, a constant pressure of 20 mmHg was applied via a syringe piston moved by compressed air to an area of 15 mm^2 of the dorsal surface of the rat paw, and discontinued (reaction time) when the animal exhibited a typical freezing reaction. The freezing reaction was indicated by brief apnea, concomitant with a retraction of the head and forepaws and a reduction in the escape movements that animals may make in order to escape from the position imposed by the hands of the experimenter. Usually, apnea was associated with successive waves of muscular tremor. For each animal, the latency to the onset of the freezing reaction (from the time of first pressure application) was measured before and after administration of the agents. The results are reported as the Δ reaction time which was calculated by subtracting the value of the measurements during the experiment from that of the first measurement (before treatment).

Drugs

Dipyrone and PGE$_2$ were purchased from Sigma (St. Louis, MO, USA). Carrageenin

was obtained from FMC Corporation (Philadelphia, PA, USA) and indomethacin from Prodome Química e Farmacêutica (São Paulo, SP, Brazil).

Carrageenin and dipyrone were diluted in sterile saline. A stock solution of PGE$_2$ was prepared in 10% ethanol, and further dilutions were made in saline; the final concentration of ethanol was 1%. Indomethacin was diluted in Tris-HCl buffer, pH 8.0, which was administered alone to the control groups.

Drug administration

Drugs were injected subcutaneously in a 50-µl volume into the plantar region of rats. A 26-G hypodermic needle was inserted into the skin of the second footpad (to avoid back flow) and the tip of the needle was placed among the five distal footpads, at the same site where filaments or the tip of the pressure-meter were applied.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times. The factors analyzed were treatments, time and time *vs* treatment interac-
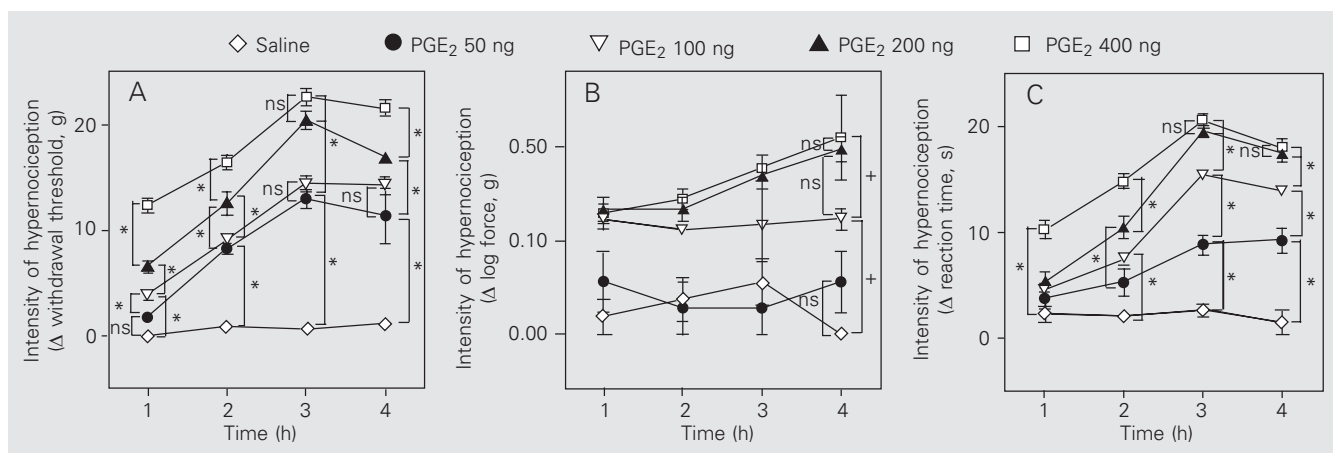

Figure 2. Dose-response curve for the hypernociception induced by intraplantar prostaglandin E$_2$ (PGE$_2$) in rats. Saline was injected in the control group. The animals were tested with the electronic pressure-meter paw test (A), von Frey filaments (B) and the rat paw constant pressure test (C). The results are reported as the mean ± SEM of 4-6 animals per group. *P < 0.05 (one-way ANOVA followed by the Tukey test) indicates significantly different time points (A and C). +P < 0.05 (one-way ANOVA followed by the Tukey test) indicates a significant difference between curves (B). ns indicates no statistically significant differences.

tion. When there was a significant time *vs* treatment interaction, one-way ANOVA followed by the Tukey test was performed for each time in order to distinguish dose effects. For nonsignificant time *vs* treatment interaction curves, the mean of repeated measures at different times for each animal was calculated and one-way ANOVA followed by the Tukey test was used to compare the doses. These same statistical tests were used for dose-response curves for a single time point. Results of statistical tests with $P < 0.05$ were considered to be significant.

Results

Comparison of the mechanical hypernociception induced by intraplantar injections of PGE$_2$ using the electronic pressure-meter test, the von Frey filaments test and the rat paw constant pressure test

Figure 2 compares the sensitivity of the electronic pressure-meter, the von Frey filaments and the rat paw constant pressure tests in detecting the hypernociception induced by intraplantar injection of PGE$_2$ (50, 100, 200 and 400 ng). Panels A, B and C in Figure 2 show a dose-dependent hypernociceptive effect after intraplantar injection of PGE$_2$ determined by these three mechanical

nociceptive methods. Statistical analysis (ANOVA) indicated that time course interacted with treatments when the electronic pressure-meter and rat paw constant pressure tests were used (panels A and C, respectively). Both methods also detected hypernociception in a time- and dose-dependent manner. However, the von Frey filaments (panel B) detected significant differences only between curves.

Comparison of the hypernociception induced by intraplantar injections of carrageenin using the electronic pressure-meter test and the rat paw constant pressure test

Figure 3 compares the use of the electronic pressure-meter and rat paw constant pressure tests to detect the hypernociception induced by carrageenin (25, 50, 100, 200 and 400 µg). Panels A and B in the figure show a dose-dependent hypernociceptive effect detected by the electronic pressure-meter and rat paw constant pressure tests, respectively. Statistical analysis (ANOVA) indicated that time course interacted with treatments when both methods were used. The electronic pressure-meter was more sensitive than the rat paw constant pressure test at early times, whereas the rat paw constant pressure test was more sensitive at later
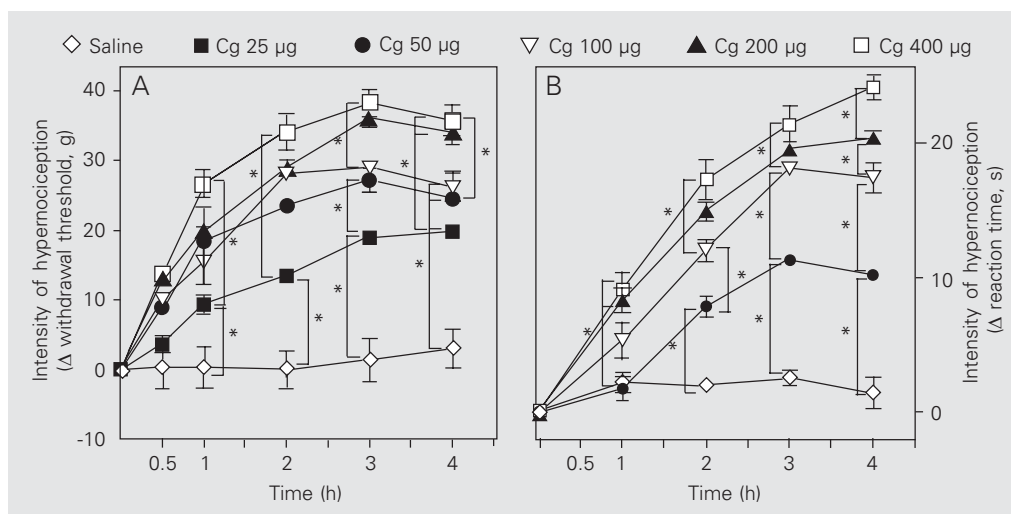

Figure 3. Dose-response curve for the hypernociception induced by intraplantar carrageenin (Cg) in rats. Saline was injected in the control group. The animals were tested with both the electronic pressure-meter paw test (A) and the rat paw constant pressure test (B). The results are reported as the mean ± SEM of 5-6 animals per group. *P < 0.05 (one-way ANOVA followed by the Tukey test) indicates significantly different time points.

times. Furthermore, the electronic pressure-meter test distinguished between the control curve and the curves of two different doses of carrageenin (200 and 400 µg), while the rat paw constant pressure test detected differences between the control and only three different doses of carrageenin (100, 200 and 400 µg) injected in the animals.

Dose-dependent antinociceptive effects of indomethacin on carrageenin-induced and of dipyrone but not of indomethacin on PGE$_2$-induced mechanical hypernociception quantified by the electronic pressure-meter test

Subcutaneous administration of indomethacin (30, 100 and 300 µg/paw) partially blocked the hypernociception induced by intraplantar injection of carrageenin (200 µg)

in a dose-dependent manner (Figure 4, panel A). The ineffectiveness of indomethacin at its maximum dose (300 µg) on PGE$_2$-induced hypernociception (100 ng; Figure 4, panel C) was detected by the electronic pressure-meter. On the other hand, the effectiveness of dipyrone (80, 160 and 320 mg/paw) in blocking PGE$_2$-induced hypernociception was detected in a dose-dependent manner. In the contralateral paw, the maximum dose of indomethacin (300 µg/paw) or dipyrone (320 µg) had no effect, excluding a systemic effect (Figure 4, panels A and B, respectively).

Discussion

In the present study, we have used hypernociception (increased nociception) to describe the behavioral response induced by the application of the von Frey filaments test, the electronic pressure-meter test and the constant pressure test. The terms allodynia and hyperalgesia describe distinct nociceptive symptoms in man (29,30). The mechanical tests have been used to measure increased experimental nociceptor sensitivity referred to either as allodynia or hyperalgesia by different investigators. In fact, thus far there is no demonstration that these symptoms describe different second messenger events in the inflammatory response. The use of the terms hypersensitivity or hyperexcitability was also avoided because they have specific meaning in immunology and electrophysiology, respectively.

Our results showed the applicability of the electronic pressure-meter test to detect nociceptor hypernociception in rats when its measurements were compared with those obtained with von Frey filaments and with the constant pressure test (our modification of the Randall and Selitto test; Ref. 2). This commercial instrument (electronic von Frey anesthesiometer) is similar to that successfully used to quantify neuropathic allodynia (8). One of the advantages of this electronic
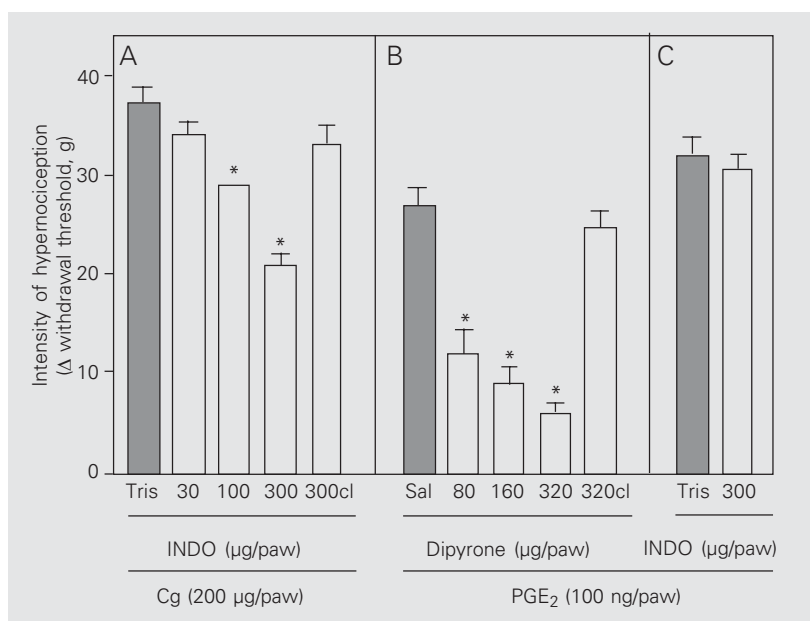

Figure 4. Effect of indomethacin on carrageenin- (Cg) and prostaglanadin E$_2$- (PGE$_2$) induced hypernociception and of dipyrone on PGE$_2$-induced hypernociception in rats. In panels *A* and *B*, animals were pretreated locally with indomethacin (INDO, 30, 100 and 300 µg in *A* and 300 µg in *C*) or Tris-HCl buffer, pH 8.0 (Tris), 30 min before injection of the hypernociceptive agent. In panel *B*, dipyrone (80, 160 and 320 µg) or saline (Sal) was injected subcutaneously into the rat paw 2 h after PGE$_2$ administration. Dipyrone (320 µg) was also injected into the contralateral (cl) paw to evaluate a possible systemic effect of the drug. Animals were tested 3 h after injection of the hypernociceptive agents. The results are reported as the mean ± SEM of 5-6 animals. *P < 0.05 compared to the respective control (one-way ANOVA followed by the Tukey test).

method over the classical von Frey filaments lies in a decrease in the number of attempts required to evaluate the nociceptive threshold and in the elimination of problems concerning the standardization of the filaments (6).

The PGE_2-induced hypernociception could be detected by the electronic pressure-meter test, von Frey filaments test and the rat paw constant pressure test. However, the von Frey filaments test did not reveal differences in time *vs* treatment interaction, which were detected by the electronic pressure-meter test and the rat paw constant pressure test. Also, the last two methods distinguished the influence of different doses on the PGE_2-induced hypernociception, but the electronic pressure-meter was more sensitive than the rat paw constant pressure test at early times, whereas the rat paw constant pressure test was more sensitive at later times.

When hypernociception was induced by carrageenin it could be detected by the electronic pressure-meter test and the rat paw constant pressure test. Moreover, both methods detected time *vs* treatment interactions in a dose- and time-dependent manner, and again, the electronic pressure-meter test was more sensitive than the rat paw constant pressure test at early times, whereas the rat paw constant pressure test was more sensitive at later times in quantifying hypernociceptive inflammatory stimuli.

Although the rat paw constant pressure test seems more discriminative in detecting differences in the effects of PGE_2 or carrageenin at later times, which are near the hypernociceptive peak, this method has a much more subjective end-point which may limit its usefulness. On this basis, it would be preferable to apply a less subjective end-point method such as the electronic pressure-meter test. These apparent discrepancies might indicate that the different tests detect the hypernociception of different sets of primary sensory neurons, which have a different time course of initiation and dura-

tion of hypernociception. In fact, using the electronic pressure-meter test, we observed that sensitization of the skin in the plantar region of the rat paw differs temporally and biochemically from that of the profound intraplantar tissues (31).

The usefulness of the electronic pressure-meter for the study of analgesia is illustrated by its ability to detect in rats the local effects of a standard COX inhibitor, indomethacin (32), and a direct blocker of hypernociception, dipyrone (33). The effect of dipyrone is mediated by the activation of the arginine/nitric oxide/cGMP pathway (34,35).

A clear temporal dissociation between nociceptor hypernociception and the behavioral response, ease of execution (clear end-point), reliability among different observers, sensitivity, reproducibility and predictivity are essential characteristics of a behavioral nociceptive test for the investigation of new analgesics. The first characteristic is quite important for investigating the contribution of the peripheral neurons to the nociceptive behavior. The use of the classical acetic acid writhing (36) or formalin (37) test does not permit the direct determination of the contribution of nociceptor sensitization to the overall nociceptive behavior. In contrast, this is a straightforward procedure with mechanical tests applied to paws pretreated with phlogogenic substances or inflammatory mediators. Stimulation with von Frey filaments has the disadvantage of activating low-threshold mechanoreceptors as well as nociceptors (28), which may be responsible for the variability of the present results, particularly when the skin of the paw is stretched with edema. This influence is probably minimized when tests are performed with anti-inflammatory drugs, which mainly affect nociceptors. One of the advantages of the electronic pressure-meter test over the von Frey filaments test may be the reduction of the variability caused by stimulating areas of different size, because increases in the diameter of the filament and the end-point are

automatically recorded (7,38). It is reasonable to assume that the electronic pressure-meter test has methodological characteristics similar to those of the other mechanical nociceptive tests, whose predictivity for development of nonsteroidal anti-inflammatory drugs should be better than that of chemical methods (28). The electronic pressure-meter test also has advantages over other mechanical tests, such as the Randall and Selitto and rat paw constant pressure tests, since it is not necessary to restrain the animals, avoiding the stress component. Furthermore, depending on the number of attempts required for an experiment, the mechanical stimulus applied by the Randall and Selitto method may be harmful to the animal inducing edema *per se*, a fact not observed with the electronic pressure-meter test.

In conclusion, we described the electronic pressure-meter test, which is a useful tool to characterize new nociceptive mediators and also to evaluate new classes of peripheral analgesics that are COX inhibitors or directly block ongoing nociceptor hypernociception.

Acknowledgments

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