



Inhibition of transient receptor potential vanilloid type 1 through α_2 adrenergic receptors at peripheral nerve terminals relieves pain

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ABSTRACT. The activation of α_2 adrenergic receptors contributes to analgesia not only in the central nervous system but also in the peripheral nervous system. We reported that noradrenaline inhibits the activity of transient receptor potential vanilloid 1 (TRPV1) evoked by capsaicin through α_2 receptors in cultured rat dorsal root ganglion (DRG) neurons. However, it is unclear whether activation of TRPV1 expressed in peripheral nerve terminals is inhibited by α_2 receptors and whether this phenomenon contributes to analgesia. Therefore, we examined effects of clonidine, an α_2 receptor agonist, on several types of nociceptive behaviors, which may be caused by TRPV1 activity, and subtypes of α_2 receptors expressed with TRPV1 in primary sensory neurons in rats. Capsaicin injected into hind paws evoked nociceptive behaviors and clonidine preinjected into the same site inhibited capsaicin-evoked responses. This inhibition was not observed when clonidine was injected into the contralateral hind paws. Preinjection of clonidine into the plantar surface of ipsilateral, but not contralateral, hind paws reduced the sensitivity to heat stimuli. Clonidine partially reduced formalin-evoked responses when it was preinjected into ipsilateral hind paws. The expression level of α_{2C} receptor mRNA quantified by real-time PCR was highest followed by those of α_{2A} and α_{2B} receptors in DRGs. α_{2A} and α_{2C} receptor-like immunoreactivities were detected with TRPV1-like immunoreactivities in the same neurons. These results suggest that TRPV1 and α_2 receptors are coexpressed in peripheral nerve terminals and that the functional association between these two molecules causes analgesia.

KEY WORDS: adrenergic system, analgesia, pain, peripheral nervous system, transient receptor potential vanilloid type 1

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Animals have mechanisms to relieve pain in addition to those to sense pain. In the central nervous system, the descending antinociceptive system (DAS) is a pain relief system that affects neurons and synapses in the spinal cord [21, 35]. The DAS includes serotonergic and adrenergic systems. The fibers descending from the raphe nucleus of the medulla oblongata to the dorsal horn of the spinal cord release serotonin, and the fibers descending from the locus ceruleus of the brain stem to the spinal cord release noradrenaline (NA). Both neurotransmitters are reported to act on the central terminals of primary nociceptive neurons and secondary neurons in the dorsal horn of the spinal cord [8, 24, 40]. NA activates α_2 adrenergic receptors expressed in the central terminals of primary sensory neurons in the dorsal horn of the spinal cord and inhibits the release of excitatory neurotransmitters from primary neurons, leading to analgesic effects [13, 23–25, 36]. On the other hand, in peripheral tissues, topical administration of clonidine, an α_2 adrenergic agonist, inhibits the nociceptive behaviors evoked by noxious heat, and yohimbine, an α_2 adrenergic receptor antagonist, blocks this analgesic effect of clonidine [7]. Since the adrenal medulla releases NA and adrenaline into the circulation and adrenergic nerve fibers are distributed under the skin [10], it is possible that NA and adrenergic receptors contribute to analgesia in peripheral tissues. However, the underlying mechanisms are not fully understood.

Transient receptor potential vanilloid 1 (TRPV1) is a nonselective cation channel expressed in primary nociceptive nerves such as A δ - and C-fibers and it acts as a sensor of noxious stimuli, including heat, acid and several chemicals, such as vanilloids [2,

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5, 39]. Recently, we reported that NA inhibits TRPV1 activity through the activation of α_2 receptors, followed by the inhibition of the adenylate cyclase/cAMP/protein kinase A pathway in dorsal root ganglion (DRG) neurons of rats [19]. Chakraborty *et al.* also reported that the activation of α_2 receptors reduces TRPV1 activity in DRG neurons of rats. Moreover, they demonstrated that the activation of α_2 receptors suppresses capsaicin-induced neurotransmitter release from the spinal terminals of primary sensory neurons [3]. Although these results indicate the interaction between α_2 receptors and TRPV1 in the cell body and central terminals of primary sensory neurons, it is unclear whether a similar interaction exists at the local peripheral nerve level where noxious stimuli are received *in vivo*. In the present study, we examined whether α_2 receptor activation inhibits the nociceptive behaviors caused by the activation of TRPV1 expressed on peripheral sensory nerves. Moreover, we investigated which subtypes of α_2 receptors are involved in the inhibition of nociceptive behaviors via immunohistochemical and real-time PCR analyses.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the guidelines of Tottori University (approval# 16-T-14 and 19-T-50), and this study was approved by the Institutional Animal Care and Use Committee, Tottori University. We used male Wistar rats (7–10 weeks old, 200–300 g) in all experiments.

Behavioral tests

Capsaicin test: The capsaicin test was performed over 2 days. Rats were placed in observation chambers (200 mm width \times 200 mm depth \times 200 mm height) for longer than 20 min to allow them to habituate to the environment before testing. On day 1, preceding the capsaicin injection, vehicle was preinjected into the hind paws of rats using a fine (30-G) needle; then, they were placed in observation chambers. Ten min after preinjection, capsaicin (5 nmol/paw in a volume of 50 μ l) was injected into the ipsilateral hind paws of rats into which vehicle was preinjected, and we observed nociceptive behaviors, such as licking, biting and flinching, for 10 min. On day 2, capsaicin was injected into the hind paw that did not receive it on day 1 to prevent tissue damage. Test drugs (50 μ l) were preinjected into the hind paws of rats; then, the rats were placed in observation chambers. Ten min after preinjection, capsaicin was injected into the same site as that of the test drug injection, and we observed their nociceptive behaviors for 10 min. Data acquisition was performed with an analog/digital converter (Power Lab; AD Instruments, Castle Hill, NSW, Australia), chart recorder software (Lab chart 7, AD Instruments) and a personal computer (Macintosh; Apple, Cupertino, CA, USA) as previously reported [9]. When nociceptive behaviors were observed, an electrical circuit, which applied a 5-V electrical signal to the analog/digital converter, was manually turned on, and the electrical signal was continuously recorded by chart recorder software. The sampling rate used was set at 100 Hz. The time that the rats showed nociceptive behaviors was calculated from the integral of the recorded electrical signal (0.2 sec/V \times sec).

Hargreave's test: We measured the latency of the withdrawal responses to heat stimuli by Hargreave's method. Paw withdrawal latency in response to radiant heat was measured using Hargreave's test apparatus (Ugo Basile, Varese, Italy). Rats were placed in observation chambers positioned on a glass surface and allowed to habituate to the environment for 20 min before testing to allow appropriate behavioral immobility. A heat source was positioned under the plantar surface of the hind paw, and the infrared beam with an intensity of 50 arbitrary units of the apparatus was turned on. A cutoff time of 30 sec was used to prevent tissue damage. When rats showed a withdrawal response, the subsequent stimulation was applied after a >10 min interval to prevent thermal sensitization and behavioral disturbances. We performed seven trials, and the mean withdrawal latency was determined from the average of five separate trials after discarding the longest and shortest response times to the stimuli. On day 1, Hargreave's test was performed on both hind paws in the absence of any test drugs (control). On day 2, the test drugs or saline as a vehicle were injected into one side of the hind paw 10 min before the beginning of Hargreave's test.

Formalin test: The nociceptive behaviors induced by the injection of 5% formalin (10 μ l) into the instep of rat hind paws were observed. In the formalin test, we used the same apparatus and method as in the capsaicin test to quantify the amount of nociceptive behaviors. Test drugs (30 μ l) and vehicle (30 μ l) were injected into the insteps of rat hind paws 10 min before formalin injection. We observed nociceptive behaviors for 90 min after formalin injection.

Real-time PCR

Total RNA used for real-time PCR analysis was isolated from DRGs from the L3–L6 segments on both sides using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Total RNA was purified using the PureLink RNA Mini Kit (Thermo Fisher Scientific) and treated with PureLink DNase (Thermo Fisher Scientific) at room temperature for 15 min to remove contaminated genomic DNA. Reverse transcription was performed with 10 μ g of total RNA and oligo dT primers using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Real-time PCR was performed with 0.5 μ l cDNA, 4 pairs of primers that were designed for 3 subtypes of α_2 adrenergic receptors (α_{2A} , α_{2B} and α_{2C}), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1), and THUNDERBIRD SYBR qPCR Mix (Toyobo). The real-time PCR protocol involved 40 cycles of PCR at 95°C for 15 sec, at 60°C for 15 sec, and at 72°C for 45 sec in a Step One system (Thermo Fisher Scientific). The amplification efficiencies of the primers were assessed using diluted cDNAs as templates. Since the amplification efficiencies of the α_{2A} , α_{2B} , α_{2C} and GAPDH primers were $96.0 \pm 2.5\%$ ($n=3$), $100.6 \pm 5.5\%$ ($n=3$), $98.4 \pm 2.1\%$ ($n=3$) and $95.4 \pm 1.7\%$ ($n=3$), respectively, the amplification efficiencies of the 4 pairs of primers were considered to be 100%. The amount of target DNA in the cDNA preparations was compared by the Δ Ct method using GAPDH as an internal control.

Table 1. Sequences of primers for RT-PCR

Target	Forward	Reverse	Product size (bp)	Accession #
α_{2A}	5'-GCACCACTTGCCCCAGTAA-3'	5'-AGCACACCCACCCGTTCTTCTT-3'	93	NM_012739.3
α_{2B}	5'-GATATAACCAGTTCACCCAGCTCAGAA-3'	5'-AGTTGGGAAGACAACCAGGAGGTAGA-3'	113	NM_138505.2
α_{2C}	5'-CGCGAGAAACGCTTCACCTT-3'	5'-CACGGCAGATGCCATACAGG-3'	103	NM_138506.1
GAPDH	5'-TTCAGGAGCGAGATCCCGCTAAC-3'	5'-TTCAGGTGAGCCCCAGCCTTCT-3'	110	NM_017008.4

Immunohistochemistry

Rats were sacrificed by decapitation under deep anesthesia with isoflurane. Bilateral L3–L6 DRGs were collected and transferred to ice-cold phosphate-buffered saline (PBS) immediately after dissection. DRG tissues were fixed in 4% paraformaldehyde in PBS for 1 hr and were embedded in paraffin. Six micrometer thick tissue sections were incubated with an appropriate blocking solution (TRPV1, 5% skim milk in PBS; α_{2A} receptor, 10% normal goat serum in PBS; α_{2C} receptor, 1% skim milk in PBS) for 30 min at room temperature. Subsequently, the sections were incubated with a guinea pig anti-TRPV1 antibody (Merck Millipore corporation, Temecula, CA, USA, Cat# AB5566, Lot# 3135959) diluted to 1:50, a rabbit anti- α_{2A} antibody (Cusabio Technology, Houston, TX, USA, Cat# CSB-PA007424, Lot# G1229Y) diluted to 1:100 or a rabbit anti- α_{2C} antibody (Cusabio Technology, Cat# CSB-PA000916, Lot# G1229Y) diluted to 1:100 overnight at 4°C. The sections were incubated with secondary antibodies, a horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG antibody (Southern Biotech, USA & Canada, Cat# 6090-05, Lot# G0014-MB76L) diluted to 1:100 in PBS for TRPV1, or an HRP-conjugated goat anti-rabbit IgG antibody (Histofine kit, Nichirei Bioscience, Tokyo, Japan, Cat# H1902) for α_{2A} and α_{2C} receptors, for 30 min. Finally, after the sections were rinsed with PBS, the HRP activity of the sections was visualized with 3,3'-diaminobenzidine tetrahydrochloride, and the sections were mounted using Eukitt mounting medium (ORSAtec GmbH, Bobingen, Germany). Negative controls, in which the primary antibodies were replaced with PBS, did not show nonspecific staining.

Drug preparations

Capsaicin test: An injection solution of 100 μ M capsaicin was prepared by dissolving capsaicin in a vehicle solution consisting of saline (0.9% NaCl), 1% DMSO and 0.5% Tween-20. Concentrated stock solutions of capsazepine (10 mM) and JNJ-17203212 (10 mM) were prepared by dissolving them in DMSO. Injection solutions of capsazepine and JNJ-17203212 at 100 μ M were prepared by diluting the stock solutions in the vehicle solution. Injection solutions of clonidine, isoproterenol, phenylephrine and NA at 500 μ M were prepared by dissolving them in the vehicle solution. The injection solution of 250 μ M yohimbine was prepared by dissolving yohimbine in the vehicle solution. All the stock solutions were stored at -30°C until use. Each injection solution of 50 μ l was administered to rat hind paws under the skin. We performed preliminary experiments to determine the dose of capsaicin used, by which nociceptive behaviors were evoked immediately after the injection and completed within 10 min to prevent tissue damage. Through preliminary experiments, we determined the dose of clonidine used. Since clonidine at 50 nmol but not 25 nmol showed a sedative effect when injected into the hind paws of rats, 25 nmol was chosen. Other adrenergic agonists were determined to be the same as clonidine. Since yohimbine at 25 nmol but not 12.5 nmol reduced capsaicin responses by itself, yohimbine at 12.5 nmol was used.

Hargreave's test: A concentrated solution of 10 mM clonidine was prepared by dissolving clonidine in saline. A concentrated solution of 10 mM capsazepine was prepared by dissolving capsazepine in DMSO. The stock solutions of clonidine and capsazepine were stored at -30°C until use. Injection solutions of clonidine (100 μ M) and capsazepine (100 μ M) were prepared by diluting the stock solutions in saline. Each injection solution of 50 μ l was administered under the skin of the plantar surface of rat hind paws. The dose of capsazepine used was determined to be 1–10 times higher than that of capsaicin because the affinities of capsaicin and capsazepine to TRPV1 molecules are reported to be almost similar (see IUPHAR/BPS Guide to Pharmacology, <http://www.guidetopharmacology.org>).

Formalin test: Formalin was diluted to 5% (1.85% formaldehyde) in saline and stored at room temperature with protection from light. A concentrated solution of 10 mM clonidine was prepared by dissolving clonidine in saline and was stored at -30°C until use. An injection solution of 1 mM capsazepine was prepared by dissolving capsazepine in saline containing 8% DMSO and 0.5% Tween-20 and was stored at -30°C until use. The stock solution of clonidine was thawed immediately before use and was diluted to 833 μ M with saline for injection. The clonidine solution (30 μ l, 25 nmol), the capsazepine solution (30 μ l, 30 nmol) and the formalin solution (10 μ l) were injected into the instep of rat hind paws.

Data analysis and statistics

Data were analyzed by Lab Chart (AD Instruments), Igor Pro (Wavemetrics, Lake Oswego, OR, USA) and Excel (Microsoft, Redmond, WA, USA). The data are presented as the mean \pm the standard error of the mean (SEM; n=the number of observations). Statistical significance was assessed by Student's *t*-test or by analysis of variance (ANOVA) with a *post hoc* Tukey's honestly significant difference (HSD) test. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Effects of adrenergic agonists on the nociceptive behaviors evoked by capsaicin

Previously, we reported that α_2 adrenergic receptor activation inhibits TRPV1 in isolated DRG neurons. To investigate whether this phenomenon could contribute to analgesia at the peripheral nerves of the primary sensory neuron, the effects of injected adrenergic agonists on capsaicin-evoked nociceptive behaviors were examined.

Rats were divided into the control, NA-, phenylephrine-, clonidine- and isoproterenol-injected groups (Fig. 1A–E). All drugs were injected under the skin of the plantar surface of the hind paw. Capsaicin (5 nmol) was injected into the same site where the adrenergic agonists were injected after a 10 min interval. Following the capsaicin injection, rats were placed in observation chambers, and their nociceptive behaviors (flinching, biting, licking and guarding) were observed for 10 min (Fig. 1A–E top and middle traces in the left panels). “ON” and “OFF” in the figure indicate the states of rats showing and not showing nociceptive behaviors, respectively. The bottom graphs in the left panel in Fig. 1A–E show the integral of the time spent in nociceptive behaviors after the capsaicin injection, and the total time spent in nociceptive behaviors over 10 min is shown in the right panels in each figure. On day 1, as a control, capsaicin induced nociceptive behaviors mainly in the first 3 min period in all groups (Fig. 1A–E). No significant difference in the total time of nociceptive behaviors was observed on days 1 and 2 in the control group rats. None of the adrenergic drugs injected in this study caused nociceptive behaviors by themselves. The relative total time spent in nociceptive behaviors over 10 min on day 2 compared to that on day 1 is summarized in Fig. 1F. The preinjection of NA (25 nmol) caused sustained capsaicin-evoked nociceptive behaviors in 2 out of 4 rats tested and significantly extended the total time of nociceptive behaviors, indicating that NA enhanced capsaicin-induced pain (Fig. 1B). Phenylephrine (25 nmol) and isoproterenol (25 nmol) had no effect (Fig. 1C, 1E). In contrast, the preinjection of clonidine (25 nmol) significantly shortened the total time of

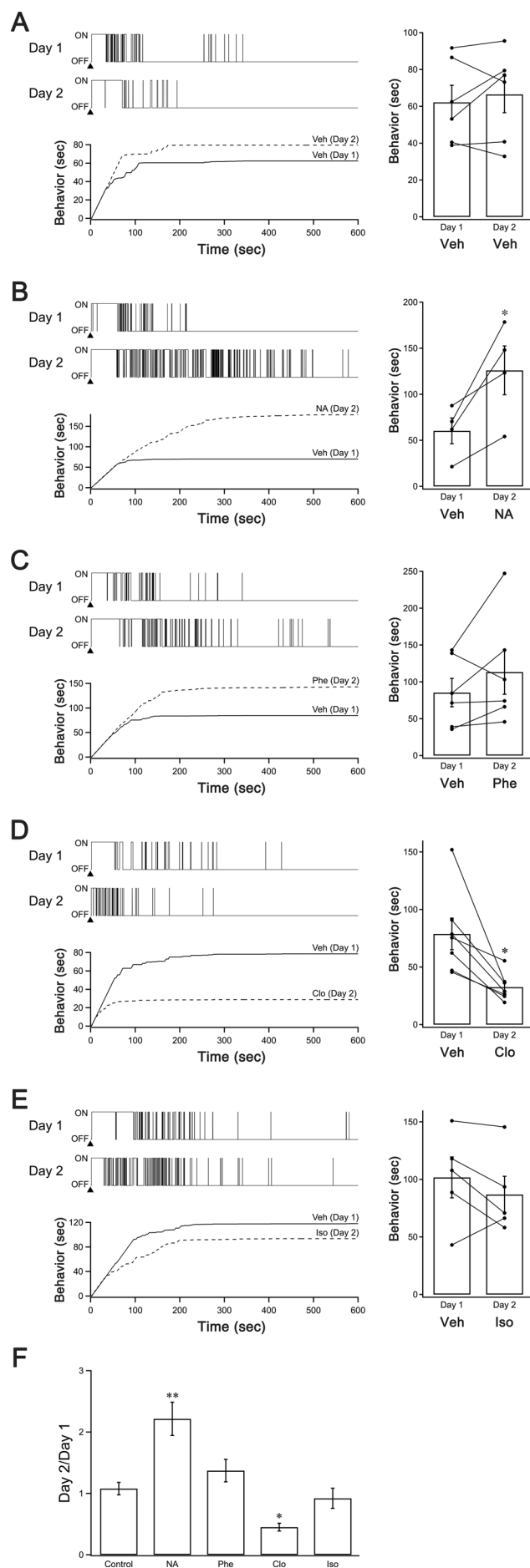


Fig. 1. Effects of adrenergic receptor agonists in the capsaicin test. A–E. Representative responses of rats into which vehicle was preinjected into the right hind paws 10 min before the capsaicin injection (5 nmol) into the same site on day 1 (top traces in each left panel) and similar responses with the preinjection of each drug (A, vehicle, n=6; B, noradrenaline, NA, 25 nmol, n=4; C, phenylephrine, Phe, 25 nmol, n=6; D, clonidine, Clo, 25 nmol, n=7; E, isoproterenol, Iso, 25 nmol, n=5) on day 2 (middle traces in each panel) are shown. The top and middle traces in the left panels describe the temporal switching of the nociceptive behavior appearance. “ON” and “OFF” in the figure indicate the states of rats showing and not showing nociceptive behaviors, respectively. The bottom graphs in each left panel show the integral of the time spent in nociceptive behaviors after the capsaicin injection. The total time spent in nociceptive behaviors during the 10 min recordings is shown in the right panels. The symbols and the lines show the responses of individual rats and the columns represent the mean \pm the standard error of the mean (SEM; * P <0.05, versus day 1 by Student’s paired t -test). F. The columns show the total time spent in nociceptive behaviors on day 2 normalized by those on day 1 (* P <0.05, ** P <0.01, versus control by ANOVA with a *post hoc* Tukey’s honestly significant difference (HSD) test).

capsaicin-evoked nociceptive behaviors, indicating that clonidine inhibited capsaicin-induced pain (Fig. 1D).

To examine whether the inhibitory effect of clonidine and the potentiating effect of NA were achieved within the restricted site on the plantar surface of the hind paw, these two drugs were preinjected into the contralateral hind paws, and we observed the nociceptive behaviors evoked by capsaicin (Fig. 2). On day 1, the nociceptive behaviors evoked by capsaicin without preinjection were observed as a control. On day 2, clonidine or NA was injected into the same hind paws that received capsaicin injection on day 1 10 min before the injection of capsaicin into the hind paws that did not receive capsaicin injection on day 1, and nociceptive behaviors induced by capsaicin were observed. The preinjection of clonidine (25 nmol) into the contralateral hind paws of which capsaicin was injected did not affect the capsaicin responses (Fig. 2A). In contrast, the preinjection of NA (25 nmol) into the contralateral hind paws on day 2 significantly extended the total time of nociceptive behaviors compared with that on day 1 (Fig. 2B). Furthermore, we investigated the effects of yohimbine, an α_2 adrenergic antagonist, on the inhibitory effect of clonidine. Clonidine (25 nmol) injected in combination with yohimbine (12.5 nmol) showed no effect on the nociceptive behaviors induced by capsaicin (Fig. 2C). The relative total time spent in nociceptive behaviors over 10 min on day 2 compared to that on day 1 is summarized in Fig. 2D.

To examine whether the inhibitory effect of clonidine on capsaicin-evoked nociceptive behaviors is caused by inhibition of TRPV1, we examined effects of clonidine on the capsaicin responses remaining in the presence of TRPV1 antagonists. In the previous study, we examined effects of TRPV1 antagonists (capsazepine, BCTC, SB366791 and 5'-IRTX) on capsaicin-evoked currents in rat DRG neurons and found that capsazepine and BCTC, but not SB366791 and 5'-IRTX, inhibit them [17]. In addition, JNJ-17203212 was used as a more specific TRPV1 antagonist than capsazepine in this study. In a series of preliminary experiments, we examined effects of capsazepine and JNJ-17203212 on capsaicin currents and voltage-gated Na⁺ currents in the whole-cell voltage clamp recordings in cultured DRG neurons isolated from adult rats and confirmed that they completely blocked capsaicin

(1 μ M)-evoked currents and had no effect on amplitudes of voltage-gated Na⁺ currents at 10 μ M (data not shown). Therefore, we used capsazepine and JNJ-17203212 to assess contribution of TRPV1 in this study. On day 2 of the capsaicin test, capsazepine (5 nmol) was injected 10 min before the capsaicin injection at the same sites. In the capsazepine-injected rats, capsaicin-evoked nociceptive behaviors for during 10 min were significantly shorter than those on day 1 with the vehicle injection (Fig. 3A). JNJ-17203212 was injected twice in each rat. JNJ-17203212 (5

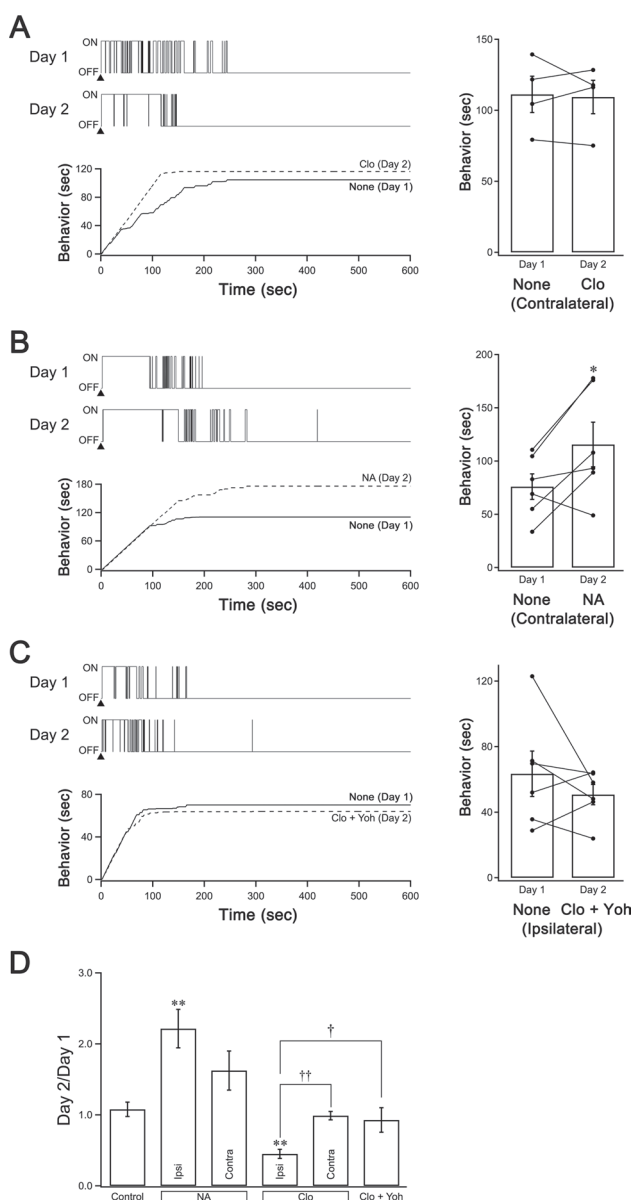


Fig. 2. Effects of noradrenaline and clonidine preinjection into the contralateral hind paws in the capsaicin test. **A, B.** Representative responses of rats into which capsaicin (5 nmol) was injected into the right hind paw without preinjection on day 1 (top traces in each left panel) and those of rats into which clonidine (Clo, 25 nmol, $n=4$) or noradrenaline (NA, 25 nmol, $n=6$) were preinjected into the right hind paws 10 min before the capsaicin injection into the left hind paws on day 2 (middle traces in each panel) are shown. The top and middle traces in the left panels describe the temporal switching of the nociceptive behavior appearance. “ON” and “OFF” in the figure indicate the states of rats showing and not showing nociceptive behaviors, respectively. The bottom graphs in each left panel show the integral of the time spent in nociceptive behaviors after the capsaicin injection. The total time spent in nociceptive behaviors during the 10 min recordings is shown in the right panels. The symbols and the lines show the responses of individual rats, and the columns represent the mean \pm the SEM (* $P < 0.05$, versus day 1 by Student's paired t -test). **C.** Representative responses of rats into which capsaicin (5 nmol) was injected into the right hind paw without preinjection on day 1 (top traces in left panel) and those of rats into which clonidine (Clo, 25 nmol) in combination with yohimbine (Yoh, 12.5 nmol) was preinjected into the left hind paws 10 min before the capsaicin injection into the same hind paws on day 2 (middle traces) are shown similarly to A and B ($n=6$). **D.** The columns show that the total time spent in nociceptive behaviors on day 2 was normalized to that on day 1. The “Control”, “NA (Ipsi)” and “Clo (Ipsi)” columns are identical to those in Fig. 1. The “NA (Contra)”, “Clo (Contra)”, “Clo + Yoh” columns are calculated from the data in panels A-C (** $P < 0.01$, versus vehicle group; † $P < 0.05$, versus clonidine ipsilateral group, †† $P < 0.01$, versus clonidine ipsilateral group by ANOVA with a *post hoc* Tukey's HSD test).

nmol) was injected 10 min before the capsaicin injection and was injected again (5 nmol) together with capsaicin. This is because preinjection of JNJ-17203212 (5 nmol) itself was not enough to affect capsaicin responses in preliminary experiments, suggesting an effective period of JNJ-17203212 was much shorter than that of capsazepine. JNJ-17203212 (totally 10 nmol) administered on day 2 significantly reduced capsaicin responses (Fig. 3C). Together with the injection of capsazepine and JNJ-17203212, clonidine (25 nmol) was injected 10 min before the capsaicin injection (Fig. 3B and 3D). Combinations of TRPV1 antagonists and clonidine also reduced capsaicin-evoked nociceptive behaviors significantly, but the relative total time spent in nociceptive behaviors over 10 min on day 2 compared to that on day 1 is not affected by the clonidine administration over the TRPV1 antagonists (Fig. 3E).

Effects of clonidine on the nociceptive behaviors evoked by thermal stimuli

TRPV1 is well known to be activated by a heat stimulus (higher than 42°C). To investigate whether the nociceptive behaviors evoked by heat are inhibited by the activation of α_2 receptors, the effects of clonidine were examined using Hargreave's test. Similar to the capsaicin test, Hargreave's test was performed on two successive days. The control responses without drugs were recorded on day 1. Some rats showed withdrawal responses with significantly different latencies between the right and left paws. We excluded such rats from the analysis. To examine whether the activation of TRPV1 triggers the nociceptive behaviors evoked by heat, we investigated the effects of a TRPV1 antagonist on the thermal responses. No differences in the withdrawal latency of paws that did not receive a drug injection between days 1 and 2 were observed. On the other hand, paw withdrawal latency increased when capsazepine (5 nmol) was preinjected (Fig. 4A). However, JNJ-17203212 (5 nmol) administered 10 min prior to the beginning of Hargreave's test on day 2 did not affect paw withdrawal latency at all (data not shown), probably because of a

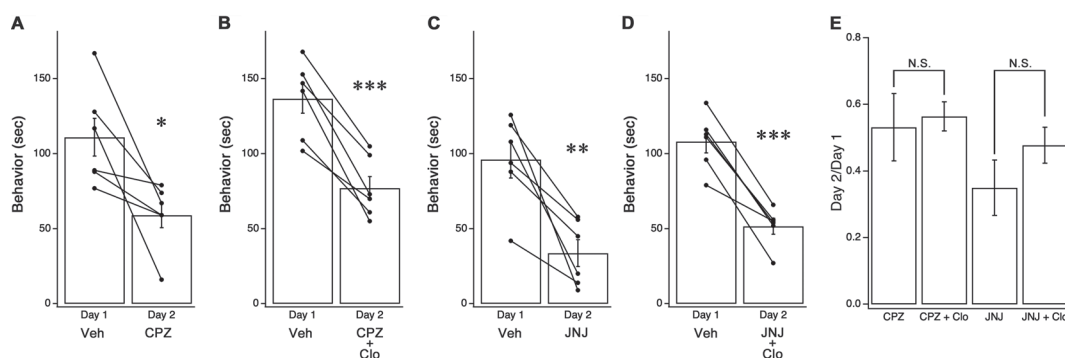


Fig. 3. Effects of clonidine on capsaicin responses remaining in the transient receptor potential vanilloid type 1 antagonists-treated rats. **A.** The total time spent in capsaicin-evoked nociceptive behaviors during the 10 min recordings is shown. The symbols and the lines show the responses of individual rats, and the columns represent the mean \pm the SEM (* P <0.05, versus day 1 by Student's paired t -test, n =6). The vehicle solution was injected on the ipsilateral side of the capsaicin injection on day 1 and capsazepine (CPZ, 5 nmol) on day 2. Ten minutes after the preinjection of these chemicals, capsaicin (5 nmol) was injected at the same sites. **B.** Clonidine (Clo, 25 nmol) was injected with capsazepine by the same protocol as **A** (** P <0.001, versus day 1 by Student's paired t -test, n =6). **C.** JNJ-17203212 (JNJ, 5 nmol) was injected 10 min before the capsaicin injection and additional JNJ-17203212 (5 nmol) was injected with capsaicin. (** P <0.01, versus day 1 by Student's paired t -test, n =6). **D.** Clonidine was injected 10 min before the capsaicin injection with JNJ-17203212 by the same protocol as **C** (** P <0.001, versus day 1 by Student's paired t -test, n =6). **E.** The columns show the total time spent in nociceptive behaviors on day 2 normalized by those on day 1 in the rats in **A**–**D** (N.S., not significant by ANOVA with a *post hoc* Tukey's HSD test).

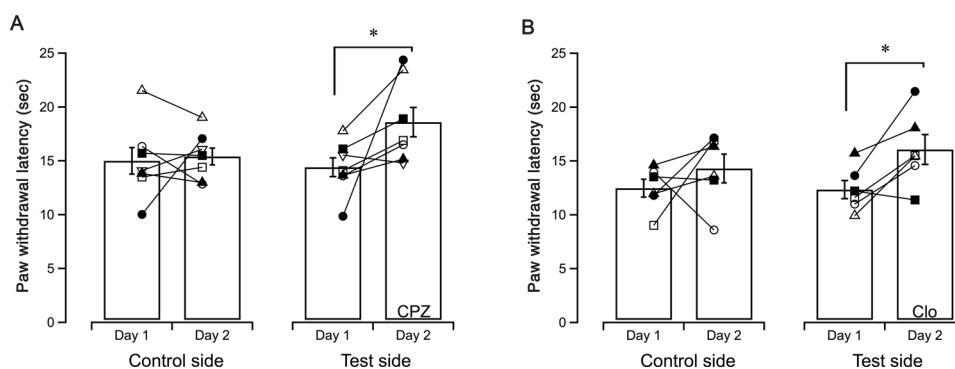


Fig. 4. Effects of capsazepine and clonidine on the nociceptive behaviors evoked by thermal stimuli. Thermal stimuli were applied to rat hind paws using Hargreave's test apparatus. The paw withdrawal latency of both sides of the hind paws on days 1 and 2 is shown. The symbols and the lines show the responses of individual rats and the columns represent the mean \pm the SEM. **(A)** Capsazepine (CPZ, 5 nmol) was injected into the right hind paws on day 2 (n =7). **(B)** Clonidine (Clo, 5 nmol) was injected into the right hind paws on day 2 (n =6). * P <0.05 by Student's paired t -test.

very short effective period of JNJ-17203212 as described above. Similar to capsaizepine, clonidine (5 nmol) was also preinjected on day 2. Clonidine extended paw withdrawal latency on the side of the injection (Fig. 4B).

Effects of clonidine on nociceptive behaviors evoked by formalin

TRPV1 is activated by several chemical substances, such as vanilloids and inflammatory mediators. However, it is not clear whether TRPV1 is involved in formalin-induced nociceptive behaviors. It is well known that formalin injection into the hind paw induces biphasic responses, so-called phase I and phase II responses, and it has been reported that phase I responses are caused by a direct effect of formalin on nociceptive nerve fibers and that phase II responses depend on inflammation [4, 6, 27, 34, 38]. Some reports have indicated that the activation of TRPV1 is involved in both phase I responses and phase II responses [31, 37], but other reports have proposed that TRPV1 activity does not contribute to the responses to formalin [1, 30]. First, we investigated the effects of capsazepine on the nociceptive behaviors induced by formalin. We observed nociceptive behaviors evoked by 5% formalin for 90 min, and we calculated the integral of the time spent in nociceptive behaviors after formalin injection (Fig. 5A). The time spent in nociceptive behaviors during every 5 min period is shown in Fig. 5B–D. In control rats that received the vehicle injection, phase I responses were observed in the initial 15 min period, and the maximum phase I response was observed in the first 5 min period. Phase II responses were observed later than 15 min, and the maximum phase II response was observed in the 30–35 min period (the black lines in Fig. 5B, 5C, 5F, 5G). The injection of capsazepine into the contralateral hind paws of the formalin injection did not show any effect on the responses in either phase (Fig. 5C). The amount of nociceptive behaviors in every 5 min period during both phases I and phase II tended to decrease by the preinjection of capsazepine (30 nmol) into the ipsilateral hind paws compared with those in control rats (Fig. 5B) and rats that received a capsazepine injection into the contralateral hind paws (Fig. 5D). The total time of the nociceptive behaviors of rats that received a capsazepine injection into the ipsilateral hind paws tended to be shorter in both phases than that of control rats and the rats that received a capsazepine injection into the contralateral hind paws (Fig. 5E). We did not examine effects of JNJ-17203212 on formalin responses because of the short effective period suggested in the capsaicin and Hargreave's tests.

The phase I responses of rats that received a clonidine injection into the contralateral hind paws were not different from those of control rats (Fig. 5G). In phase II, the preinjection of clonidine into the contralateral hind paws shifted the period, in which the maximum responses were observed, to later and decreased the amount of the maximum responses, but the total amount of nociceptive behaviors was not different from that of control rats (Fig. 5I). The preinjection of clonidine (25 nmol) into the ipsilateral hind paws tended to reduce the phase I response (Fig. 5F). In contrast, the phase II responses of rats that received a clonidine injection into the ipsilateral hind paws developed later than 25 min after the formalin injection and were sustained during the 90 min recording. The preinjection of clonidine into the ipsilateral hind paws shifted the period, in which the maximum responses were observed, to later and reduced the total time of the phase II response. Compared with rats that received a clonidine injection into the contralateral hind paws, the amount of nociceptive behaviors of rats that received a clonidine injection into the ipsilateral hind paws every 5 min tended to be shorter until 80 min (Fig. 5H). The total amount of nociceptive behaviors of rats that received a clonidine injection into the ipsilateral hind paws was shorter, but not significantly, than that of rats that received a clonidine injection into the contralateral hind paws in both phases (Fig. 5I; $P=0.18$ for phase I, $P=0.073$ for phase II by a *post hoc* Tukey's HSD test). These results show that the shift of the peak of the phase II responses induced by clonidine depends on some systemic effect. In addition, clonidine injected into the ipsilateral hind paws causes analgesia depending on the peripheral effect.

Subtypes of α_2 adrenergic receptor mRNA expressed in DRGs

It is known that α_2 receptors are classified into three major subtypes: α_{2A} , α_{2B} and α_{2C} . To investigate which subtype of α_2 receptor mRNA is expressed at the highest level in DRGs, we assessed the relative levels of α_{2A} , α_{2B} and α_{2C} mRNA using real-time PCR. Figure 5A presents representative amplification curves of fragments of α_2 receptor cDNAs and GAPDH cDNA. The threshold cycle (Ct) of the α_{2C} receptor is smallest followed by those of the α_{2A} and α_{2B} receptors. The Δ Ct of the α_{2A} , α_{2B} and α_{2C} receptors against GAPDH is shown in the right panel of Fig. 6A. It is suggested that α_{2C} receptor mRNA is much more abundant than α_{2A} or α_{2B} in DRGs. The differences in Δ Ct values of the α_{2A} and α_{2B} receptors compared with the α_{2C} receptor are 3.21 ± 0.37 ($n=8$) and 6.24 ± 0.26 ($n=8$), respectively (mean \pm SD).

Coexpression of TRPV1 and α_2 receptors in DRGs

The expression level of α_{2C} receptor mRNA was highest in DRGs according to real-time PCR. To examine whether TRPV1 and α_2 receptors are expressed in the same neurons, we performed immunostaining. It is generally known that DRG neurons are classified into small-, medium- and large-sized neurons. DRG neurons displayed immunoreactivities to the anti-TRPV1 antibody and the anti- α_{2A} and α_{2C} receptor antibodies in consecutive sections as shown in Fig. 6B, 6C. Obvious TRPV1-like immunoreactivity was detected in small- and medium-sized neurons. Small- and medium-sized neurons showed obvious α_{2C} receptor-like immunoreactivity. In small- and medium-sized neurons, TRPV1- and α_{2C} receptor-like immunoreactivities were often detected in the same neurons. Obvious α_{2A} receptor-like immunoreactivity was also detected in small- and medium-sized neurons. Small- and medium-sized neurons showing both TRPV1- and α_{2A} receptor-like immunoreactivities also existed.

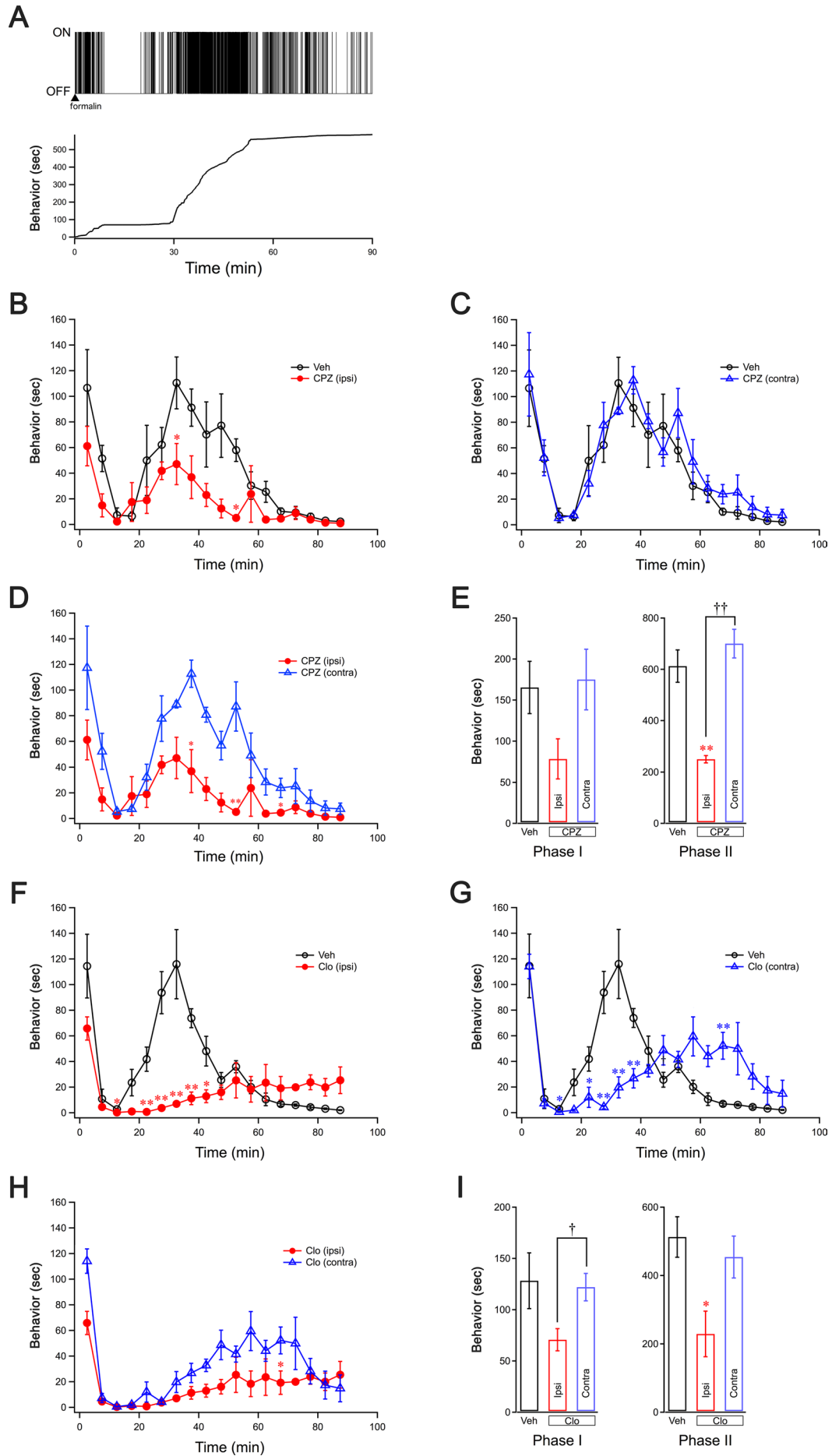


Fig. 5.

Fig. 5. Effects of capsazepine and clonidine on the nociceptive behaviors evoked by formalin. **A.** The representative response of rats to vehicle preinjection into the hind paw 10 min before the formalin injection is shown. Nociceptive behaviors were monitored for 90 min after formalin injection (upper). The upper trace describes the temporal switching of the nociceptive behavior appearance. “ON” and “OFF” in the figure indicate the states of rats showing and not showing nociceptive behaviors, respectively. The cumulative time spent in nociceptive behaviors is plotted against the time after the formalin injection (lower). **B–D.** The time of nociceptive behaviors during every 5 min period is shown by the symbols. As the control experiment, vehicle (Veh, black open circles, n=4) was injected into the hind paws of the formalin injection side. Capsazepine was injected into the ipsilateral (CPZ, ipsi, red closed circles, n=4) or the contralateral (CPZ, contra, blue open triangles, n=4) hind paws of the formalin injection side. * $P < 0.05$, ** $P < 0.01$ by ANOVA with a *post hoc* Tukey’s HSD test in each 5 min period. **E.** The total times spent in nociceptive behaviors in phase I (0–15 min) and phase II (15–90 min) evoked by formalin in rats that received the vehicle injection and capsazepine injection into the ipsilateral and contralateral hind paws. The columns represent the mean \pm the SEM (** $P < 0.01$, † $P < 0.01$ by ANOVA with a *post hoc* Tukey’s HSD test). **F–H.** The time of nociceptive behaviors during every 5 min period is shown by the symbols. As the control experiment, vehicle (Veh, black open circles, n=4) was injected into the hind paws of the formalin injection side. Clonidine was injected into the ipsilateral (Clo, ipsi, red closed circles, n=4) or the contralateral (Clo, contra, blue open triangles, n=4) hind paws of the formalin injection side. * $P < 0.05$, ** $P < 0.01$ by ANOVA with a *post hoc* Tukey’s HSD test in each 5 min period. **I.** The total times spent in nociceptive behaviors in phase I and phase II evoked by formalin with vehicle injection and clonidine injection into the ipsilateral and contralateral hind paws. The columns represent the mean \pm the SEM (* $P < 0.05$, † $P < 0.05$ by ANOVA with a *post hoc* Tukey’s HSD test).

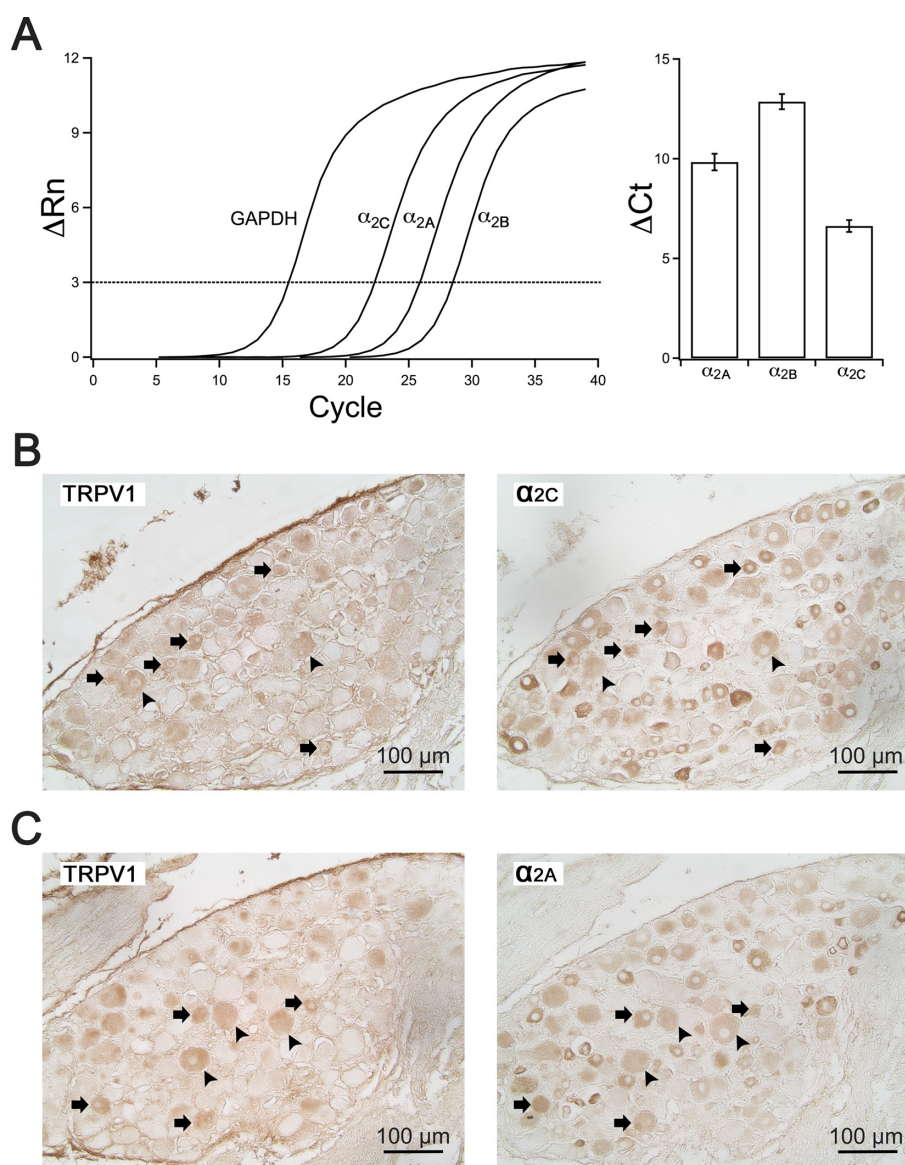


Fig. 6. Subtypes of the α_2 adrenergic receptors detected in dorsal root ganglia. **A.** The real-time PCR amplification curves are shown in the left panel. A threshold was set for 3, and the Ct values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α_2 receptors were obtained. The ΔCt values of α_{2A} (n=8), α_{2B} (n=8) and α_{2C} (n=8) receptors against GAPDH were calculated and are shown in the right panel. The columns represent the mean \pm the standard error of the mean. **B, C.** Images of the immunoreactivities of TRPV1, α_{2A} and α_{2C} receptors in rat dorsal root ganglia. The TRPV1-, α_{2A} receptor- and α_{2C} receptor-like immunoreactivities were visualized by 3,3'-diaminobenzidine. Arrows, positively stained small-sized neurons; arrowheads, positively stained medium-sized neurons.

DISCUSSION

In this study, we examined the effects of adrenergic drugs on several kinds of nociceptive behaviors induced by capsaicin, noxious heat and formalin. It has been reported that the nociceptive behaviors induced by capsaicin are inhibited by capsazepine [32]. We demonstrated that capsazepine and JNJ-17203212, a more specific TRPV1 antagonist, reduced capsaicin-evoked responses in this study, indicating that capsaicin-evoked nociceptive behaviors depend on the activation of TRPV1. Although, we could not find inhibitory effects of JNJ-17203212 on thermal and formalin responses, unfortunately, nociceptive behaviors induced by noxious heat and formalin were inhibited by capsazepine, suggesting a possibility that TRPV1 activation was involved in the nociceptive behaviors evoked by these stimuli. However, since capsazepine has been reported to have inhibitory effects on other molecules, such as low voltage-activated Ca^{2+} channels [20] and HCN channels [28], we cannot exclude the possibility that nonspecific effects of capsazepine contribute to inhibition of these nociceptive responses to heat and formalin. In the capsaicin test, the inhibitory action of clonidine was reversed by yohimbine. Moreover, the injection of clonidine into the contralateral side did not inhibit the nociceptive behaviors evoked by capsaicin. These results indicate that the activation of α_2 receptors at peripheral nerve terminals under the skin of the hind paw suppresses nociceptive behaviors. We tried to assess the effects of dexmedetomidine and ST-91, other α_2 agonists, in the capsaicin test. Since lower doses of dexmedetomidine and ST-91 than those showing analgesic effects caused strong and sustained sedation, we could not assess the analgesic effects of these drugs. Although clonidine has been reported to inhibit tetrodotoxin-resistant voltage-gated Na^+ channels in sensory neurons of rats [14, 22], we could not detect inhibitory effects of clonidine on total Na^+ currents in cultured DRG neurons isolated from adult rats. Moreover, the inhibitory effect of clonidine was not detected on capsaicin responses in the presence of the inhibitory effect of the TRPV1 antagonists, capsazepine and JNJ-17203212. Therefore, we consider that the analgesic action of clonidine on capsaicin responses was caused mainly by its inhibitory action on TRPV1 rather than that on voltage-gated Na^+ channels.

Against our expectations, NA enhanced the nociceptive behaviors evoked by capsaicin, which is inconsistent with results from our previous study showing that NA inhibits TRPV1 currents in cultured rat DRG neurons [19]. On the other hand, Sawynok *et al.* reported that NA enhances capsaicin-evoked nociceptive behaviors [32]. It has also been reported that phentolamine, a nonselective α_1/α_2 antagonist, produces a significant reduction in flinches evoked by capsaicin [32] and that both phentolamine and prazosin, an α_1 antagonist, reduce the mechanical allodynia produced by an intradermal injection of capsaicin [16]. It is likely that the increase in capsaicin responses induced by NA observed in the present study may reflect enhancing effects of α_1 receptors on TRPV1 activity. Since the injection of NA into the contralateral hind paws also increased capsaicin-evoked nociceptive behaviors, we consider that NA injected into the hind paw affects nociceptive behaviors depending on some systemic effect. A potentiate effect of phenylephrine observed in a part of the rats used may also be caused by the systemic effect on α_1 receptors.

TRPV1 is activated by noxious heat ($>42^\circ\text{C}$) [2, 39]. It has been reported that clonidine applied topically via tail immersion showed an analgesic effect in the radiant heat tail-flick test in mice [7]. In the Hargreave's test in this study, both on days 1 and 2, there was no significant difference between the responses of the right and left paws, regardless of the injection of capsazepine or clonidine. Since we found rats showing withdrawal responses with significantly different latencies between the right paws and the left paws, it is possible that variations in the sensitivity of individual paws mask the effects of the applied drugs when comparing responses of the right and left paws statistically. Capsazepine extended the withdrawal latency of the hind paw on the capsazepine-injected side but not on the other side. Although we also tried to examine effects of JNJ-17203212 on responses to heat stimuli, JNJ-17203212 showed no effect on them at all. The reason may be that JNJ-17203212 has a shorter effective period than capsazepine, as found in the capsaicin test. It has been reported that capsazepine had inhibitory effects on other channels than TRPV1, e.g. voltage-gated cation channels, at higher doses. However, since TRPV1 is well known to respond to noxious heat and it is widely accepted to be involved in behavioral responses to heat stimuli and we confirmed that capsazepine at $1\ \mu\text{M}$ does not affect amplitudes of voltage-gated Na^+ currents in rat DRG neurons, we consider that the results in the Hargreave's test suggest that the nociceptive behaviors evoked by heat are likely to be caused, at least partially, by TRPV1 activation in the peripheral sensory nerve. Clonidine also extended the latency of the paw withdrawal responses to heat stimuli on the clonidine-injected side. As described above, we could not detect an inhibitory effect of clonidine on voltage-gated Na^+ channels in electrophysiological recordings. Although we cannot exclude the possibility that inhibitory effects of clonidine on other molecules than TRPV1 contribute to reduction of nociceptive thermal responses, we consider that clonidine may have a peripheral analgesic effect under the skin at least partially depending on the inhibition of TRPV1 activated by noxious heat.

Formalin induces biphasic (phase I and phase II) nociceptive behaviors. Phase I responses are caused by formaldehyde, a principal component of formalin, which directly activates nociceptive molecules on sensory neurons, and phase II responses are induced by secondary inflammation [12, 26, 38]. In this study, capsazepine tended to inhibit nociceptive behaviors in both phases I and phase II. These inhibitory effects were observed unilaterally, implying the possibility that the activation of TRPV1 expressed in peripheral nerve fibers under the skin where formalin is injected is involved in the phase I and II responses. Clonidine also tended to inhibit phase I responses. This analgesic effect was observed in rats that received a clonidine injection into the ipsilateral but not the contralateral hind paws that received the formalin injection, suggesting that the activation of α_2 receptors at the local site of formalin injection caused this analgesic effect. In addition, the injection of clonidine into the contralateral hind paws shifted the maximum responses of the phase II behavior to a later period. This shifting effect was observed regardless of the side of the clonidine injection, suggesting that some systemic effect of clonidine may cause this phenomenon. Some effect on central nervous systems higher than the spinal cord may contribute to this systemic effect. However, the lower amount of phase II responses when clonidine was injected into ipsilateral hind paws than when clonidine was injected into contralateral hind paws seems to be

caused by the peripheral effect. Because of the nonspecific effects of capsazepine and effects of clonidine on other molecules than TRPV1, it is not certain whether unilateral effects of clonidine on phase I and II responses are solely caused by inhibitory actions on TRPV1. However, it may be possible that these peripheral effects observed in the formalin test partially depend on the inhibition of TRPV1 through the activation of α_2 receptors, and that all of the results of the three types of behavioral tests are not inconsistent with the hypothesis that α_2 receptors expressed on peripheral nerve terminals inhibit TRPV1 activity, resulting in analgesia.

The results of real-time PCR are consistent with a report indicating that the α_{2C} receptor is most common, followed by the α_{2A} receptor, and the α_{2B} receptor is rare in DRGs [33]. In addition, immunohistochemical images showed that α_{2A} and α_{2C} receptors are coexpressed with TRPV1 in small- and medium-sized DRG neurons. We tried to immunostain DRG preparations by using an anti- α_{2B} receptor antibody. However, we could not detect obvious α_{2B} receptor-like immunoreactivity. Given these results, in combination with the results of real-time PCR, the expression level of α_{2B} receptors is likely too low to be detected by immunohistochemistry. We also tried to perform immunohistochemical staining of sciatic nerve fibers, but we could not obtain images showing TRPV1- and α_2 receptor-like immunoreactivities with a reliable contrast. In sciatic nerves, the expression density of TRPV1 and α_2 receptors may be too low to be detected with the antibodies used in this study. However, since TRPV1 and α_2 receptors were coexpressed in the neuronal cell bodies in DRGs, it is possible that both TRPV1 and α_2 receptors are transported along the dendrites of the same neurons and are expressed on membranes of the peripheral nerve terminal.

Many studies are found in the literature that indicate that α_2 receptors contribute to analgesia in artificial pain models of rats. For example, clonidine reduces nociceptive responses to noxious mechanical stimuli in rats whose sciatic nerve is injured [18, 29], and venlafaxine blocks thermal hyperalgesia through α_2 receptors in chronic constriction injury model rats [11]. However, the detailed mechanisms of the analgesic effects that occur through α_2 receptors in normal animals have not been well studied. In this study, we found that clonidine inhibited nociceptive behaviors related to the activation of TRPV1 at the peripheral nerve terminals of the primary sensory neuron. We consider that the analgesic mechanism based on the inhibition of TRPV1 by α_2 receptors at the peripheral nerve terminals of the primary sensory neuron is working as well as the central DAS. This novel analgesic mechanism seems to work topically at peripheral nerve terminals under the skin.

From this study, it is clear that an exogenous α_2 agonist (clonidine) reduces nociceptive behaviors depending on its action at the peripheral site of injection. However, it is still unclear whether endogenous ligands, such as NA and adrenaline, utilize the same mechanism and where the adrenergic agonists that activate α_2 receptors at peripheral nerve fibers come from. It has been reported that sympathetic nerves expressing tyrosine hydroxylase, which is a key enzyme in the synthesis of catecholamines, are distributed under the skin [10]. Thus, it may be possible that NA released from sympathetic nerves distributed under the skin acts on primary sensory neurons expressing TRPV1 and α_2 receptors and that NA inhibits TRPV1 activity through α_2 receptors. In addition, adrenaline and/or NA released from the adrenal medulla and circulating in the blood may also contribute to α_2 receptor-mediated analgesia. In this study, we could not demonstrate that NA injected under the skin inhibited the nociceptive behaviors evoked by capsaicin because of some systemic effect *in vivo*. Previously, we reported that NA at concentrations lower than 1 pM inhibits capsaicin-induced TRPV1 currents in cultured DRG neurons [19]. In addition, it has been reported that plasma NA concentrations range between 0.9 and 1.5 nM in rats [15]. Taken together, we consider that it may be possible that adrenalin and/or NA derived from circulating blood inhibit pain caused by TRPV1 activation. We empirically know that the threshold to sense pain rises when the adrenaline concentration in the blood increases under conditions such as physical exercise or emotional excitement. In combination with the central DAS, the peripheral analgesic mechanism proposed in this study may contribute to this phenomenon.

CONFLICT OF INTERESTS. The authors have declared that no competing interests exist.

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