ABSTRACT
We examined the current stimulus threshold in rats with the Neurometer, a device used clinically for measuring perception and pain thresholds. Although many studies have indicated the usefulness of this device in the quantification of nerve dysfunction in patients, we have found no published reports on the use of the Neurometer in animals. Transcutaneous nerve stimuli of the three sine-wave pulses produced by the Neurometer (at 2000, 250, and 5 Hz) were applied to plantar surface of rats. The intensity of each stimulation at which rats vocalized or were hardly startled was defined as the current stimulus threshold. With repeated stimulation, the thresholds were almost constant. Repeated topical application to the area around the stimulating electrode of a high concentration of capsaicin, which acts on small-diameter fibers, increased the thresholds at 250 and 5 Hz, but did not affect the 2000-Hz threshold. Intravenous morphine (2–5 mg/kg) increased all three thresholds, whereas intrathecal morphine (20 or 80 μg) increased only the 5-Hz threshold. Intravenous injection of a minor tranquilizer, diazepam, at 1 mg/kg raised the thresholds at 2000 and 250 Hz, but did not affect the 5-Hz threshold. Higher dose of diazepam increased all three thresholds. These results suggest that the Neurometer makes possible selective examination of subsets of nerve fibers that differ in diameter not only in humans but also in animals. The present study in rats, in which we established a method of measurement, may provide helpful suggestions for the interpretation of data in humans.

Materials and Methods

Animals. A total of 87 male Sprague-Dawley rats weighing 290 to 360 g was used. They were maintained on ordinary laboratory chow and tap water ad libitum in a constant 13:11-h light/dark cycle. For some experiments, a jugular vein catheter (PE-50) or an intrathecal (i.t.) catheter (Yaksh and Rudy, 1976) was inserted for drug administration under pentobarbital sodium (50 mg/kg i.p.) anesthesia. The experiments were performed at least 5 days after surgery. Each rat with an i.t. catheter was used three times at 4- or 5-day intervals. All experiments were performed in compliance with the regulations of the Animal Ethical Committee of Yamanouchi Pharmaceutical Co., Ltd.

Experimental Protocol. A small electrode (ATE1925; Neurotron Inc.) was applied to the left side of the back of the body, where the hair had been removed with an animal hair clipper, and was secured in place by wrapping with gauze. Each rat was kept in a Ballman cage (Natsume, Tokyo, Japan) suitable for light restraint. Transcutaneous nerve stimuli of each of the three sine-wave pulses (at 2000, 250, and 5 Hz) were applied to the plantar surface of the rats, using the animal response test mode of the Neurometer CPT/C (Neurotron Inc.). In this mode, the intensity of each stimulation was gradually increased automatically (increments of 0.4, 0.2, and 0.1.
mA for 2000, 250, and 5 Hz, respectively). The minimum intensity (mA) at which each rat vocalized or was hardly startled without vocalization was defined as the current stimulus threshold, at which point the stimulus was immediately stopped. The threshold at all three frequencies in each rat was determined within 2 min, with the procedure repeated at 10-min intervals. Because the threshold values obtained at first and second measurement fluctuated slightly (Fig. 1A), the values obtained after the third measurement were used in the following experiments. The value obtained at the third measurement was defined as the prevalence except for daily measurement.

In the experiment to examine day-by-day fluctuation of the current stimulus threshold, data were reported as the means of the values obtained at three consecutive (third, fourth, and fifth) measurements. Drugs were administered 10 min before the next measurement.

**Drugs.** Capsaicin (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in 50% ethanol and a 100-μl volume of solution was gradually applied to the skin of the right foot with a syringe. This treatment was repeated four times at 10-min intervals without removing the stimulating electrode. Morphine HCl (Takeda Chemical Industries, Osaka, Japan) dissolved in saline or diazepam (Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan) was intravenously administered in a cumulative manner, in volumes of 1 ml/kg followed by saline flushing for each injection. Naloxone HCl was also administered intrathecally in a volume of 5 μl, followed by 5 μl of saline to flush. Naloxone (Sigma Chemical Co., St. Louis, MO) dissolved in saline was intravenously administered. All drug doses are stated in terms of the free base.

**Statistical Analysis.** Data are expressed as means ± S.E.M. Statistical analysis of data was performed with Dunnett’s test using within subject error to conform to the repeated measurement. That is, Dunnett’s multiple comparison test was used in the following experiments. The rats showed hardly any variation in the current stimulus threshold with respective stimulations over a 3-day period (Fig. 1B).

**Effect of Topical Application of Capsaicin.** Topical repeated application of a high concentration of capsaicin (150 mM, 100 μl × 4) to the area around the stimulating electrode significantly increased the thresholds at 250 and 5 Hz, but did not affect that at 2000 Hz (Fig. 2). The maximal increases in threshold at 250 and 5 Hz were 126 ± 8 and 142 ± 14%, respectively. The threshold at 5 Hz tended to decrease 10 min after the application of capsaicin was completed.

**Effect of Morphine Injection.** Repeated i.v. injection of saline (1 ml/kg × 6) did not affect the current stimulus thresholds (Fig. 3). Intravenous morphine (2–5 mg/kg) significantly and dose dependently increased the thresholds at all frequencies (Fig. 4). At 5 mg/kg, the increase in threshold at 2000, 250, and 5 Hz was 162 ± 12, 147 ± 10, and 279 ± 45%, respectively. At 1 mg/kg, morphine tended to increase the thresholds at 250 and 5 Hz. Naloxone (1 mg/kg i.v.) completely reversed the effects of morphine (3 mg/kg i.v.) on the thresholds at all frequencies, but saline did not affect the effects of morphine (n = 10, data not shown). Intrathecal saline injection did not change the current stimulus threshold (n = 9, data not shown). Intrathecal morphine (20 μg) significantly increased the current stimulus threshold at 5 Hz, but did not affect those at 2000 and 250 Hz (Fig. 5). A higher dose (80 μg) of morphine (n = 9) did not raise the threshold at 5 Hz above that by 20 μg of morphine (154 ± 22 and 155 ± 21% for 20 and 80 μg, respectively at 40 min after administration).

**Effect of Diazepam Injection.** Intravenous diazepam at 1 mg/kg significantly increased the thresholds at 2000 and 250 Hz, but did not affect that at 5 Hz (Fig. 6). Diazepam at 3 mg/kg significantly increased the threshold at 5 Hz, as well as those at 2000 and 250 Hz. The increases in threshold at 2000, 250, and 5 Hz were 162 ± 10, 140 ± 11, and 141 ± 14%, respectively.

**Discussion**

In the present study, the current stimulus threshold was measured in rats with the Neurometer for clinical use. The Neurometer can transcutaneously stimulate nerve fibers,
with the three different sine-wave pulses (at 2000, 250, and 5 Hz) produced, providing selective stimulation for three subsets of nerve fibers that differ in diameter (Baquis et al., 1999). In human studies, it is reported that sine-wave pulses at 2000, 250, and 5 Hz primarily stimulate Aβ-, Ad-, and C-fibers, respectively (Masson et al., 1989; Masson and Boulton, 1991; Pitei et al., 1994; Liu et al., 1995; Tay et al., 1997). In the present study, we investigated whether the Neurometer also permits selective examination of three subsets of nerve fibers that differ in diameter in animals using pharmacological tools. Capsaicin is reported to act on C-fibers selectively (Yeomans et al., 1996) or both Aδ- and C-fibers (Holzer, 1991). In the present study, repeated topical application of a high concentration of capsaicin increased the thresholds at 250 and 5 Hz, but did not affect that at 2000 Hz. This indicates that pulses at 250 and 5 Hz stimulate small-diameter fibers in animals as well as in humans. It is reported that repeated topical applications of a high concentration of capsaicin selectively increase the latency of foot withdrawal responses for low rates of noxious foot heating, without affecting the high rates of heating (Yeomans et al., 1996). The present results indicate that pulses at 250 and 5 Hz stimulate a subset of nerve fibers that respond to both capsaicin and low rates of heating. Recently, a capsaicin receptor (vanilloid receptor), VR-1, and a capsaicin receptor homolog, VRL-1, were reported (Caterina et al., 1997, 1999). VR-1 is reported to be predominantly expressed in small-diameter, unmyelinated neurons (C-fibers), and to be activated by moderate thermal stimuli, protons, and capsaicin. In contrast, VRL-1 is most prominently expressed in a subset of medium- to large-diameter sensory neurons (Aδ-fibers), and is activated by high temperatures but not by capsaicin. These results seem to show that pulses at both 250 and 5 Hz stimulate small-diameter nociceptive C-fibers in the same way, but the time course of the increase in threshold at 250 and 5 Hz was different. Some reports show the possibility that VR-1 is expressed not only in C-fibers but also in Aδ-fibers (Caterina et al., 1999; Michael and Priestley, 1999) and that different vanilloid receptor subtypes exist (Helliwell et al., 1998). Taken together, these results suggest that there is one subset of nerve fibers that is stimulated by pulses at 250 Hz and another at 5 Hz, one probably consisting of Aδ-fibers and the other of C-fibers. The possibility that the properties of vanilloid receptors in these two subsets of nerve fibers are different is also indicated.

In the present study, i.t. injection of morphine, one of the most common antinociceptive drugs, specifically increased threshold at 5 Hz. The i.t. morphine dosages used in this study are considered sufficient because these doses abolish the nociceptive response or maximally inhibit C-fiber-evoked activity (Yaksh and Rudy, 1976; Jurna et al., 1996). It is reported that i.t. morphine more efficiently inhibits C-fiber-evoked response than Aδ-fiber-evoked activity, without affecting A-fiber-evoked response (Doi and Jurna, 1982; Wilcox...
et al., 1987). It is also reported that when A\(\delta\)- and C-fibers are coactivated, 20 \(\mu\)g i.t. morphine inhibits C-fiber-evoked response but not A\(\delta\)-fiber-evoked response (Doi and Jurna, 1982). In a human study with the Neurometer, epidural administration of fentanyl, an opioid receptor agonist that shows selective inhibitory effect on C-fiber reflex versus A\(\delta\)-fiber reflex (Wang et al., 1992), selectively increased the threshold at 5 Hz only (Liu et al., 1995). Based on these reports, i.t. morphine efficiently inhibits C-fiber-mediated response, moderately inhibits A\(\delta\)-fiber response, and hardly inhibits A\(\beta\)-fiber-mediated response. It is suggested that the increase in the threshold at 5 Hz with the Neurometer in rats preferably reflected the inhibition of C-fiber-mediated transmission. The ineffectiveness of i.t. morphine to the 250-Hz threshold is considered to reflect the fact that i.t. morphine does not sufficiently inhibit the response mediated by A\(\delta\)-fibers.

Intravenous morphine increased the thresholds at all frequencies. This result apparently shows that the increase in the threshold at 2000 Hz by i.v. morphine reflects the inhibition of nociceptive transmission mediated by capsaicin-insensitive fibers, such as A\(\beta\)-fibers. However, this idea is not consistent with results showing that only pulses at 250 and 5 Hz and not at 2000 Hz, cause pain sensation in humans (Liu et al., 1996; Dinh et al., 1997), and i.v. morphine does not affect A\(\beta\)-fiber-evoked activity at the spinal level in animals (Jurna and Heinz, 1979). In the present study, i.v. morphine at 1 mg/kg tended to increase the thresholds at 250 and 5 Hz. We therefore hypothesize that the increase in threshold at 2000 Hz by i.v. morphine did not reflect an antinociceptive effect. Intravenous injection of 1 mg/kg of the minor tranquilizer diazepam, which is considered to induce a sedative effect (Giusti et al., 1993) but not an antinociceptive effect, increased thresholds at 2000 and 250 Hz without affecting that at 5 Hz. It is reported that diazepam 2 or 2.5 mg/kg i.v. inhibits C-fiber-evoked activity or increases in pain threshold (Wüster et al., 1980; Jurna, 1984). The 3-mg/kg dose of diazepam that we used in this study is therefore considered to have not only a sedative but also an antinociceptive effect, and to have increased threshold at all frequencies. It is possible that the increase in threshold at 2000 and 250 Hz reflects the sedative effect of drugs, such as morphine and
diazepam. In the normal condition, Aβ-fibers contribute to the transmission of tactile sensations, in addition to nociceptive transmission, and Aβ-fibers take part in the transmission of tactile but not pain sensation (Guyton and Hall, 1996). Because morphine does not inhibit tactile sensation in humans (Reisine and Pasternak, 1995), it is suggested that the sedative effect of the drugs reduces the discomfort caused by stimulation of nerves that transmit tactile sensation in rats. Taken together, these results suggest that the increase in threshold at 2000 Hz does not reflect the inhibition of nociceptive transmission, as has been shown in human studies. From the results with capsaicin, morphine, and diazepam, the increase in threshold at 250 Hz reflects the inhibition of nociceptive and probably the other sensations, again suggesting that pulses at 250 Hz with the Neurometer in rats stimulate Aβ-fibers. Further investigation is desirable to confirm that pulses at 2000 Hz stimulate Aβ-fibers.

The maximal effect of i.v. morphine on threshold at 5 Hz is much greater than that of i.t. morphine. These results suggest the possibility that morphine acts on sites other than the spinal cord, including the supraspinal central nervous system, or that the increase in threshold at 5 Hz by i.v. morphine reflects not only the inhibition of nociceptive sensation but also the sedative effect of morphine, or that both processes take place.

In conclusion, we established a method of measuring the current stimulus threshold with the Neurometer in animals. Our study indicates that the Neurometer can provide selective examination for subsets of nerve fibers that differ in diameter not only in humans but also in animals. Further studies such as recording from nerve fibers are desirable to clarify these findings. This study in rats may be useful for understanding the different physiological characteristics of specific neurons as well as in evaluating antinoceptive chemicals, and provides information that will probably be useful to the interpretation of clinical data using the Neurometer.

References


Send reprint requests to: Dr. Tetu Kiso, Neuroscience Research, Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co. Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan. E-mail: kiso@yamanouchi.co.jp