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 neurons of rats with inflammation induced by intraplantar injection of carrageenan into the hind paw. Controls were treated with saline. Paw withdrawal and threshold forces in response to tactile stimuli were determined using von Frey filaments. [Ca^{2+}]i in C-fiber-like neurons derived from dorsal root ganglia was visualised using a calcium fluorescence probe. Changes in neuronal [Ca^{2+}]i were assessed as relative fluorescence intensity (F/F_0). One day after carrageenan injection, the paw withdrawal response to tactile stimuli and the paw withdrawal threshold were increased and reduced, respectively. KCl loading of 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, but not 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 of rats with inflammation.

Keywords: orexin receptor; orexin-A; intracellular calcium ion level; carrageenan; dorsal root ganglion; rat.

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 both (2).

Orexin-A has been shown to play a stimulatory role in feeding regulation (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; intravenous injection of orexin-A exerts anti-nociceptive and anti-hyperalgesic effects in mice and rats through stimulation of OX_1 receptors (8). Intrathecal administration of orexin-A also reduces 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 into the rat hind paws is a method used to induce experimental inflammatory symptoms, including nociceptive behaviour (9). Our preliminary study (11) has shown that potassium chloride (KCl) loading of C-fiber-like small neurons isolated from the dorsal root ganglion (DRG) of rats, either with or without intraplantar injection of carrageenan, increases the intracellular level of calcium ions ([Ca^{2+}]) in these neurons. Furthermore, we have shown that in the presence of orexin-A, the KCl-induced increase of [Ca^{2+}] in these small neurons of rats receiving carrageenan is strongly inhibited (11). Interestingly, in rats without carrageenan treatment, orexin-A failed to alter the KCl-induced increase of [Ca^{2+}] in these C-fiber-like small neurons. These results clearly suggest that orexin-A may inhibit depolarisation of C-fiber-like neural cells in the DRG of rats with inflammation, but not in those from rats without inflammation. Neural cells in the DRG were found to express OX1 (3,8) and OX2 (4) receptors. However, the roles of orexin receptor subtypes in the inhibitory effects of orexin-A on the KCl-induced increase of [Ca^{2+}], in neural cells derived from the DRG of carrageenan-treated rats have remained unclear. Therefore, we studied the effects of selective antagonists of orexin receptor subtypes on orexin-A-induced inhibition of the KCl-provoked increase of [Ca^{2+}], in cultured DRG cells from carrageenan-treated rats. The null hypothesis of the present study was that orexin receptor subtype-selective antagonists would fail to alter orexin-A-induced inhibition of the KCl-provoked increase of [Ca^{2+}], in cultured DRG cells from carrageenan-treated rats.

First, we carried out a detailed analysis of changes in hind paw withdrawal behaviour of rats 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 the withdrawal threshold and withdrawal response once a day for one week. As early as 1 day after intraplantar injection of carrageenan, rats showed a significant reduction and increase of the paw withdrawal threshold and paw withdrawal response to tactile stimulation of the hind paws, respectively. Therefore, we next isolated neuronal cells from the DRG of rats 24 h (1 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+}], were visualised using confocal laser scanning microscopy with a calcium fluorescent probe. In particular, we focused on small neuronal cells measuring approximately 20 μm in diameter, because these cells are suggested to be C-fibers (12). Changes of [Ca^{2+}], in these small neurons were assessed by determining the relative fluorescence intensity. Finally, we investigated the effects of orexin-A in the presence or absence of MK-4305, an antagonist of OX1, and OX2, receptor subtypes, and SB 334867, a selective antagonist of the OX1 receptor subtype, on KCl-induced increases of 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 a constant room temperature (23 ± 2°C) and relative humidity (55 ± 5%) under a 12-h day:night cycle (lights on: 0700 h), 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 the both hind paws via a 27-gauge needle. In control rats, the same volume of saline (0.25 mL) was injected subcutaneously into the plantar surface of the hind paws.

These experiments were approved by the Animal Experimentation Committee of Nihon University School of Dentistry at Matsudo (AP14MD006) 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 the behavioural experiments, 1-7 days after carrageenan treatment, rats were placed individually on an elevated nylon mesh floor (50 cm square, 30 cm high) at least 1 h 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, USA). Sensitivity to a tactile stimulus was evaluated using von Frey filaments
with different bending forces (10 and 26 g). Each von Frey filament was applied to the centre of the plantar surface of the hind paw for 3 s, and this was repeated on three occasions 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 was determined as the mean score 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 induced a slow and/or slight paw withdrawal in response to the stimulus was also measured daily immediately before carrageenan treatment (day 0); subsequently, one to seven days after carrageenan treatment, the paw withdrawal threshold was determined as the mean threshold force (g) obtained from the hind paws of each rat.

**Cell culture**

Twenty-four hours after subcutaneous injection of carrageenan into the plantar surface of the hind paws, the bilateral sciatic ganglia associated with the sciatic nerve were carefully removed from the rats after euthanasia by carbon dioxide inhalation. The DRG was dissected from the 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 the DRG were incubated at 37°C in Ca2+-Mg2+-free Hank's balanced salt solution containing trypsin (1 mg/L) for 15 min with shaking. The neurons were then incubated at 37°C in fresh HBSS (−) containing trypsin and collagenase (3 mg/mL), penicillin (1,000 units/mL) and streptomycin (10,000 μg/mL), 5% fetal bovine serum (FBS, 10%), 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. The 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 kept in an incubator for 60 min at 37°C. This procedure allowed placement of neurons onto the glass slips. The preparation was used for real-time monitoring of neuronal [Ca2+], (see next section).

**[Ca2+]i measurement**

Real-time alteration of [Ca2+]i, in small neurons (approximately 15-25 μm in diameter) derived from the DRG was visualised by means of confocal laser scanning microscopy using the fluorescent calcium probe fluo-4-acetoxymethyl ester (Fluo-4/AM, Dojindo Laboratories, Kamimashiki, Japan). Changes in [Ca2+], were assessed as the relative fluorescence intensity (F/F0; F: fluorescence intensity at different time points after KCl treatment, F0: baseline fluorescence intensity).

Briefly, neurons isolated from the DRG kept in an acrylic chamber (see previous section) were incubated at 37°C in HBSS (+) containing Fluo-4/AM (2.2 μM) and 0.1% pluronic F127 for 60 min. Subsequently, the 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 the fluorescence intensity of the images was determined at 510 nm. Images were captured by a ZEN lite 2011 image analyser (Carl Zeiss Micro Imaging Co. Ltd.) at intervals of 1 s. These images were further analysed off-line.

Real-time measurement of [Ca2+]i in each isolated neuron started with determination of basal fluorescence 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 and then changes in fluorescence intensity subsequent to basal fluorescence intensity were recorded for 90 s.

After completing the fluorescence measurements for each neural cell, 1 μM ionomycin, an ionophore, was applied for confirmation of 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-NH2, Peptide Institute, Inc., Osaka, Japan); MK-4305 (([7R]-4-(5-chloro-1,3-benzoazol-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); an 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 the 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 have enhanced neuronal fluorescence intensity through non-specific mechanisms, including 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 to indicate statistical significance.

Results
Intraplantar injection of carrageenan induces an increase in the paw withdrawal response to tactile stimulation and a decrease in the paw withdrawal threshold
Rats treated with carrageenan showed an increase in the 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 the mean paw withdrawal response persisted for 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 days 1 to 5 all differed ($P < 0.05$) from the baseline paw withdrawal responses (day 0). Post hoc Scheffé’s test also showed that the paw withdrawal responses obtained on days 1 to 7 did not differ from each other. In control rats, the baseline paw withdrawal responses to the tactile stimulus applied using the filament with a 10-g bending force remained unchanged over the observation period (from day 0 to day 7; Fig. 1A).

Fig. 1 (A and B) Changes in the paw withdrawal response to a tactile stimulus in carrageenan-treated rats. Data are expressed as means of the following scores used to evaluate the 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 using filaments with two different bending forces, 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 the hind paws. Control rats received subcutaneous administration of the same volume of saline (0.25 mL) into the plantar surface of the hind paws. (C) Changes in the paw withdrawal threshold (g) of carrageenan-treated rats. Data are expressed as the mean paw withdrawal threshold (g) of the hind paws after subcutaneous administration of carrageenan (5 mg) suspended in 0.25 mL saline and administered subcutaneously into the plantar surface. Control rats received subcutaneous administration of the same volume of saline (0.25 mL) into the plantar surface of the hind paws. Vertical bars indicate S.E.M.
The mean paw withdrawal response to a filament with a 26-g bending force was also increased in rats 1 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 the mean paw withdrawal response persisted for seven days (Fig. 1B; one-way ANOVA, F(7, 48) = 168000, P < 0.001). Post hoc Scheffé’s test revealed that the thresholds obtained on days 1 to 7 all differed (P < 0.05) from the baseline paw withdrawal responses (day 0). Post hoc Scheffé’s test also showed that the paw withdrawal responses obtained on days 1 to 7 did not differ significantly. In control rats, the baseline paw withdrawal responses to the tactile stimulus applied by a filament with a 26-g bending force were also unchanged over this period (Fig. 1B).

Subcutaneous injection of carrageenan (5 mg) into the plantar surface of the hind paws induced a large decrease in the mean paw withdrawal threshold 1 day after carrageenan treatment (Fig. 1C; day 0: 26.0 ± 0.0 g [n = 7], day 1: treatment: 8.6 ± 1.3 g [n = 7]). This carrageenan-induced decrease in the mean paw withdrawal threshold persisted for seven days (Fig. 1C; one-way ANOVA, F(7, 48) = 55.9, P < 0.001). Post hoc Scheffé’s test revealed that the thresholds obtained on days 1 to 7 differed (P < 0.05) from the baseline paw withdrawal threshold (day 0). Post hoc Scheffé’s test also showed that the paw withdrawal thresholds obtained on days 1 to 7 did not differ from each other. The 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 approximate diameters of the analysed neurons from control and carrageenan-treated rats were 23.7 ± 0.8 μm (n = 22) and 23.3 ± 0.7 μm (n = 20), respectively.

KCl (25 mM) increased the relative fluorescence intensity in isolated neurons derived from the 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) (Fig. 2A, B).

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

Figures 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 those of vehicle \((P < 0.05)\) and that the effects of co-administration of orexin-A and MK-4305 differed from those of orexin-A alone \((P < 0.05)\).

**Effects of ionomycin**

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

**Discussion**

Our behavioural analyses revealed that the paw withdrawal response was increased 1 day after carrageenan treatment (Fig. 1A, B) and that the paw withdrawal threshold was 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) removed from the DRG of rats 1 day after intraplantar injection of carrageenan, as these cells are thought to be C-fibers (12). Ionomycin, a selective calcium ionophore that mobilises intracellular calcium ions, markedly enhanced the relative fluorescence intensity in all of the cells 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 investigated the effects of orexin-A on excitation of C-fiber-like small neural cells derived from the DRG of rats treated with a low dose of orexin-A and/or orexin receptor antagonists that did not alter the baseline fluorescence intensity (basal \([Ca^{2+}]_i\)). Two orexin receptor subtypes are known: OX1 and OX2 (2). Orexin-A activates both OX1 and OX2 receptor subtypes (2). Doses of MK-4305 (200 pmol), an antagonist of both OX1 and OX2 receptors, and SB 334867 (2 pmol), a selective antagonist of OX1 receptors, were the highest that did not alter the basal fluorescence 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 the DRG of control rats, and this was not affected by orexin-A or orexin receptor antagonists. In contrast, orexin-A strongly inhibited the KCl loading-induced increases in \([Ca^{2+}]_i\) in neural cells derived from carrageenan-treated rats. The present results suggest that orexin-A stimulates OX2, but not OX1 receptors on the surface of neural cells and inhibits KCl loading-induced increases of \([Ca^{2+}]_i\) in neural cells derived from carrageenan-treated rats. This is because MK-4305, which antagonises both OX1 and OX2 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 OX1 receptors, failed to reduce the inhibitory effects of orexin-A on the KCl loading-induced increases of [Ca\(^{2+}\)] in neural cells. In order to provide direct evidence that OX2 receptors mediate the inhibitory effect of orexin-A on KCl-induced increases in neuronal [Ca\(^{2+}\)], further studies using selective OX2 receptor antagonists will be necessary.

As described above, orexin-A failed to alter the KCl-induced increases of [Ca\(^{2+}\)] in cells isolated from control rats, but suppressed such increases in neurons derived from carrageenan-treated rats via activation of OX2 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 the OX2 receptor induced by decreased stimulation of OX1 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 OX2 receptors on neural cells derived from DRG, is decreased by carrageenan-induced inflammation.

Spinally applied orexin-A has been shown to induce antinociceptive effects through activation of spinal OX2 receptors in diabetic rats (19) and carrageenan-treated rats (9). The precise synaptic location of these OX2 receptors in the context of spinal nociceptive and/or antinociceptive neural transmission remains unknown. The present results suggest that OX1 receptors, putatively localised on the central terminals of primary sensory neurons, are not involved in the production of spinally applied orexin-A-induced antinociceptive effects because orexin-A inhibited the excitation of C-fiber-like neural cells via OX2, and not OX1 receptors. In agreement with these notions, OX2, but not OX1 receptors have been thought to play an inhibitory role in spinal nociceptive transmission in neonatal rats (20).

In summary, the present results show that orexin-A inhibits KCl loading-induced increases of [Ca\(^{2+}\)], in C-fiber-like small neurons from the DRG of rats 1 day after intraplantar injection of carrageenan into the hind paws of rats. These results suggest that OX2, but not OX1 receptors mediate the inhibitory effect of orexin-A on KCl-induced increases of [Ca\(^{2+}\)] in neurons derived from the DRG of carrageenan-treated rats.

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**Conflict of interest**
The authors have no conflict of interest to declare.

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