Orexins Activates Protein Kinase C-Mediated Ca^{2+} Signaling in Isolated Rat Primary Sensory Neurons

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Summary

Previous results have suggested that orexins causes a rise of intracellular free calcium ($[Ca^{2+}]_i$) in cultured rat dorsal root ganglion (DRG) neurons, implicating a role in nociception, but the underlying mechanism is unknown. Hence, the aim of the present study was to investigate whether the orexins-mediated signaling involves the PKC pathways in these sensory neurons. Cultured DRG neurons were loaded with 1 µmol Fura-2 AM and $[Ca^{2+}]_i$ responses were quantified by the changes in 340/380 ratio using fluorescence imaging system. The orexin-1 receptor antagonist SB-334867-A (1 µM) inhibited the calcium responses to orexin-A and orexin-B (59.1±5.1 % vs. 200 nM orexin-A, n=8, and 67±3.8 % vs. 200 nM orexin-B, n=12, respectively). The PKC inhibitor chelerythrine (10 and 100 µM) significantly decreased the orexin-A (200 nM)-induced $[Ca^{2+}]_i$ increase (59.4±4.8 % P<0.01, n=10 and 4.9±1.6 %, P<0.01, n=9) versus response to orexin-A). It was also found that chelerythrine dose-dependently inhibited the $[Ca^{2+}]_i$ response to 200 nM orexin-B. In conclusion, our results suggest that orexins activate intracellular calcium signaling in cultured rat sensory neurons through PKC-dependent pathway, which may have important implications for nociceptive modulation and pain.

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Introduction

Orexin A and B (or Hypocretins 1 and 2) are two new neuropeptides synthesized by neurons in the lateral hypothalamus and characterized as ligands of two "orphan" receptors (Sakurai *et al.* 1998). They were originally believed to play an important role in appetite control, but widespread projections of their axons in the brain led to the idea and later discovery that these neuropeptides have multiple physiological functions including regulation of food intake, neuroendocrine system, energy metabolism, blood pressure, body temperature and sleep-wake cycle (Matsuki *et al.* 2008). Evidence emerges that orexins may also modulate pain transmission (Bingham *et al.* 2001, Holland and Goadsby 2007, Yan *et al.* 2008).

To date there are two identified receptors, orexin 1 (OX_1R) and orexin 2 (OX_2R) (also known as hypocretin-1 and -2 receptors) (Sakurai *et al.* 1998, Ammoun *et al.* 2003), which are distributed throughout the areas of the body innervated by orexinergic neurons (Matsuki *et al.* 2008). The OX_1R and OX_2R are coupled to G proteins (Sakurai *et al.* 1998) and ligand binding generally leads to excitatory effects. The rat and human receptors display 94 % and 95 % homology for the OX_1R and OX_2R, respectively, suggesting that they are highly conserved between mammalian species (Sakurai *et al.* 1998). Radioligand-binding studies using ^{125}J-orexin-A have shown a higher affinity to the OX_1R for orexin A, whereas the binding affinity of the two peptides to the OX_2R is in the same order of magnitude (Sakurai *et al.* 1998). Activation of either receptor induces an increase in free intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration (Smart *et al.*

1999), although the mechanism involved is still controversial. There are studies reporting phospholipase C (PLC)-mediated mobilization of calcium from intracellular stores (Sakurai *et al.* 1998), while PKC-mediated calcium influx has also been reported in neurons (De Lecea *et al.* 1998, Van den Pol *et al.* 1998, Uramura *et al.* 2001).

Dorsal root ganglion (DRG) neurons are primary sensory neurons that (alongside with trigeminal neurons) constitute the first link in the chain of neurons making up somatosensory pathways. DRG neurons selectively express structures and signals associated with pain processing such as multiple-type sodium channels (Kostyuk *et al.* 1981, Wood *et al.* 2002) and purine receptors (for review see Norenberg and Illes 2000, Donnelly-Roberts *et al.* 2008). Under normal circumstances, DRG neurons are relatively quiescent and when stimulated they produce series of action potentials that convey their messages to the brain. Since cultured rat DRG neurons functionally express a variety of ion channels and receptors for several agents including those involved in pain transmission, they have been successfully used as an *in vitro* model for studying cellular mechanisms of nociception (Vyklický *et al.* 1996). The majority of small and medium diameter neurons corresponds to the primary sensory neurons that carry nociceptive information, whilst the large diameter neurons are normally responsible for mechanosensory processing (Harper *et al.* 1985, Lu and Gold 2008).

DRG neurons express both OX_1R and OX_2R (Bingham *et al.* 2001). Moreover, both, OX_1R and OX_2R are expressed throughout the spinal cord being abundantly present in lamina I of the dorsal horn and in lamina X surrounding the central canal and on C-fibers in the spinal cord (Van den Pol 1999). The presence of orexinergic neuronal projections and orexin receptors in the DRG and the observation that orexins stimulate excitability and cause $[Ca^{2+}]_i$ increase (Ayar *et al.* 2008, Yan *et al.* 2008) indicate that orexinergic system may play role in the regulation of pain transmission.

However, the intracellular signaling pathways mediating the orexin-induced $[Ca^{2+}]_i$ response have not been studied in the rat primary sensory neurons. In the present study, we therefore investigated the contribution of the PKC-mediated pathways to orexin-A- and orexin-B-induced intracellular calcium signaling in rat DRG neurons, with special consideration of their nociceptive subtypes.

Methods

Preparation and primary culture of rat DRG neurons

The protocol of this study was approved by the local Ethic Committee. Short-term primary cultures of dorsal root ganglia neurons were obtained from 1- to 2-day old Wistar rats. The animals were decapitated, their spinal cords were removed, and DRGs at the cervical, thoracic, lumbar and sacral levels were removed (~45-50/pup) and temporarily collected in culture medium containing neurobasal medium with B27 (Invitrogen), 5 mM glutamine, and penicillin (5000 IU/ml)-streptomycin (5000mg/ml). Afterward they were treated enzymatically with collagenase (0.125 % in culture medium for 13 min) and trypsin (0.25 % in PBS for 6 min). Then, the cells were mechanically dissociated by trituration and plated onto 12 mm round, poly-D-lysine/laminin-coated glass coverslips (BD BioCoat, Bedford, MA, USA). Cells were maintained in the culture medium supplemented with nerve growth factor (NGF 2.5 S, Sigma-Aldrich, Germany) at 37 °C in a 95 % air/5 % CO_2 humidified incubator (Heracell, Kendro Lab GmbH, Germany). DRG neurons were incubated for at least 3-4 h before being used for imaging experiments. Coverslips with cell cultures were taken for calcium imaging experiments from 3 hours after plating up to 24 hours in culture.

Ratiometric intracellular calcium imaging

DRG neurons were loaded with the Ca^{2+}-sensitive dye Fura-2 acetoxymethyl ester (Fura 2-AM, 1 μM, Invitrogen) for about 60 min at 37 °C in a 5 % CO_2 humidified incubator. De-esterification and removal of unincorporated Fura 2-AM was accomplished by washing the cells 2-3 times with Fura-2 free standard solution for at least 10 min. The imaging bath solution contained (in mM): 130.0 NaCl, 3.0 KCl, 0.6 $MgCl_2$, 2.0 $CaCl_2$, 1.0 $NaHCO_3$, 5.0 glucose, and 10.0 HEPES. The pH was adjusted with NaOH to 7.4 and the osmolarity was adjusted to 310-320 mOsm by sucrose. All imaging experiments were performed in the dark at room temperature.

Glass coverslips with Fura-2-loaded cells were mounted in an imaging/perfusion chamber equipped with perfusion valve system (Warner Instruments, Hamden, CT, USA) which was mounted and viewed through an inverted microscope (Nikon TE2000S, Japan). The bath volume of the chamber was 600 μl.

The Fura-2 loaded DRG cells were alternately

illuminated with 340 nm and 380 nm wavelengths from a 175-W Xenon ozone-free lamp source (Sutter Instruments Co., Novato, CA, USA) optically coupled to the microscope with a liquid guide. A computer-controlled filter wheel (Lambda-10; Sutter Instruments, Novato, CA) was used to switch filters of 340 and 380 nm into the light path. Emitted light was passed through a S-flour 40x objective (oil: N.A. 1.30, W.D. 0.22 mm, Nikon) attached to a Nikon TE2000S inverted microscope, through a 510 nm band-pass emission filter (Fura-2 filter set, Semrock Brightline, Invitrogen), and finally into a cooled CCD (charge-coupled device) camera (C4742-95; Hamamatsu Photonics, Japan). The exposure time to excitation varied between experiments and was between 150 to 400 msec for each wavelength. Image pairs were captured and digitized every 5 s.

The camera, shutter duration and speed of filter changes by the filter wheel, and image acquisition were controlled with "Simple PCI" advanced imaging software (sPCI, Compix, USA). In order to minimize photobleaching, a computer-controlled shutter was used for allowing exposure of the cells to the excitation light only when required for imaging.

Calculations and analysis of intracellular calcium concentrations were performed off-line on a computer with an image processor and data-analysis software (sPCI, Hamamatsu Photonics). Fluorescence intensity of individual cells was determined over time by selecting a region of interest using the imaging software. An estimate of $[Ca^{2+}]_i$ was calculated from the ratio of 340/380 nm fluorescence intensity values (after correction by subtraction of background fluorescence) and expressed as a ratio (F340/F380). Only cells with resting 340/380 fluorescence intensity ratio of 0.50-0.90 were included.

The area under the curve (AUC) of the Ca^{2+} response was obtained from the integral of the area of the response for each cell, using SigmaPlot (version 9.0). Results are calculated as mean peak amplitude and AUC. The maximum Fura-2 emission ratio (340/380 nm) response to the application of 200 nM orexin-A or orexin-B was used as 100 % to normalize the effects of ORX_1R antagonist SB-334867-A and PKC inhibitor chelerythrine chloride (ChCl) for each cell. To avoid variations, control and drug treatment values were collected from the same cells, so that the data from the same cells could be compared directly. Responses with different concentrations of ChCl were obtained from different cells.

Fura-2 AM was obtained from Invitrogen (Switzerland). Fura-2-AM and SB334867A (Tocris Bioscience, Bristol, UK) were dissolved in dimethyl-sulfoxide (DMSO). The final concentration of DMSO in the bathing solution did not exceed 0.2 % (v/v), which did not elicit any change in $[Ca^{2+}]_i$ by itself in control experiments.

Orexin-A and -B were obtained from Bachem AG (Bubendorf, Switzerland). Chelerythrine chloride (Tocris Bioscience) and orexin-A and -B were dissolved in the imaging bath solution and aliquots were frozen. Each stock solution was diluted to the required concentration few minutes before bath application. Orexin-A (200 nM) and -B (200 nM) were delivered in the imaging bath solution for a total application time of 1 min.

Statistical analysis

Data are expressed as mean ± S.E.M. Differences between means were evaluated using unpaired Student's t-test with Origin 6.0 software (Microcal, Northampton, USA). The difference was accepted as significant when P<0.05.

Results

Two consecutive applications of orexin-A and -B, separated by a rinse of 5-min intervals, elicited similar $[Ca^{2+}]_i$ responses: second response averaged 94.1±3.6 % of first response, n=7 for orexin A and 104.0±2.7 % of first response, n=8 for orexin-B, respectively (Fig. 1B). Thus the comparisons were made between the first response to the only orexin-A or -B administration and the second response when orexins were applied in the presence of the orexin-1 receptor antagonist SB-334867-A or the PKC inhibitor ChCl.

Effects of orexin-A on the Fura-2 340/380 nm fluorescence ratio due to changes in the intracellular Ca^{2+} concentration was tested over a dose range of 1 and 200 nM (data not shown). A significant increase was observed even at 1 nM concentration, but >70 % of the small-sized nociceptive DRG neurons responded to the 200 nM orexin A and -B with an increase in $[Ca^{2+}]_i$. The 200 nM concentration was routinely used in all experiments reported in this paper.

Stimulation of cultured DRG neurons with orexin-A (200 nM) and -B resulted in a rapid elevation of $[Ca^{2+}]_i$. No return to baseline level could be seen during orexin application but $[Ca^{2+}]_i$ concentration begun to decline almost immediately when the application was discontinued, and returned to baseline (Fig. 1).
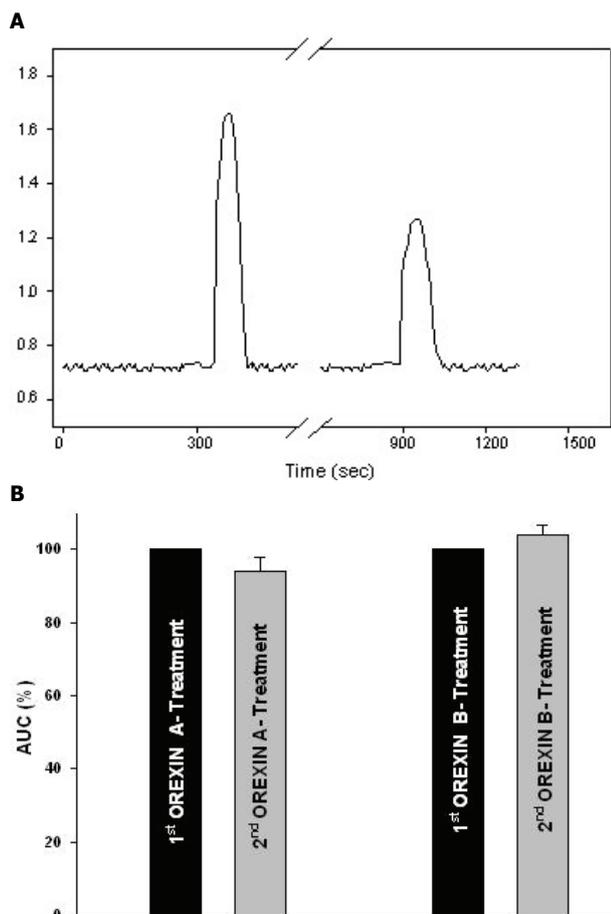
A

B

Fig. 1. A. Time-based plot for inhibition of orexin A-induced $[Ca^{2+}]_i$ by orexin-1 receptor antagonist SB-334867-A. The representative trace showing change in Ca^{2+} levels plotted against time in response to application of orexin-A alone and in the presence of SB-334867-A. Open bars indicate duration of perfusion of orexin-A and solid bar perfusion of SB-334867-A. **B**. Two consecutive applications of orexin-A (n=7) and –B (n=8) produced similar $[Ca^{2+}]_i$ responses.
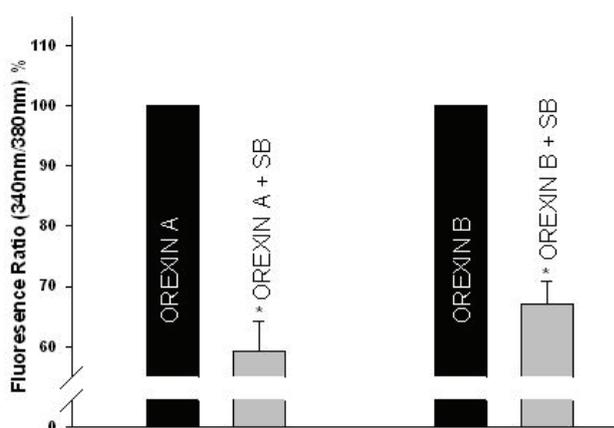

Fig. 2. Effects of orexin-1 receptor antagonist, SB-334867-A, on Ca^{2+} signals evoked by orexin-A and -B. Cells were stimulated by 200 nM orexin-A and -B, and after the induction of $[Ca^{2+}]_i$ response the SB-334867-A was applied. $[Ca^{2+}]_i$ responses are presented as the mean percentage of the peak amplitude after orexin application to the baseline in the 340/380 nm fluorescence ratio of Fura-2, where *n*=8. *: p<0.01 vs. orexin-A; *n*=12. *: p<0.01 vs. orexin-B, unpaired *t* test.
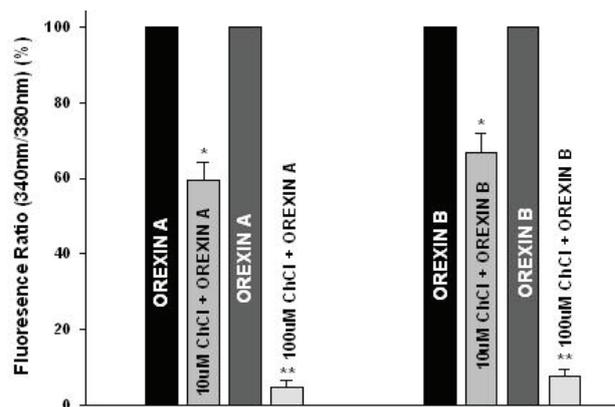

Fig. 3. Effects of PKC inhibitor chelerythrine chloride (ChCl) on orexin-A- and orexin-B-induced fluorescence calcium responses in cultured rat DRG neurons. Fluorescence calcium responses are graphed as a percentage of mean Fura-2 fluorescence ratio increase at the peak level compared to the baseline after stimulation with 200 nM orexin-A and -B. ChCl caused a dose dependent significant decrease of orexin-A- and orexin-B-induced fluorescence ratio. Results are expressed as mean ± S.E.M. *: p<0.01, **: p<0.001 vs. respective orexin response, unpaired t test.

We used SB-334867-A, the orexin-1 receptor antagonist, for pharmacological characterization of the orexin-A induced Ca^{2+} response. Peak $[Ca^{2+}]_i$ response to orexin-A (200 nM) was reduced to 59.1±5.1 % by the application of 1 μM SB-334867-A (P<0.01, n=8) (Figs 1 and 2). The SB-334867-A also inhibited OX_2R-mediated calcium responses, but with a lower potency (causing reduction to 67±3.8 % (n=12) at 1 μM of the orexin-B (200 nM)-induced responses (P<0.01) (Fig. 2).

One of the possible mechanisms of the orexin-A-induced $[Ca^{2+}]_i$ rise could be the activation of PKC. The ability of the PKC receptor antagonist, chelerythrine chloride, to affect the $[Ca^{2+}]_i$ response to orexin A was examined. As can be seen in Figure 3, chelerythrine chloride (10 and 100 μM) caused a significant, concentration-dependent inhibition of the 200 nM orexin-A-induced increase in $[Ca^{2+}]_i$ response (Fig. 3). The $[Ca^{2+}]_i$ response was reduced to 59.4±4.8 % (P<0.01, n=10) and 4.9±1.6 % (*P*<0.01, n=9) of mean peak response to orexin-A (Fig. 3). In the presence of chelerythrine chloride the mean increase in fluorescence ratio by orexin-B decreased to 60.6±6.2 % (n=16) and 8.3±2.6 % (n=11) for 10 μM and 100 μM, respectively. Apparently PKC-mediated signaling pathways play an important role in the orexin-A- and orexin-B-induced $[Ca^{2+}]_i$ response in this sensory neurons.

When the area under the curve (AUC) of the Ca^{2+} response was considered, consistent with the mean peak amplitude of the data, the AUC of $[Ca^{2+}]_i$ increase

evoked by 200 nM orexin-A was significantly and dose-dependently reduced to 52.1±4.6 % (n=10, P<0.01) and 3.1±0.7 % (n=9, P<0.001) after application of 10 and 100 μM chelerythrine chloride, respectively (Fig. 4). Chelerythrine chloride also inhibited orexin-B-induced $[Ca^{2+}]_i$ responses, causing a 39.4±6.0 (n=16) and 91.7±2.6 % (n=11) inhibition of AUC values of the orexin-B (200 nM)-induced responses at 10 μM and 100 μM, respectively (Fig. 4).
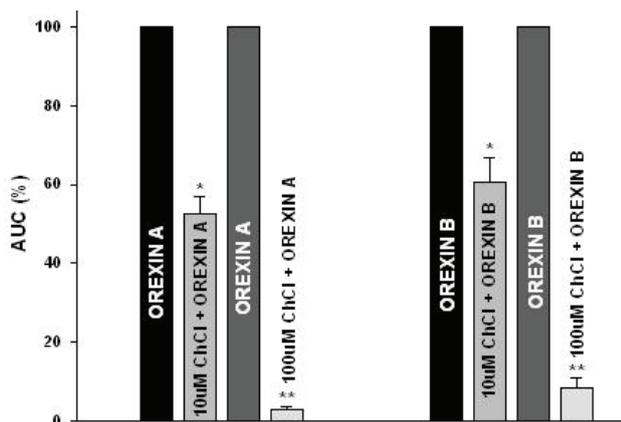

Fig. 4. Inhibition of orexin-A responses by chelerythrine chloride (ChCl) in terms of AUC. Data points were derived by calculating the difference between $[Ca^{2+}]_i$ AUC at each ChCl concentration as percentages of the responses to 200 nM orexin-A and -B. Results are expressed as mean ± S.E.M. *: p<0.01, **: p<0.001 vs. respective orexin response, unpaired t test.

Discussion

The novel finding of this study is that PKC participates in the signaling pathway for orexin A-induced calcium response in cultured rat sensory neurons. Using ratiometric intracellular calcium imaging approach, we demonstrate for the first time that the specific inhibitor of PKC, chelerythrine chloride, significantly inhibited orexin-A and -B induced intracellular calcium responses in rat nociceptive DRG neurons.

Previous studies including from our own laboratory have demonstrated that the application of orexin-A at nanomolar concentrations caused depolarization of the membrane and stimulated $[Ca^{2+}]_i$ increase, consistent with the increased excitability, in cultured rat DRG neurons (Ayar *et al.* 2007, 2008, Yan *et al.* 2008). But the mechanism underlying the orexin effect is not studied yet. The aim of this study was to understand the signal transduction pathways that mediate the orexin-A-induced intracellular calcium responses in

cultured rat sensory neurons.

To verify that the orexin-A-induced increase in $[Ca^{2+}]_i$ was mediated through its receptor, we performed antagonist study to evaluate the ability of the OX_1R antagonist, SB-334867-A, to inhibit orexin-A-induced increases in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was effectively inhibited by this OX_1R antagonist, suggesting that the effect was mediated through the OX_1R. It should also be noted that the inhibition of orexin-A-mediated $[Ca^{2+}]_i$ increase in cultured rat DRG neurons by SB-334867-A is consistent with the previous report by Yan *et al.* (2008) in this sensory neurons. It is known that the orexin receptors are coupled to the PKC, PLC and AC, and cause the elevation of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response to orexin A was abolished, when calcium was omitted from the external medium, indicating that the entry of calcium from the extracellular source is more important in this response (data not shown). Next, we examined whether the PKC-mediated signaling pathway is involved in the orexins-induced increase in $[Ca^{2+}]_i$. We have demonstrated that the inhibition of PKC by chelerythrine chloride resulted in a significant inhibition of $[Ca^{2+}]_i$, suggesting that the intracellular calcium response to orexin-A in this rat primary sensory neurons is mediated by the activation of PKC.

The $[Ca^{2+}]_i$ responses to orexin-A were significantly reduced in the presence of the selective OX_1R antagonist SB-334867-A at a concentration of 1 μM. SB-334867-A also inhibited orexin-B (OX_2R-mediated) induced calcium responses, but with a lower efficiency, causing at 1 μM concentration about 33 % (n=12) inhibition of the orexin-B (200 nM)-induced response. These data suggest that both OX_1R and OX_2R are involved in the orexin-mediated calcium signaling in DRGs. This finding also confirms that both of orexin receptors are functional in these sensory neurons in which their expressions have been shown (Bingham *et al.* 2001). In a previous study in rat DRG neurons the SB-334867-A has been shown to abolish the orexin-A-induced increase in $[Ca^{2+}]_i$, but our results differ from those of Yan *et al.* (2008). Although the results of the present study do not confirm a complete inhibition of orexin-induced $[Ca^{2+}]_i$ increase as in the above mentioned study, our results are compatible (even the obtained rate of inhibition) with other reports showing significant but incomplete inhibition of orexin-induced $[Ca^{2+}]_i$ responses in rat sympathetic preganglionic neurons (Van den Top *et al.* 2003) and in Chinese hamster ovary cells (Smart *et al.* 2001). We have no explanation for the discrepancy of our

results with those of Yan *et al.* (2008), but they used freshly isolated cells from adult Sprague-Dawley rats, whereas our short-term cultured cells were isolated from neonatal Wistar rats.

Our results are in agreement with previous findings demonstrating that orexin-A and -B cause neuronal membrane depolarization and increase in cytosolic calcium levels, which leads to increased neuronal excitability in different neuronal types including the rat DRG neurons (Ayar *et al.* 2007, 2008, Yan *et al.* 2008), neurons in paraventricular nucleus (Ishibashi *et al.* 2005), histaminergic neurons of the tuberomammillary nucleus (Eriksson *et al.* 2001), sympathetic preganglionic neurons (Van den Top *et al.* 2003) and neurons in the arcuate nuclei and the ventral tegmental area (Uramura *et al.* 2001). Our observations are also in agreement with previous findings that orexin-A increased $[Ca^{2+}]_i$ *via* the PKC pathway, leading to elevated activities of the neurons in arcuate nuclei and the ventral tegmental area (Uramura *et al.* 2001).

Several cellular mechanisms including adenylyl cyclase (AC) (Holmqvist *et al.* 2005), phospholipase C (PLC) (Holmqvist *et al.* 2005, Johansson *et al.* 2007) and PKC signaling pathways (Van den Pol *et al.* 1998), as well as the co-involvement of PLC and PKC pathways in (Uramura *et al.* 2001, Narita *et al.* 2007) have been suggested to be involved in the orexin-induced $[Ca^{2+}]_i$ increase. To the best of our knowledge, this is the first study to provide evidence that the activation of PKC-mediated signaling is involved in orexin-A-induced $[Ca^{2+}]_i$ increase in cultured rat DRG neurons.

Our results are also in agreement with previous findings which reported the involvement of PKC signaling cascade in orexin-A-induced $[Ca^{2+}]_i$ increase (Van den Pol *et al.* 1998, Uramura *et al.* 2001, Narita *et al.* 2007). In a previous study, calcium response to orexin-A in the lateral hypothalamus and perifornical area neurons was completely blocked by PKC inhibition (Van den Pol *et al.* 1998), whereas in our study the effect was highly significant but incomplete. This may be due to the fact that we used different PKC inhibitor with short application duration as well as using higher concentration of orexin A (they used 1 nM orexin A). Consistent with our study, same dose of chelerythrine chloride provided

significant but incomplete inhibition of intracellular calcium response to orexin in midbrain dopamine neurons (Narita *et al.* 2007).

The findings of this study are in line with the emerging role of orexinergic system in modulation of nociceptive processing and pain transmission. Activation of PKC is suggested to be involved in pain sensation (Velázquez *et al.* 2007). Indeed, the anatomical localization of protein kinase C isoenzymes in both peripheral and central nervous system sites that process pain further highlights the potential master regulatory role in pain and analgesia (Velázquez *et al.* 2007). Furthermore, it has been shown that activation PKC sensitizes the transient receptor potential vanilloid 1 (TRPV1) channels (Bhave *et al.* 2003), which are Ca^{2+}-permeant nonspecific cation channels specifically expressed in nociceptive DRG neurons. This effect may underline many chronic pain conditions. Therefore, PKC inhibitors are currently being considered as a therapeutic approach for the treatment of certain painful conditions including diabetic neuropathy (Adis International Ltd 2007, Das and King 2007). Considering the DRG cells used in this study as a cellular model for studies of modulation of nociceptive transmission (Vyklický *et al.* 1996), the finding that PKC-mediated signaling is involved in orexin-induced activation of this sensory neurons, is of importance as novel therapeutic target for the treatment of pain.

In conclusion, we demonstrated for the first time that the activation of PKC signaling pathway is involved in orexin-A- and orexin-B-mediated intracellular calcium response in rat nociceptive DRG neurons. These results provide a new insight into the role and mechanism of the effect of orexins in pain signaling which may present a novel therapeutic target for the treatment of pain.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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