Novel Expression of Vanilloid Receptor 1 on Capsaicin-Insensitive Fibers Accounts for the Analgesic Effect of Capsaicin Cream in Neuropathic Pain

MD HARUNOR RASHID, MAKOTO INOUE, SAORI KONDO, TOSHIKO KAWASHIMA, SHIHO BAKOSHI, and HIROSHI UEDA

Division of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan (M.H.R., M.I., S.K., T.K., H.U.); and Central Research Laboratories, Maruishi Pharmaceutical Co. Ltd., Osaka, Japan (S.B.)

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ABSTRACT

Here, we investigated the mechanism of the antihyperalgesic effect of capsaicin cream in the nerve injury-induced neuropathic pain model in mice. In naive mice, application of capsaicin cream onto footpad caused no significant changes in the thermal latency in contrast to the severe thermal hyperalgesia induced by a capsaicin ointment. On the other hand, application of the cream 3 h before test concentration dependently reversed both thermal and mechanical hyperalgesia observed after partial sciatic nerve injury in mice. In algogenic-induced nociceptive flexion (ANF) test, application of 0.1% capsaicin cream in naive mice blocked intraplantar (i.pl.) nociceptin- and ATP-induced flexion responses, whereas prostaglandin I₂ (PGI₂) agonist-induced responses were unaffected. After nerve injury PGI₂ agonist-induced flexion responses were also hypersensitized in such injured mice. Capsaicin cream completely reversed both i.pl. capsaicin- or i.pl. PGI₂ agonist-induced hyperalgesia in neonatal capsaicin-treated injured mice. Finally, novel expression of VR1 receptors on neonatal capsaicin-insensitive neurons after nerve injury was confirmed by immunohistochemistry. The newly expressed VR1 receptors after nerve injury were mainly confined to A-fibers. Together, our results suggest that novel expression of capsaicin receptors in neuropathic condition contributes to the analgesic effects of the capsacin cream.

Capsaicin, [(E)-N-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamidyl], the pungent ingredient of hot pepper, has been recognized for almost 150 years to produce pain relief (Turnbull, 1850). Capsaicin stimulates the vanilloid receptor 1 (VR1) located mainly on polymodal C-fibers and initiates a complex cascade of events, including neuronal excitation and release of proinflammatory mediators, desensitization of the receptor, and neuronal toxicity (Caterina et al., 1997). Topical application of capsaicin has been shown to be effective in a variety of chronic painful conditions, including postherpetic neuralgia (Watson et al., 1988), painful diabetic neuropathy (The Capsaicin Study Group, 1991), chronic distal painful polyneuropathy (Low et al., 1995), and surgical neuropathic pain (Ellison et al., 1997). Desensitization of the capsaicin receptors has been demonstrated to play a major role in the analgesic actions of vanilloids (Jancso and Jancso, 1949; Holzer, 1991; Szallasi and Blumberg, 1999). On the other hand, it has been hypothesized that capsaicin exerts pain relief through reversible depletion of the neurotransmitter substance P (SP) from the sensory nerve endings (Fitzgerald, 1983). However, loss of SP immunoreactivity in small-diameter dorsal root ganglion (DRG) neurons and spinal dorsal horn has been reported in many experimental neuropathy models (Garrison et al., 1993; Malmberg and Basbaum, 1998). Moreover, peripheral axotomy caused down-regulation of neuropeptide SP at the nerve ending, which also explains why SP antagonists are ineffective in neuropathic pain. Therefore, the mechanism of the analgesic effect of capsaicin cream is still poorly understood.

ABREVIATIONS: VR1, vanilloid receptor 1; SP, substance P; DRG, dorsal root ganglion; ANF, algogenic-induced nociceptive flexion; N/OFQ, nociceptin/orphanin FQ; PGI2, prostaglandin I₂; i.pl., intraplantar; PBS, phosphate-buffered saline; BK, bradykinin; ONO-54918-07, 15-cis-(4-N-propylcyclohexyl)-16,17,18,19,20-pentanor-9-deoxy-6,9α-nitriloprostaglandin F₁.
pain (Hökfelt et al., 1994). Thus, the exact mechanism of the
analgesic action of topical capsaicin in neuropathic pain state
has not been fully understood.

Recently, we developed a new technique in mice to study
the mechanism of signal transduction of different receptor
ligands at the peripheral nerve endings (Ueda, 1999). This
“peripheral nociception test or algogenic-induced nociceptive
flexion (ANF) test” has been proved to be advantageous for
the study of in vivo molecular mechanism of nociceptive
transmission (Inoue et al., 1998; Ueda, 1999; Ueda and In-
oue, 2000). With this technique, we also proposed the pres-
eence of at least three distinct types of nociceptive fibers
depending on their pharmacological sensitivity to different
peripheral receptor ligand stimuli and neonatal capsaicin
treatment, which degenerates small-diameter primary affer-
ants (Ueda et al., 2000). Utilizing the ANF test as well as
immunohistochemical study of DRG neurons, in the present
report, we studied the possible mechanism of the antihyper-
algic effect of topical capsaicin cream in mice partial sciatic
nerve injury-induced neuropathic pain model.

Materials and Methods

Animals. Male ddY mice weighing between 20 and 25 g were used
together throughout the experiments. They were kept in a room maintained
at 21 ± 2°C with free access to a standard laboratory diet and tap
water. Procedures were approved by Nagasaki University Animal
Care Committee and complied with the recommendations of the Interna-
tional Association for the Study of Pain (Zimmermann, 1983).

Drugs. The following drugs were used: nociceptin/orphanin FQ
(N/OFQ; Sawady Technology, Tokyo, Japan), AT P (Nalcaí Tesque,
Kyoto, Japan), and capsaicin (Nalcaí Tesque). ONO-54918-07 [a
stable prostaglandin E2 (PGE2) agonist; Iguchi et al., 1989] was a kind
gift from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Capsaicin
cream, base cream, and capsaicin ointment were prepared at the
Central Research Laboratories of the Muriishi Pharmaceutical Co.,
Ltd. (Osaka, Japan). The capsaicin cream labeled 0.01, 0.025, 0.05,
and 0.1% contained 0.1, 0.25, 0.5, and 1 mg of capsaicin in 1 g of
hydrophilic cream base, respectively (Minami et al., 2001). The cap-
saicin ointment (0.1%) was prepared in a conventional hydrophilic
ointment base. N/OFQ, AT P, and ONO-54918-07 were dissolved in
physiological saline. Capsaicin solution for intraplantar application
(i.pl., 1 ng/20 g) was prepared by dilution with physiological saline of
a 5 mg/ml capsaicin stock solution dissolved in 10% ethanol and 10%
Tween 80 in physiological saline. The vehicle was found to be innoc-
uous in ANF test. The cream was applied in a volume of 0.1 ml/10 g
and then gently rubbed over the mouse footpad skin. The footpad
was then covered with adhesive tape to prevent the mice from licking
off the cream.

Partial Ligation of Sciatic Nerve. Partial ligation of the sciatic
nerve of the mice was performed under pentobarbital (50 mg/kg i.p.)
anesthesia, following the methods of Malmberg and Basbaum (1998).
Briefly, the common sciatic nerve of right hind limb was exposed at
high thigh level through a small incision. The nerve was carefully
cleared off the surrounding connective tissues. A silk suture was
inserted into the nerve with a 3/8 curved, reversed-cutting mini-
needle and tightly ligated so that the dorsal 1/3 to 1/2 of the nerve
thickness was held within the ligature. The wound was closed with
a single muscle silk suture and antibiotic powder was dusted over
the wound area after surgery. Sham operation was performed sim-
larly except without touching the sciatic nerve. Immediately after
surgery, the animals were kept in a soft bed cage with some food
inside so that the animals could feed themselves without difficulty in
standing. The wound healed within 1 to 2 days and the animals
behaved normally. Experiments were carried out at 7 days postliga-
tion.

Thermal and Mechanical Nociception Tests. In the thermal
paw withdrawal test, antinociception or analgesia was measured from
the latency to withdrawal evoked by exposing the right hind
(10% to 15°C) or thermal stimulus (Hargens et al., 1988). Tailimmunized
animals were placed in Plexiglas cages on top of a glass sheet and an
adaptation period of 1 h was allowed. The thermal stimulus (IITC,
Inc., Woodland Hills, CA) was positioned under the glass sheet to
focus the projection bulb exactly on the middle of plantar surface of
the animals. A mirror attached to the stimulus permitted visualization
of the undersurface of the paw. A cut-off time of 20 s was set to prevent
tissue damage. The paw pressure test was performed as described previously (Rashid and Ueda, 2002). Briefly, mice were
placed into a Plexiglas chamber on a 6 x 6-mm wire mesh grid floor and
were allowed to accommodate for a period of 1 h. The mechanical
stimulus was then delivered onto the middle of the plantar surface of
the right hind paw using a transducer indicator (model 1601; IITC,
Inc.). With this apparatus, a control response of 10 g was earlier
adjusted for naive mice. A cut-off pressure of 20 g was set to avoid
tissue damage.

ANF Test. Experiments were performed essentially as described previously (Inoue et al., 1998; Ueda, 1999; Ueda and Inoue, 2000;
Dobolyi et al., 2002). All experiments were performed in compliance with
the relevant laws and institutional guidelines. Briefly, mice were lightly anesthetized with ether and held in a cloth sling with their four limbs hanging free through holes. The sling was suspended on a metal bar. All limbs were tied with soft thread strings. Then three limbs were fixed to the floor, while the other one (right hind limb) was connected to an isometric transducer and recorder. A poly-
ethylene cannula (0.61 mm outer diameter) filled with drug solution was connected to a microsyringe and then carefully inserted into the undersurface of the right hind paw. Nociceptive flexor responses induced by intraplantar injection (2 ml) of algogenic substances (N/OFQ, ATP, ONO-54918-07, or capsaicin) were evaluated. All exper-
iments were started after complete recovery from the light ether
anesthesia and when i.pl. injection of saline did not show any signif-
icient flexor response. The nociceptive activity was represented as
percentage of maximal reflex. The biggest response among the spon-
taneous and nonspecific flexor responses that occurred immediately
after cannulation was considered as the maximal reflex.

Neonatal Capsaicin Treatment. For the degeneration of small-
diameter afferent sensory neurons, capsaicin solution was injected
subcutaneously into newborn (P4) ddY mice at a dose of 50 mg/kg
(Hirata and Ishizuka, 1989; Inoue et al., 1999). As a control, vehicle
(10% ethanol and 10% Tween 80 in physiological saline) was in-
jected. No gross behavioral changes were observed in such treated
mice. Partial sciatic nerve injury in neonatal capsaicin-treated mice was
performed as described in the previous section.

Immunohistochemistry. For immunohistochemical experi-
ments, naive mice, neonatal capsicain-treated mice, or neonatal cap-
saicin-treated nerve-injured mice at 7 days after nerve injury were
used. Mice were deeply anesthetized with sodium pentobarbital (50
mg/kg i.v.) and perfused transcardially with 50 ml of 0.1 M potas-
sium free phosphate-buffered saline (K+-free PBS, pH 7.4), followed
by 50 ml of 4% paraformaldehyde in K+-free PBS. The L4-L5 DRGs
were removed, postfixed, and cryoprotected overnight in 25% sucrose
with a cryostat, thaw-mounted on silane-coated glass slide, and air-dried overnight at room tempera-
ture. For immunolabeling, DRG sections were first washed with
K+-free PBS 3 times for 5 min each and then incubated with 50%
methanol 10 min and 100% methanol for 10 min each, washed with
K+-free PBS, and incubated with excess blocking buffer containing
2% bovine serum albumin in 2% NaCl, 0.1% Triton X-100 in K+-free
PBS for 60 min. The sections were then reacted overnight at 4°C with
goat polyclonal antibody raised against the C-terminal of vanilloid
receptor 1 (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in
blocking buffer containing 2% bovine serum albumin in 2% NaCl,
0.1% Triton X-100 in K⁺-free PBS. After three 5-min washings in K⁺-free PBS, the sections were placed in Texas Red-conjugated anti-goat IgG secondary antibody (1:200; Rockland, Gilbertsville, PA) for 60 min at room temperature. For double immunolabeling with Alexa-ber marker N-52, sections were rinsed and first incubated with anti-mouse IgG (1:200; Cappel, Aurora, OH) for 60 min and then reacted with mouse anti-N52 (1:30,000; Sigma-Aldrich, St. Louis, MO) overnight at 4°C. The sections were then placed in fluorescein isothiocyanate-conjugated anti-mouse IgG (1:200; Cappel) for 60 min. After washing, the sections were coverslipped with PermaFluor (Thermo Shandon, Pittsburgh, PA) and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

**Statistical Analysis.** Statistical evaluations were performed using the Student’s t test after one-way analysis of variance performing the Bonferroni’s test. In the time-course experiments, statistical evaluations were also performed using the Student’s t test after one-way analysis of variance at each time point (5, 10, 20, or 30 min) after drug application. Data were expressed as mean ± S.E.M. Significance was established at *p < 0.05.

**Results**

**New Type Nonirritating Capsaicin Cream.** The new type capsaicin cream, when applied onto mouse footpad, was nonirritating compared with a capsaicin ointment, which produced some biting-licking behavior immediately after application (data not shown). Moreover, effects of the capsaicin cream on the thermal latency at different time points after application in naive mice was measured. A thermal latency of ~10 s was adjusted for naive mice before initiation of experiments. At 30 min after application, the capsaicin ointment (0.1%) induced a profound thermal hyperalgesia that persisted throughout all time points tested within 3 h after application. On the other hand, application of 0.1% capsaicin cream did not produce significant changes in the thermal latency at any time point within 3 h after application (Fig. 1).

**Reversible and Concentration-Dependent Analgesic Action of the Capsaicin Cream against the Bradykinin-Induced Nociceptive Flexion Responses in Naive Mice.** The effect of the cream was next examined with the more sensitive ANF test. When the nociceptive flexion responses induced by 2 pmol of bradykinin (BK) was measured at different time points after a single application of 0.1% capsaicin cream onto the footpad of naive mice, the BK-induced nociception was significantly blocked by the capsaicin cream at 1, 3, and 6 h after application compared with control BK response at 0 h before returning to baseline level at 12 h (Fig. 2A). The maximal blockade of BK nociception was observed at 3 h after application. Thus, the next experiments were performed at 3 h after the application of the capsaicin cream. On the other hand, capsaicin cream produced a concentration-dependent analgesic action from 0.01 to 0.1% against the 2 pmol of BK-induced nociceptive flexion responses measured at 3 h after topical application (Fig. 2B). The maximal blockade of the BK nociception was observed with 0.1% capsaicin cream (Fig. 2B). Thus, this concentration has been used in most of the subsequent experiments.

**Effects of the Capsaicin Cream on Different Peripheral Receptor Ligand-Induced Nociceptive Flexion Responses in Naive and Injured Condition.** Using the ANF test, effect of the capsaicin cream on different peripheral receptor ligands-induced nociceptive flexion responses in naive and injured condition was next studied. With this test in naive mice, previously we have proposed the presence of three distinct types of nociceptors, depending on their stimulation by specific receptor ligands. The nociceptors called
neonatal capsaicin-sensitive type I, were stimulated by i.pl. injection of bradykinin, nociceptin/orphanin FQ, or substance P; the nociceptors called neonatal capsaicin-sensitive type II were stimulated by i.pl. P2X3 receptor agonists; the nociceptors called neonatal capsaicin-insensitive type III, were stimulated by i.pl. PGI2 agonist ONO-54918-07 (Ueda et al., 2000). In the present study, we observed the effect of the capsaicin cream on the nociceptive flexor responses induced by the above-mentioned receptor ligands in naive mice. Prior application of the cream (0.1%) onto mouse footpad 3 h before testing almost completely blocked the nociceptive responses induced by i.pl. injection of nociceptin/orphanin FQ (type I response) and ATP (type II response) (Fig. 3, A and B). The bradykinin and substance P-induced nociceptive (type I) responses were also blocked by capsaicin cream (Fig. 2, A and B; data not shown). However, the nociceptive flexor responses induced by i.pl. injection of PGI2 agonist ONO-54918-07 (type III response) were not affected by the cream in naive mice (Fig. 3C).

After peripheral nerve injury in mice, the type I nociceptive responses were found to be functionally lost and the type II nociceptive responses remained functionally unchanged (Fig. 4, A and B). However, PGI2 agonist-induced type III nociceptive responses were sensitized after nerve injury (Fig. 4C). Thus, experiments for the effect of capsaicin cream on nociceptin/orphanin FQ- or ATP-induced responses were not performed in injured mice. When the effect of the capsaicin cream on PGI2 agonist-induced type III hyper-responses in injured mice was examined, a concentration-dependent reversal of the hyperalgesic responses to the level in naive mice was observed (Fig. 4C).

Effects of the Capsaicin Cream on Nerve Injury-Induced Thermal and Mechanical Hyperalgesia. The effect of the capsaicin cream was also evaluated in nerve injury-induced thermal and mechanical hyperalgesia. The cream was applied 3 h before examining the thermal latency or pressure threshold. In the thermal paw withdrawal test, capsaicin cream concentration dependently reversed the hyperalgesia from 0.01 to 0.1% concentration (Fig. 5A). The cream also concentration dependently reversed the mechanical hyperalgesia in injured mice (Fig. 5B). The cream was slightly more potent in the mechanical than in the thermal test with an EC50 value of 0.042 and 0.066% in mechanical and thermal tests, respectively.

Effects of Neonatal Capsaicin Treatment on Capsaicin Solution-Induced Nociceptive Responses. Next, we utilized the neonatal capsaicin treatment technique to investigate the mechanism of the antihyperalgesic action of the capsaicin cream. Neonatal capsaicin treatment is reported to destroy the majority of the small-diameter C fiber primary afferents in rodents (Jancso et al., 1977; Hiura and Ishizuka, 1989; Inoue et al., 1999). Previously, we have reported that neonatal capsaicin treatment in mice abolished the type I and type II nociceptive responses in the ANF test (Ueda et al., 2000), indicating that these nociceptors falls under the category of small-diameter primary afferents. However, the type III response was insensitive to neonatal capsaicin treatment, indicating their presence on capsaicin-insensitive large-diameter afferents (Ueda et al., 2000). In the present experiment, we observed the effect of nerve injury on the sensitivity of i.pl. capsaicin solution (1 ng/2 μl) or i.pl. PGI2 agonist in such mice. Neonatal capsaicin treatment almost
completely abolished the i.pl. capsaicin solution (1 ng/2 μl)-induced flexor responses in sham-operated mice, whereas the responses substantially increased after nerve injury in such mice, indicating novel expression of capsaicin receptors after injury (Fig. 6A). PGI2 agonist-induced type III responses were also sensitized after nerve injury in neonatal capsaicin-treated mice (Fig. 6B). Application of 0.1% capsaicin cream completely blocked the hypersensitized responses to i.pl.
PGI₂ agonist or i.pl. capsaicin solution observed after nerve injury in neonatal capsaicin-treated mice (Fig. 6, A and B).

Novel Expression of VR1 on Neonatal Capsaicin-Insensitive Fibers after Nerve Injury. To confirm our speculation that partial sciatic nerve injury caused novel expression of capsaicin receptors on neonatal capsaicin-insensitive fibers, immunohistochemical double-labeling was performed on DRG neurons with antibodies to VR1, the putative capsaicin receptor, and N-52, a marker of the myelinated A-fiber. As shown in Fig. 7A, in naive DRG VR1-immunoreactive neurons were not colocalized with N-52, indicating their presence on unmyelinated primary afferents. After neonatal capsaicin treatment, the VR1-immunoreactive neurons almost completely disappeared (Fig. 7B). However, after partial sciatic nerve injury in neonatal capsaicin-treated mice, large numbers of VR1-immunoreactive neurons were observed that were mainly colocalized with N-52 (Fig. 7C).

Discussion

Therapeutic value of topical capsaicin in various chronic pain states has always been under question. Moreover, its pungent and strong irritating nature makes it unacceptable to many patients in clinic. However, clinical evidence suggests its usefulness in chronic pain relief (Watson et al., 1988; The Capsaicin Study Group, 1991; Low et al., 1995; Ellison et al., 1997). In the present report, a new-type capsaicin cream was nonirritating in nature when applied to mouse footpad. Moreover, the cream did not produce thermal hyperalgesia in contrast to the capsaicin ointment (Fig. 1). We speculate that the water-based formulation of the cream may underlie such differences due to slower absorption of the cream than the ointment. Because the capsaicin cream contained a more hydrophilic-type cream base than the capsaicin ointment, the rate of absorption of the capsaicin ointment from skin surface is expected to be considerably faster than the capsaicin cream. This might cause a rapid and lasting sensitization of the nociceptors by the ointment, giving pronounced thermal hyperalgesia. However, a more detailed pharmacokinetic study for the apparent differences between the two formulations remains to be done. Another important finding of the present report is that although the cream was nonanalgesic to thermal stimulation in naive mice, it was analgesic to peripheral receptor ligand-induced nociception in ANF test (Figs. 2, A and B, and 3, A and B). It might be due to the fact that capsaicin cream-induced desensitization in naive mice was not sufficient to prevent the intense thermal stimulation-induced excitation of the sensory neurons, whereas it was sufficient to prevent the flexion reflex induced by very low concentrations of peripheral receptor ligands in the more sensitive ANF test. In the ANF test, the neonatal capsaicin-sensitive type I nociceptive responses induced by BK and N/OFQ were blocked by the capsaicin cream (Figs. 2, A and B, and 3A). The neonatal capsaicin-sensitive type II nociceptive responses induced by ATP were also blocked the cream in naive mice (Fig. 3B). The analgesic action of the cream diminished by 12 h after application, indicating the reversible nature of the desensitizing effect (Fig. 2A). Moreover, the effect of the cream was not due to tachyphylaxis, which represents gradually diminishing effects due to repeated stimulations by an agonist (Liu and Simon, 1996). Although the type I (BK and N/OFQ) and type II (ATP) nociceptive responses induced by ATP were also blocked by the capsaicin cream (Figs. 2, A and B, and 3A). The neonatal capsaicin-sensitive type II nociceptive responses induced by PGI₂ agonist ONO-54918-07 could not be blocked, indicating that these responses were mediated through capsaicin-insensitive sensory neurons in naive conditions (Fig. 3C). On the other hand, PGI₂-induced sensitization of capsaicin-sensitive sensory neurons has also been reported elsewhere (Pitchford and Levine, 1991; Hingtgen and Vasko,
Nevertheless, there has been very limited information on the ability of prostaglandin I<sub>2</sub> receptor agonists to produce nociception by directly activating the sensory neurons. In our experiments, the PGI<sub>2</sub> agonist ONO-54918-07 produced potent nociceptive flexion responses at extremely low doses (attomolar to picomolar ranges). These responses could not be abolished by neonatal capsaicin treatment, indicating their mediation through capsaicin-insensitive neurons (Ueda et al., 2000). Moreover, intrathecal pretreatment of mice with antisense oligodeoxynucleotide for prostaglandin I<sub>2</sub> receptor completely abolished the nociceptive flexion responses of ONO-54918-07 (our unpublished data). This result clearly indicates that the nociceptive flexion responses of ONO-54918-07 were mediated through the prostaglandin I<sub>2</sub> receptor at the peripheral nerve ending. We speculate that the sensitizing and the nociceptive effects of PGI<sub>2</sub> might involve different mechanisms at the nerve endings. However, further biochemical and molecular experiments will only clarify the exact mechanism of the PGI<sub>2</sub> agonist-induced nociception.

We examined the effect of the capsaicin cream under neuropathic pain condition. After partial sciatic nerve injury in mice, both the thermal and mechanical hyperalgesia were concentration dependently blocked by the capsaicin cream (Fig. 5, A and B). The potency of the cream was a little higher with the mechanical nociception test (mechanical test, EC<sub>50</sub> value of 0.042%; thermal test, EC<sub>50</sub> value of 0.066%).
results indicate that the effect of the cream on mechanosensitive fibers was more pronounced in injured condition. Thus, possible involvement of large-diameter mechanosensitive fiber could be speculated for the effect of the capsaicin cream in injured mice. On the other hand, with the ANF test in injured mice, N/OFQ-induced type I nociceptive response was lost (Fig. 4A) and ATP-induced type II nociceptive response was unaffected (Fig. 4B), whereas the PGL₂ agonist-induced nociception was hypersensitized (Fig. 4C). Thus, peripheral nerve injury in mice caused contrasting functional changes in type I and type III responses. We have previously reported that type I responses of N/OFQ is mediated through substance P release from peripheral nerve endings (Inoue et al., 1998). N/OFQ-induced flexion responses were also blocked by intrathecal substance P receptor antagonist (Inoue et al., 1999). On the other hand, there have been reports of drastic decrease in the SP immunoreactivity in DRG and spinal cord after peripheral nerve injury (Malmberg and Basbaum, 1998; Lee et al., 2001). Thus, the loss of type I responses of N/OFQ in nerve-injured mice might be due to functional loss of SP after injury. On the other hand, we observed novel expression of VR1 in neonatal capsaicin-insensitive type III neurons after nerve injury (Fig. 7C). PGL₂ agonist produces nociceptive responses acting on the neonatal capsaicin-insensitive type III neurons through activation of G₂-coupled IP receptor. Thus, the hyperalgesic responses of PGL₂ agonist after nerve injury could be due to a protein kinase A-mediated transactivation of the newly expressed VR1 receptors, which has been reported elsewhere (De Petrocellis et al., 2001). Application of the capsaicin cream concentration dependently reversed the PGL₂ agonist-induced type III nociceptive hyperresponses to the level in naive mice (Fig. 4C). Thus, although the cream was ineffective to thermal and type III nociception in naive mice, it blocked the thermal, mechanical, and type III nociceptive hyperalgesia observed after nerve injury (Figs. 1, 3C, 4C, and 5, A and B). These results indicate a selective increase in capsaicin-sensitive sites due to peripheral nerve injury. To examine the mechanism of the antihyperalgesic effect of capsaicin cream, we used the neonatal capsaicin treatment technique. Neonatal capsaicin treatment in rodent has long been regarded to destroy the majority of small-diameter sensory neurons, mainly the C-fibers (Jancso et al., 1977; Hiura and Ishizuka, 1989). Neonatal capsaicin treatment is thus used as a good tool to identify capsaicin-sensitive pathways and to explore their contributions to pathophysiological processes (Buck and Burks, 1986; Holzer, 1991). In the present study, the i.pl. capsaicin solution-induced nociceptive responses in the ANF test were almost completely abolished after neonatal capsaicin treatment in mice, indicating the destruction of the capsaicin-sensitive primary afferents (Fig. 6A, column 2). However, partial sciatic nerve injury of neonatal capsaicin-treated mice caused marked increase in the i.pl. capsaicin solution-induced nociceptive responses (Fig. 6A, column 4), and topical application of capsaicin cream (0.1%) completely blocked these newly induced responses (Fig. 6A, column 5). Nerve injury in neonatal capsaicin-treated mice also caused hyperalgesia to PGL₂ agonist-induced responses, which were blocked by the capsaicin cream (Fig. 6B). These results clearly suggest a novel increase in capsaicin receptors on previously capsaicin-insensitive fibers due to nerve injury treatment. We next confirmed the novel expression of putative capsaicin receptor, VR1 on neonatal capsaicin-insensitive fibers by immunohistochemistry. As shown in Fig. 7A, the VR1-positive neurons in naive mice were unlabeled by A-fiber marker N-52, indicating their presence on unmyelinated C-fibers. Consistent to the previous reports (Hiura and Ishizuka, 1989), neonatal capsaicin treatment caused almost complete loss of VR1-immunoreactive neurons (Fig. 7B). However, partial sciatic nerve injury in neonatal capsaicin-treated mice caused novel expression of VR1 receptors, which were mainly colocalized with A-fiber marker N-52 (Fig. 7C). These results confirm the speculation that capsaicin cream worked after nerve injury by desensitizing the newly expressed VR1 receptors mainly located on A-fibers. Recently, increase in VR1 protein expression on undamaged large-diameter DRG neurons has been reported after partial nerve injury in the rat (Hudson et al., 2001). Our results as well as these previous observations suggest that the analgesic effects of capsaicin cream in nerve injury-induced neuropathic pain might be due to desensitization of the newly expressed capsaicin receptors on previously capsaicin-insensitive fibers. On the other hand, our pharmacological classification of the nociceptive fibers into three distinct types does not exclude the presence of other functional types of fibers. Moreover, physiological role of these three types of fibers is yet to be studied. Nonetheless, the neonatal capsaicin sensitivity of type I and type II responses indicates their actions through small-diameter unmyelinated C-fibers, whereas the neonatal capsaicin insensitivity of the type III responses indicates these responses through large-diameter myelinated A-fibers. Moreover, our immunohistochemical results clearly support these propositions.

In conclusion, we demonstrate that the new-type capsaicin cream could be a better formulation than the currently available preparations to treat neuropathic pain because of its nonirritating and nonhyperalgesic effects. Our results indicate that the novel expression of VR1 receptors on previously capsaicin-insensitive fibers after peripheral nerve injury may account for the analgesic action of capsaicin cream in neuropathic pain.

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References


Address correspondence to: Dr. Hiroshi Ueda, Division of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan. E-mail: ueda@net.nagasaki-u.ac.jp.