Role of orexin receptor subtypes in the inhibitory effects of orexin-A on potassium chloride-induced increases in intracellular calcium ion levels in neurons derived from dorsal root ganglion of carrageenan-treated rats

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Abstract

We analysed the roles of orexin receptors in the effects of orexin-A on KCl-induced increases in intracellular calcium ion levels ($[Ca^{2+}]_i$) in C-fiber-like small size neurons of rats with experimentally induced inflammation. Carrageenan was injected into the plantar of hind paws. Controls were treated with saline. Paw withdrawal and threshold forces in response to tactile stimuli were determined using von Frey filaments. The $[Ca^{2+}]_i$ in C-fiber-like neurons derived from dorsal root ganglia were visualised by a calcium fluorescent probe. Changes in neuronal $[Ca^{2+}]_i$ were assessed as relative fluorescence intensity (F/F_0). One day after carrageenan injection, paw withdrawal response and paw withdrawal threshold to tactile stimuli were increased and reduced, respectively. KCl loading to neurons from either carrageenan-treated or control rats increased F/F_0 to about 2.0. KCl-induced increases in F/F_0 of carrageenan-treated rats, but not of control rats, were inhibited by orexin-A. The OX_1 and OX_2 receptor antagonist MK-4305, but not the OX_1 receptor antagonist SB334867, counteracted the effects of orexin-A on the KCl-induced increase in F/F_0. These results suggest that OX_2 but not OX_1 receptors mediate the inhibitory effect of orexin-A on KCl-induced increases in $[Ca^{2+}]_i$ in C-fiber-like neurons from rats with inflammation.

Keywords: Orexin receptor; Orexin-A; Intracellular calcium ion levels; Carrageenan; Dorsal root ganglion; Rats

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium ion levels; ANOVA, analysis of variance; EDTA, ethylendiaminetetraacetic acid

Introduction

Orexin-A and orexin-B are neuropeptides that were originally isolated from rat hypothalamus (1, 2). They are known to regulate the sleep/wakefulness cycle and feeding behavior. There are at least two types of orexin receptor subtypes, namely OX_1 and OX_2 receptors. OX_1 (3) and OX_2 (4) receptors are distributed throughout the central nervous system, including the spinal cord. In contrast to orexin-B, which shows lower affinity for OX_1 receptors than for OX_2 receptors, orexin-A shows similar affinities for OX_1 and OX_2 receptors (2).

Orexin-A has been shown to play a stimulatory role in regulating feeding (5) and could be involved in eating disorders such as anorexia nervosa (6). Orexin-A is also known to regulate energy homeostasis (7). In addition to the above effects, orexin-A shows analgesic properties. Thus, intravenous injection of orexin-A induces anti-nociceptive and anti-hyperalgesic effects in mice and rats through stimulation of OX_1 receptors (8). Intrathecal administration of orexin-A also reduced experimentally induced mechanical allodynia in rats through activation of spinal OX_1 receptors (9). These results clearly suggest that orexin-A may inhibit spinal nociceptive neural transmission. However, the mechanisms of action of orexin-A on excitation of sensory neuronal cells derived from experimental animals under nociceptive stimulation remain unknown. The spinal cord receives input from primary sensory neurons, including C-fibers that are excited to transmit peripheral mechanical stimuli (10). Therefore, intrathecally-administered orexin-A could affect the input of primary sensory neurons to the spinal cord.

Intraplantar injection of carrageenan to the rat hind paws is used to induce experimental inflammatory symptoms, including nociceptive behaviour (9). Our preliminary report (11) has shown that potassium chloride (KCl) loading to C-fiber-like small size neurons isolated from dorsal root ganglion (DRG) of rats,

either with or without the intraplantar injection of carrageenan, increased the intracellular calcium ion levels ($[Ca^{2+}]_i$) of these neurons. Furthermore, we have shown that in the presence of orexin-A, the KCl-induced increase in $[Ca^{2+}]_i$ of these small size neurons in rats receiving carrageenan treatment was strongly inhibited (11). Interestingly, orexin-A failed to alter the KCl-induced increase in $[Ca^{2+}]_i$ of these C-fiber-like small size neurons in rats without carrageenan treatment. These results clearly suggest that orexin-A may inhibit depolarisation of C-fiber-like neural cells in DRG derived from rats with inflammation, but not from those without inflammation. Neural cells in DRG were found to express OX_1 (3, 8) and OX_2 (4) receptors. However, the roles of orexin receptor subtypes in the inhibitory effects of orexin-A on KCl-induced increases in $[Ca^{2+}]_i$ in neural cells derived from DRG of carrageenan-treated rats remain unknown. The present study investigates which of these orexin receptor subtypes mediates the effect of orexin-A on excitation of primary sensory neurons from rats with inflammation. Therefore, we studied the effects of selective antagonists at orexin receptor subtypes on orexin-A-induced inhibition of KCl-provoked increases in $[Ca^{2+}]_i$ in cultured DRG cells from carrageenan-treated rats. Our research question was which orexin receptor subtype-selective antagonists counteract orexin-A-induced inhibition of KCl-provoked increases in $[Ca^{2+}]_i$ in cultured DRG cells in this model system.

First, we carried out a detailed analysis of changes in withdrawal behaviour of rat hind paws after carrageenan had been injected subcutaneously into the plantar surface. We used von Frey filaments to apply a tactile stimulus to the centre of the plantar surface of hind paws and evaluated withdrawal threshold and withdrawal response once a day for one week. One day after intraplantar injection of carrageenan, rats already showed significant reduction and increase in paw withdrawal threshold and paw withdrawal response to tactile stimulation of the hind

paws, respectively. Therefore, we next isolated neuronal cells in DRG from rats 24h (one day) after carrageenan treatment. These neuronal cells were placed on glass slips coated with an adhesive on the bottom of an acrylic analytical chamber. Then, KCl loading-induced increases in $[Ca^{2+}]_i$ were visualised by means of confocal laser scanning microscopy using a calcium fluorescent probe. In particular, we focused on small size neuronal cells, a size of approximately 20 μm, because these cells are suggested to be C-fibers (12). Changes in $[Ca^{2+}]_i$ in these small size neurons were assessed by determining relative fluorescence intensity. Finally, we investigated the effects of orexin-A in the presence or absence of MK-4305, an antagonist at OX_1 and OX_2 receptor subtypes, and SB 334867, a selective antagonist at the OX_1 receptor subtype, on KCl-induced increases in relative fluorescence intensity in these neurons.

Materials and methods

Animals

Male Wistar rats (Sankyo Laboratory Service Co., Ltd. Tokyo, Japan), weighing between 225 and 275 g at the start of the experiments, were used. Rats were kept at constant room temperature (23 ± 2 ºC) and relative humidity (55 ± 5%) under a 12 h day:night cycle (light on: 0700 a.m.), with *ad libitum* access to food and water.

Carrageenan treatment

Rats were anaesthetised with sodium pentobarbital (50 mg/kg i.p.) and carrageenan (5 mg; lambda carrageenan; Sigma-Aldrich, St. Louis, MO, USA) suspended in 0.25 ml saline was injected subcutaneously into the plantar surface of hind paws via a 27-gauge needle. The same volume of saline (0.25 ml) was injected subcutaneously into the plantar surface of hind paws in control rats.

These experiments were approved by the Animal Experimentation Committee of Nihon University School of Dentistry at Matsudo (AP14MD006, 2014) and were performed in accordance with national and international guidelines for the care and welfare of animals. All efforts were made to minimise animal suffering and to reduce the number of animals used.

Evaluation of paw withdrawal in response to a tactile stimulus

On the day of behavioural experiments, 1-7 days after intraplantar injection (control: n = 7; carrageenan: n = 7), rats were placed individually on an elevated nylon mesh floor (50 cm square, 30 cm high) at least 1h before application of the tactile stimulus.

Paw withdrawal in response to a tactile stimulus and threshold forces were assessed using von Frey filaments (DanMic Global, San Jose, CA). Thus, sensitivity to a tactile stimulus was evaluated using von Frey filaments with different bending forces (2, 4, 6, 8, 10, 15 and 26 g). Each von Frey filament was applied to the centre of the plantar surface of the hind paws for 3 s, and this was repeated on three occasion per paw. Each of the hind paws of rats was tested individually.

Paw withdrawal behaviour in response to a tactile stimulus was scored as follows: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. Paw withdrawal in response to each filament (10 and 26 g) was determined as the mean scores obtained from hind paws of each rat. Paw movements associated with locomotion or weight shift were not recorded as a response.

The threshold force (g) that induces a slow and/or slight paw withdrawal to the stimulus was also measured daily immediately before carrageenan-treatment (day 0); subsequently, one to seven days after carrageenan-treatment, paw withdrawal threshold was determined as the mean threshold force (g) obtained from hind paws of each rat.

Cell culture

Twenty-four hours after subcutaneous injection of carrageenan into the plantar surface of hind paws, bilateral sciatic ganglia associated with sciatic nerve were carefully removed from rats euthanized by carbon dioxide inhalation. DRG were dissected from lumbar vertebrae between L4 and L6 of the spinal column. Nerve fibers extending from the ganglia were removed under a stereoscopic microscope. The neurons derived from DRG were incubated at 37 ^{o}C in

Ca^{2+}-Mg^{2+}-free Hank's balanced salt solution (HBSS (−)) containing 10% fetal bovine serum (FBS, 5 mg/ml, Sigma-Aldrich Co. Ltd., Tokyo, Japan), 5% penicillin (1000 units/ml) and streptomycin (10000 μg/ml) and collagenase (3 mg/ml, Collagenase Type 1, Wako Ltd., Osaka, Japan) for 120 min with shaking. These neurons were centrifuged at 700 rpm for 3 min and rinsed with fresh HBSS (−) in order to remove enzymes. Subsequently, the neurons derived from DRG were incubated at 37 ºC in fresh HBSS (−) containing trypsin (1 mg/l) for 15 min with shaking. Then, these neurons were centrifuged at 700 rpm for 3 min and rinsed with fresh HBSS (−) in order to remove enzymes.

The isolated neurons were suspended in Hank's balanced salt solution containing Ca^{2+} and Mg^{2+} (HBSS (＋)). The suspension (1 ml) was collected in an acrylic chamber equipped with glass slips on the bottom coated with an adhesive (Cell-Tak, BD Bioscience, Franklin Lakes, USA) and kept in an incubator for 60 min at 37 ºC. This procedure allowed placement of neurons onto the glass slips. The preparation was used for real-time monitoring of neuronal $[Ca^{2+}]_i$ (see next section).

$[Ca^{2+}]_i$ measurement

Real-time alternation of $[Ca^{2+}]_i$ in small size neurons (approximately 15-25 μm in diameter) derived from DRG was visualised by means of confocal laser scanning microscopy using the calcium fluorescent probe fluo-4-acetoxymethyl ester (Fluo-4/AM, Dojindo laboratories, Kumamoto, Japan). Changes in $[Ca^{2+}]_i$ were assessed as the relative fluorescence intensity (F/F_0 %; F: fluorescence intensity at different time points after KCl treatment, F_0: baseline fluorescence intensity).

Briefly, neurons isolated from DRG kept in a acrylic chamber (see previous section) were incubated at 37 ºC in HBSS (＋) containing Fluo-4/AM (2.2 μM) and 0.1% pluronic F127 for 60 min. Subsequently, supernatants were removed and fresh

HBSS (+), used as a bath solution, was added to the acrylic chamber. The chamber was placed on the stage of a confocal microscope (LSM5Exciter-ZEN 2007, Carl Zeiss Micro Imaging Co. Ltd., Jena, Germany) and fluorescence measurements of small size neurons (approximately 15-25 μm in diameter) were carried out at room temperature (20-22 ^{o}C). The excitation wavelength of dye in the cytoplasm of selected neuronal cells was 488 nm, with the fluorescent intensity of images determined at 510 nm. Images were captured by a ZEN lite 2011 image analyser (Carl Zeiss Micro Imaging Co. Ltd., Jena, Germany) with an interval of 1 s. These images were further analysed off-line.

Real-time measurement of $[Ca^{2+}]_i$ in an isolated neuron started with determination of basal fluorescent intensity during a period of at least 60 s. Vehicle, orexin-A, orexin-A + SB 334867 or orexin-A + MK-4305 were added to the bath solution. Then, after 60 s, KCl was added to the bath solution. Changes in fluorescence intensity subsequent to basal fluorescent intensity were recorded for 90 s.

After finishing fluorescence measurements for each neural cell, 1 μM of ionomycin, an ionophore, was applied to confirm neuronal viability.

Drugs

Drugs applied to the isolated neurons were: orexin-A (Pyr-Pro-Leu-Pro-Asp-Cys-Cys-Arg-Gln-Lys-Thr-Cys-Ser-Cys-Arg-Leu-Tyr-Glu-Leu-Leu-His-Gly-Ala-Gly-Asn-His-Ala-Ala-Gly-Ile-Leu-Thr-Leu-NH_2, Peptide Institute, Inc., Osaka, Japan); MK-4305 ([(7R)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl], ChemScene, LLC, NJ, USA); SB 334867 (N-(2-Methyl-6-benzoxazolyl)-N'-1,5-naphthyridin-4-yl urea, Tocris Bioscience, Ellisville, MO, USA); ionomycin (Sigma-Aldrich, St. Louis, MO, USA). These

drugs were dissolved in HBSS (+) with a small amount of dimethyl sulfoxide (<0.1%) added to the bath solution. Each dose indicates the total amount (nmol) in the 2 ml analytical chamber. Doses of KCl and ionomycin indicate the concentration (mM) in the analytical chamber. Doses used were determined by a series of pilot experiments based on the outcome of previously reported studies (13-17). The vehicle for selected doses of orexin-A (20 pmol), MK-4305 (200 pmol) and SB 334867 (2 pmol) in the present study did not alter baseline fluorescence intensity. Doses of orexin-A higher than 20 pmol, MK-4305 higher than 200 pmol, and SB 334867 higher than 2 pmol were not used in the present study because these doses might enhance neuronal fluorescence intensity through non-specific mechanisms that include influences on osmotic pressure.

Statistical analysis

Data were expressed as mean ± S.E.M. Comparisons of paw withdrawal response, paw withdrawal threshold and relative fluorescence values were carried out by one-way analysis of variance (ANOVA) followed by post hoc Scheffé's test, where appropriate. A probability level of $P < 0.05$ was taken as statistically significant.

Results

Intraplantar injection of carrageenan induces an increase in paw withdrawal response and a decrease in paw withdrawal threshold to tactile stimulation

Rats treated with carrageenan showed an increase in mean paw withdrawal response to tactile stimulation applied by a filament with 10 g bending force at one day after carrageenan treatment [Fig. 1A; day 0 (control): 0.0 ± 0.0 (n = 7); day 1: treatment: 2.3 ± 0.4 (n = 7)]. The carrageenan-induced increase in mean paw withdrawal response lasted over an observation period of seven days (Fig. 1A; one-way ANOVA, $F_{(7, 48)} = 10.9$, $P < 0.001$). Post hoc Scheffé's test revealed that responses obtained on day 1 to day 5 each differed ($P < 0.05$) from baseline paw withdrawal responses (day 0). Post hoc Scheffé's test also showed that paw withdrawal responses obtained on day 1 to day 7 did not differ from each other. Baseline paw withdrawal responses of control rats to the tactile stimulus applied by the filament with 10 g bending force remained unchanged over the observation period (from day 0 to day 7; Fig. 1A).

Mean paw withdrawal response to a filament with a 26g bending force was also increased in rats one day after carrageenan treatment [Fig. 1B; day 0: 1.0 ± 0.0 g (n = 7); day 1: treatment: 3.0 ± 0.0 (n = 7)]. The carrageenan-induced increase in mean paw withdrawal response lasted over an observation period of seven days (Fig. 1B; one-way ANOVA, $F_{(7, 48)} = 168000$, $P < 0.001$). Post hoc Scheffé's test revealed that thresholds obtained on day 1 to day 7 each differed ($P < 0.05$) from baseline paw withdrawal responses (day 0). Post hoc Scheffé's test also showed that paw withdrawal responses obtained on day 1 to day 7 were not significantly different. Baseline paw withdrawal responses of control rats to the tactile stimulus applied by the filament with 26 g bending forces were also unchanged over this period (Fig.

1B).

Subcutaneous injection of carrageenan into the plantar surface of hind paws of rats induced a large decrease in mean paw withdrawal threshold one day after carrageenan treatment [Fig. 1C; day 0: 26.0 ± 0.0 g (n = 7), day 1: 8.6 ± 1.3 g (n = 7)]. This carrageenan-induced decrease in mean paw withdrawal threshold lasted over an observation period of seven days (Fig. 1C; one-way ANOVA, $F_{(7, 48)} = 55.9$, $P < 0.001$). Post hoc Scheffé's test revealed that thresholds obtained on day 1 to day 7 differed ($P < 0.05$) from baseline paw withdrawal threshold (day 0). Post hoc Scheffé's test also showed that paw withdrawal thresholds obtained on day 1 to day 7 did not differ from each other. Paw withdrawal thresholds of control rats were unchanged throughout the observation period (26.0 ± 0.0 g, n = 7, Fig. 1C).

Orexin-A inhibits KCl-induced increases in relative fluorescence intensity in isolated neurons from carrageenan-treated rats

The diameter of analysed neurons derived from control and carrageenan-treated rats were 23.3 ± 0.7 μm (n = 20) and 23.7 ± 0.8 μm (n = 22), respectively.

KCl (25 mM) increased relative fluorescence intensity in isolated neurons derived from DRG of control rats to 2.0 ± 0.1 (Fig. 2A) and this was not affected by orexin-A (20 pmol) in the presence of MK-4305 (200 pmol) and SB334867 (2 pmol) (Figs. 2A and B).

KCl (25 mM) also increased relative fluorescence intensity in isolated neurons derived from DRG of carrageenan-treated rats to 2.1 ± 0.1 (Fig. 3A) but, in contrast, this increase was inhibited by orexin-A (20 pmol) to 1.3 ± 0.02 [Figs. 3A and B; n = 6; one-way ANOVA, $F_{(3, 18)} = 24.6$, $P < 0.001$].

Figs. 3A and B show that the inhibitory effect of orexin-A on the KCl (25

mM)-induced increase in relative fluorescence intensity in neurons from carrageenan-treated rats was inhibited (n = 6; one-way ANOVA, $F_{(3, 18)} = 24.6$, $P < 0.001$) by co-administration of MK-4305 (200 pmol), but not by SB334867 (2 pmol). Post hoc Scheffé's test revealed that the effects of orexin-A differed from vehicle ($P < 0.05$) and that the effects of co-administration of orexin-A and MK-4305 differed from the effects of orexin-A alone ($P < 0.05$).

Effects of ionomycin

Ionomycin (1 μM) strongly enhanced relative fluorescence intensity in all tested neuronal cells to 3.4-5.0 (data not shown).

Discussion

The behavioural analyses revealed that the paw withdrawal response was increased one day after carrageenan treatment (Figs. 1A and B) and that the paw withdrawal threshold decreased (Fig. 1C) in response to a tactile stimulus delivered to the hind paws. The present study focused on small neuronal cells (approximately 20 μm) derived from DRG of rats one day after intraplantar injection of carrageenan because these cells are thought to be C-fibers (12). Ionomycin, a selective calcium ionophore that mobilises intracellular calcium ions, markedly enhanced relative fluorescence intensity in all the cells that were tested, demonstrating that they were viable throughout the duration of the experiments.

Orexin-A is known to increase the number of action potentials evoked by a series of current pulses in rat DRG neurons (13). The present study analysed the effects of orexin-A on excitation of C-fiber-like small size neural cells derived from rat DRG. The doses of orexin-A and/or orexin receptor antagonists that did not alter baseline fluorescence intensity (basal $[Ca^{2+}]_i$). Two orexin receptor subtypes are known, OX_1 and OX_2 (2). Orexin-A activates both OX_1 and OX_2 receptor subtypes (2). Doses of MK-4305 (200 pmol), an antagonist at both OX_1 and OX_2 receptors, and SB 334867 (2 pmol), a selective antagonist at OX_1 receptors, were the highest that did not alter the basal fluorescent intensity of neuronal cells. Infusion of a medium containing a high dose of KCl (25 mM) increased $[Ca^{2+}]_i$ in isolated neural cells derived from DRG of control rats and this was not affected by orexin-A or orexin receptor antagonists. In contrast, orexin-A strongly inhibited the KCl-induced increase in $[Ca^{2+}]_i$ in isolated neural cells derived from DRG of carrageenan-treated rats. The present results suggest that orexin-A stimulates OX_2 but not OX_1 receptors on the surface of neural cells and inhibits KCl loading-induced increases in $[Ca^{2+}]_i$ in neural cells derived from carrageenan-treated rats. This is because MK-4305, which

antagonises both OX_1 and OX_2 receptors, reduced the inhibitory effect of orexin-A on KCl loading-induced increases in $[Ca^{2+}]_i$ in neural cells derived from carrageenan-treated rats. Furthermore, SB 334867, which selectively antagonises OX_1 receptors, failed to reduce the inhibitory effects of orexin-A on KCl loading-induced increases in $[Ca^{2+}]_i$ in neural cells. In order to provide direct evidence that OX_2 receptors mediate inhibition of orexin-A on KCl-induced increases in neuronal $[Ca^{2+}]_i$, further studies are necessary using selective OX_2 receptors antagonist.

As already mentioned, orexin-A failed to alter KCl-induced increases in $[Ca^{2+}]_i$ in cells isolated from control rats but suppressed such increases in neurons derived from carrageenan-treated rats via activation of OX_2 receptors. Thus, the present results further indicate that the sensitivity of C-fiber-like small neurons to orexin-A was enhanced by carrageenan treatment. The increase in sensitivity to exogenously applied orexin-A in neural cells derived from carrageenan-treated rats could be explained by supersensitivity of OX_2 receptor induced by decreased stimulation of OX_2 receptors by endogenous orexin-A and/or orexin-B. Indeed, the spinal cord has been shown to receive a robust orexin-containing neural projection from the hypothalamus (18). Bingham et al. (8) have suggested that central terminals of primary afferents in the spinal cord express orexin receptors that could be activated by orexin-A and/or orexin-B released from hypothalamo-spinal fibers. They further suggest that orexin-containing fibers projecting from the hypothalamus to the spinal cord operate as a descending inhibitory system for spinal nociceptive neural transmission. The present findings support the suggestion of Bingham et al. (8) that activity in a descending orexinergic inhibitory system, involving OX_2 receptors on neural cells derived from dorsal root ganglia, is decreased by carrageenan-induced inflammation.

Spinally applied orexin-A has been shown to induce antinociceptive effects through activation of spinal OX_1 receptors in diabetic rats (19) and carrageenan-treated rats (9). The detailed synaptic location of these OX_1 receptors in spinal nociceptive and/or antinociceptive neural transmission remains unknown. The present results suggest that OX_1 receptors, putatively localised on central terminals of primary sensory neurons, are not involved in the production of spinally applied orexin-A-induced antinociceptive effects because orexin-A inhibited excitation of C-fiber-like neural cells via OX_2 and not OX_1 receptors. In agreement with these notions, OX_2 but not OX_1 receptors have been thought to play an inhibitory role in spinal nociceptive transmission of neonatal rats (20).

In summary, the present results show that orexin-A inhibits KCl loading-induced increases in $[Ca^{2+}]_i$ in C-fiber-like small neurons derived from DRG of rats one day after the intraplantar injection of carrageenan to the hind paws. These results suggest that OX_2 but not OX_1 receptors mediate the inhibitory effect of orexin-A on KCl-induced increases in $[Ca^{2+}]_i$ in neurons derived from DRG of carrageenan-treated rats.

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Figure legends

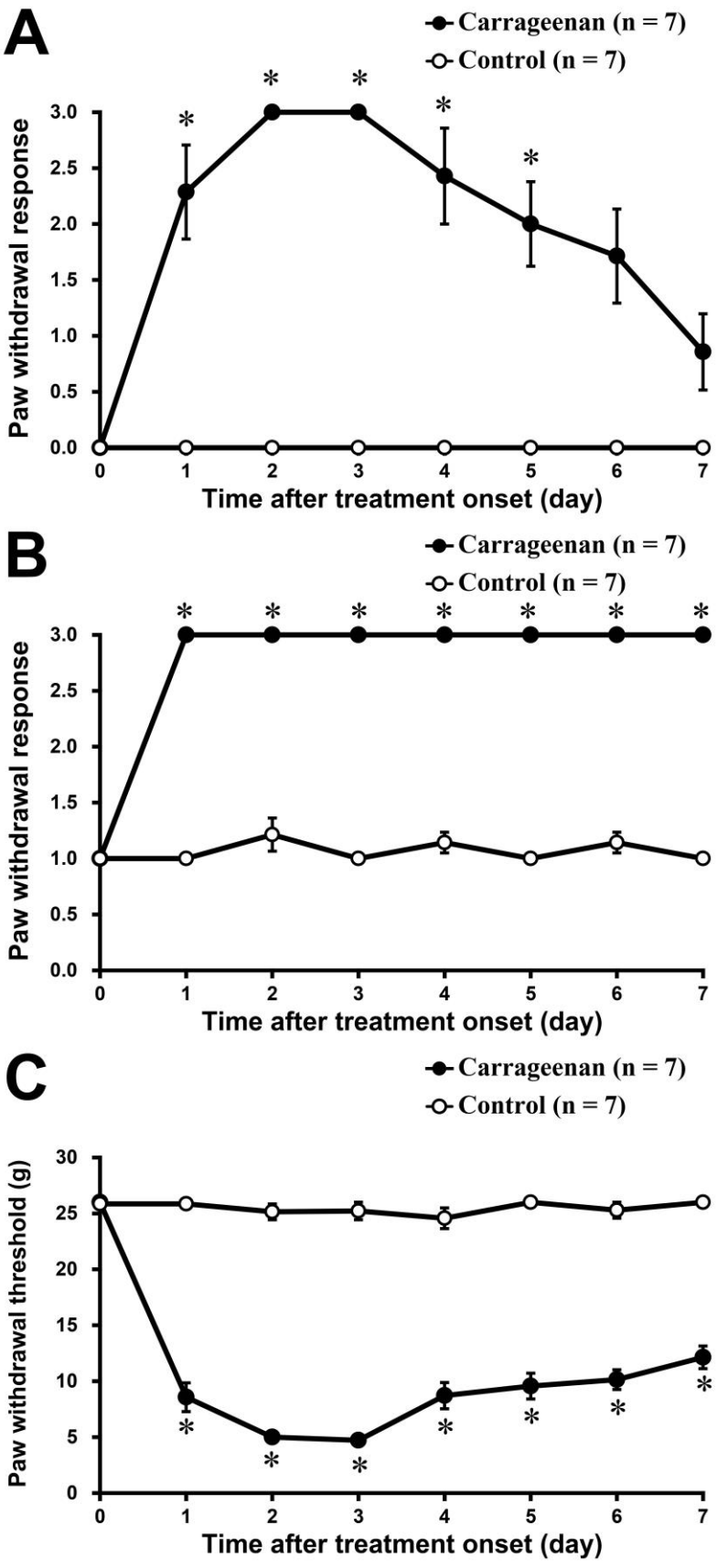

Fig. 1

(A and B) Changes in paw withdrawal in response to a tactile stimulus of carrageenan treated-rats. Data are expressed as means of following scores to evaluate paw withdrawal response: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. Vertical bars indicate S.E.M. The tactile stimulus was applied by using filaments with two different bending forces, namely 10 g (A) and 26 g (B). Carrageenan (5 mg) was suspended in 0.25 ml saline and administered subcutaneously into the plantar surface of hind paws. Control rats received subcutaneous administration of the same volume of saline (0.25 ml) into the plantar surface of hind paws. Asterisks indicate those comparisons per time point between baseline paw withdrawal response (day 0) and responses obtained on days 1 to 7 that are statistically significant (* $P < 0.05$, post hoc Scheffé's test).

(C) Changes in paw withdrawal threshold (g) of carrageenan treated-rats. Data are expressed as mean paw withdrawal threshold (g) of hind paws after subcutaneous administration of carrageenan (5 mg) suspended in 0.25 ml saline into the plantar surface of hind paws. Control rats were received subcutaneous administration of the same volume of saline (0.25 ml) into the plantar surface of hind paws. Vertical bars indicate S.E.M. Asterisks indicate those comparisons per time point between baseline paw withdrawal threshold (day 0) and thresholds obtained on days 1 to 7 that are statistically significant (* $P < 0.05$, post hoc Scheffé's test).

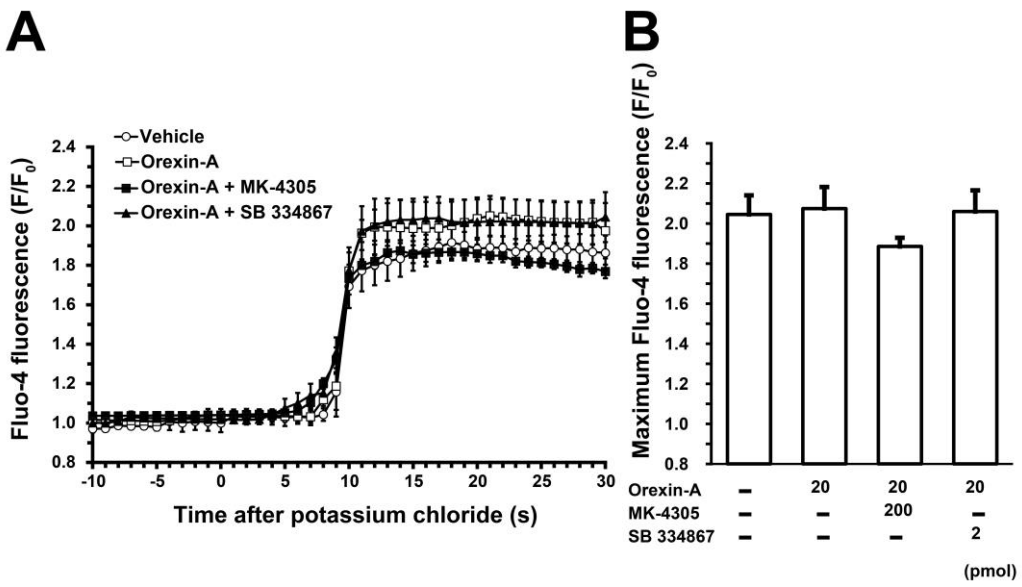

Fig. 2

(A) Effects of vehicle and orexin-A (20 pmol) with or without MK-4305 (200 pmol) or SB 334867 (2 pmol) on KCl (25 mM)-induced increases in relative fluorescence values F/F_0 detected in DRG neurons from control rats. F denotes neuronal fluorescence intensity at each time point; F_0 denotes baseline neuronal fluorescence intensity. Data are expressed as mean changes in 1 s lapse time after addition of KCl (25 mM) to the bath solution in the analytical chamber. Vertical bars indicate S.E.M. KCl was applied to the bath solution in the analytical chamber at 0 s. Vehicle, orexin-A, orexin-A + SB 334867 or orexin-A + MK-4305 were applied to the bath solution in the analytical chamber 60 s before the KCl treatment. (B) Mean values of maximum F/F_0 shown in Fig. 2A. Vertical bars indicate S.E.M.

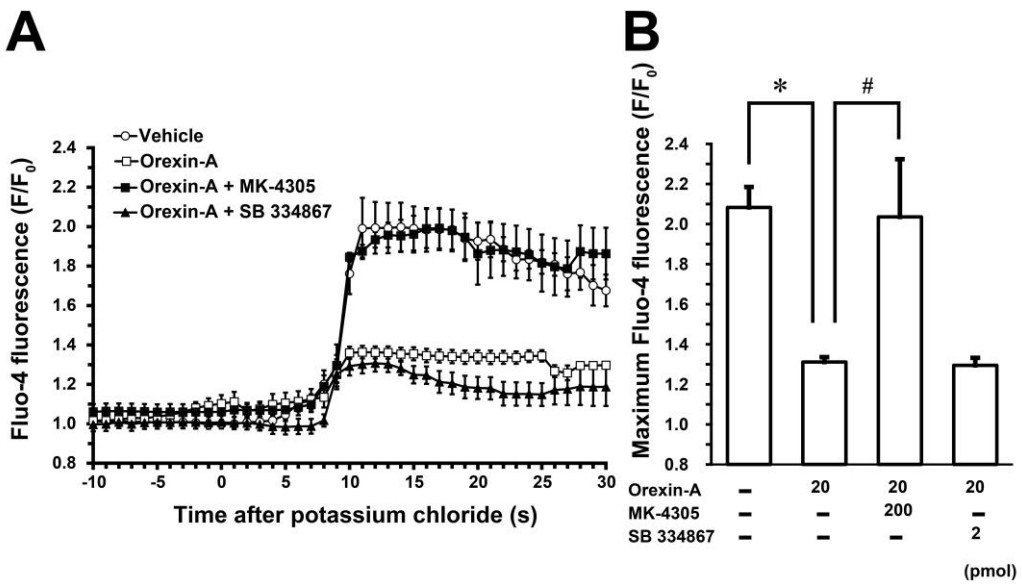

Fig. 3

(A) Effects of vehicle and orexin-A (20 pmol) with or without MK-4305 (200 pmol) or SB 334867 (2 pmol) on KCl (25 mM)-induced increase in relative fluorescence values F/F_0 detected in DRG neurons from carrageenan-treated rats. F denotes neuronal fluorescence intensity at each time point; F_0 denotes baseline neuronal fluorescence intensity. Data are expressed as mean changes in 1 s lapse time after addition of KCl (25 mM) to the bath solution in the analytical chamber. Vertical bars indicate S.E.M. KCl was applied to the bath solution in the analytical chamber at 0 s. Vehicle, orexin-A, orexin-A + SB 334867 or orexin-A + MK-4305 were applied to the bath solution in the analytical chamber 60 s before the KCl treatment. (B) Mean values of maximum F/F_0 shown in Fig. 3A. Vertical bars indicate S.E.M. Asterisk indicates the comparison between vehicle and orexin-A that is statistically significant (* $P < 0.05$, post hoc Scheffé's test). Hash sign indicates the comparison between orexin-A and co-administration of orexin-A + MK-4305 that is statistically significant ($^{\#}$ $P < 0.05$, post hoc Scheffé's test).