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

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Short title: PKC-Mediate Ca^{2+} response to Orexins in rat sensory neurons

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Summary

Previous results have suggested that orexins causes a rise in 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. All data were analyzed by using unpaired *t* test, $P < .05$ defining statistical significance. The orexin-1 receptor antagonist SB-334867-A (1 μM) inhibited the calcium responses to orexin-A and -B (59.1±5.1% versus 200 nM orexin-A, n=8, and 67±3.8% versus 200 nM orexin-B, n=12, respectively). The PKC inhibitor chelerythrine chloride (ChCl, 10 and 100 μM) significantly decreased the orexin-A (200 nM)-induced increase in $[Ca^{2+}]_i$ (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 ChCl dose dependently inhibited the $[Ca^{2+}]_i$ response to 200 nM orexin-B. In conclusion, the results suggest that orexins activates intracellular calcium signaling in cultured rat sensory neurons through PKC-dependent pathway, which may have important implications for nociceptive modulation and pain.

Key words: Orexin A, Protein Kinase C, calcium signaling, nociception, sensory neuron

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 food intake, regulation of the neuroendocrine system, energy metabolism, blood pressure, body temperature, and the sleep-wake cycle (Matsuki *et al.* 2008). Evidence emerges now that orexins may 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 125J-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 controversial. There are studies reporting phospholipase C (PLC)-mediated mobilization of calcium from intracellular stores (Sakurai *et al.* 1998) while on the other hand PKC-mediated calcium

influx have also been reported in neurons (van den Pol *et al*. 1998, de Lecea *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 purin receptors (for review: 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 (Vyklicky *et al.* 1996). The majority of small and medium diameter neurons correspond to the primary sensory neurons that carry nociceptive information, whilst the large diameter neurons are normally responsible for mechano-sensory 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 its receptors in the DRG and reports of previous studies demonstrating that orexin stimulates excitability and cause increase in $[Ca^{2+}]_i$, (Yan *et al.* 2008, Ayar *et al.* 2008) indicates that orexinergic system may play role in the regulation of pain transmission.

But to date, the intracellular signaling pathways mediating the orexin-induced $[Ca^{2+}]_i$ response has not been studied in the rat primary sensory neurons. In the present study, therefore we investigated the contribution of the PKC-mediated pathways to orexin-A and

- 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-hour 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 minutes 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. 0.22mm, 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 sec.

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 (ROI) 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 -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 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). The fura-2-AM and SB334867A (Tocris Bioscience, Bristol, UK) were dissolved in dimethylsulfoxide (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 minutes before bath applied. 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 ± SEM. 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, seperated 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 1. B. So that comparisons were made between the first response to the only orexin-A or -B and the second response when they applied in the presence of the orexin-1 receptor antagonist SB-33487-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 were 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 begun to decline almost immediately after when application was discontinued, and returned to baseline (Fig. 1).

We used SB-33487-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 significantly reduced to 59.1±5.1 % by application of 1 μM SB-33487-A (Fig. 1 and 2; $P<0.01$, n=8). The SB-334867-A also inhibited OX_2R-mediated calcium responses, but with lower potency (causing reduction to 67±3.8% (n=12) at 1 μM of the orexin- B (200 nM)-induced responses, Fig. 2; $P<0.01$).

One of the possibilities of the mechanism 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 Fig 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 -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).

Discussion

The novel finding of this study is that PKC lies on the signaling pathway for orexin A-induced calcium response in rat cultured 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 nociceptive rat 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 increased excitability, in cultured rat DRG neurons (Ayar *et al.* 2007, Ayar *et al.* 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 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 and significantly inhibited by the OX_1R antagonist, SB-334867-A, suggesting that the effect was through the OX_1R. It is also a point to note that 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 couple to the PKC, PLC and AC, and cause 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 entry of the ion 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 $[Ca^{2+}]_i$ and we have demonstrated that the inhibition of PKC by chelerythrine chloride resulted in 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 was significantly reduced in the presence of the selective OX_1R antagonist SB-334867-A at concentrations of 1 uM. The SB-334867-A also inhibited orexin-B (OX_2R-mediated) induced calcium responses, but with lower efficiency, causing about 33% (n=12) inhibition at 1 μM 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 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 $[Ca^{2+}]_{i,}$ where 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 latest study, our results are compatible (even the obtained rate of inhibition) with other reports showing significant but not complete 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 the Yan *et al.*'s (2008) but the difference might arise from that 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 causes 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, Ayar *et al.* 2008, Yan et al. 2008) 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 in the arcuate nuclei and the ventral tegmental area (Uramura *et al.* 2001). Our observations were 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 co-involvement of the PLC and PKC pathways

in (Uramura *et al.* 2001, Narita *et al.* 2007) have been suggested to be involved in the orexin-induced increase in $[Ca^{2+}]_i$. But, to the best of our knowledge, this is the first study to provide evidence that 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 report involvement of PKC signaling cascade in orexin A induced increase in $[Ca^{2+}]_i$ (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 not complete. This may be due to 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).

We have no direct evidence for clinical indication and interpretation of the findings of this study, but they 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 and shown to be specifically expressed in nociceptive DRG neurons, an effect may underline many chronic pain conditions. Therefore, PKC inhibitors are currently being considered as a therapeutical approach for the treatment of certain painful

conditions including diabetic neuropathy (Adis International Limited, 2007, Das and King 2007). Considering the DRG cells used in this study presents a cellular model for studies of modulation of nociceptive transmission (Vyklický *et al.* 1996), the finding of 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 demonstrate for the first time that activation of PKC signaling pathway is involved in orexin-A and -B mediated intracellular calcium response in rat nociceptive DRG neurons. Thus, these results provide 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.

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References

ADIS INTERNATIONAL LIMITED: Ruboxistaurin: LY 333531. *Drugs RD* **8**: 193-199, 2007.

AMMOUN S, HOLMQVIST T, SHARIATMADARI R, OONK HB, DETHEUX M, PARMENTIER M, AKERMAN KE, KUKKONEN JP: Distinct recognition of OX1 and OX2 receptors by orexin peptides. *J Pharmacol Exp Ther* **305**: 507-514, 2003.

AYAR A, OZCAN M, HAAS HL: Effects of orexins/hypocretins on excitability of isolated rat sensory neurones, *Life Sciences 2007. Proc Life Sciences***,** PC179 (8-12 July 2007, Glasgow, UK). 2007.

AYAR A, OZCAN M, SERHATLIOGLU I, SAHIN Z: Orexin/hypocretin-A induces intracellular calcium transients in rat cultured dorsal root ganglia neurones. *Proc Physiol Soc* **11** (University of Cambridge 14-16 July 2008), PC154, 2008.

BHAVE G, HU HJ, GLAUNER KS, ZHU W, WANG H, BRASIER DJ, OXFORD GS, GEREAU RW 4TH: Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1) *Proc Natl Acad Sci U SA* **100**: 12480-12485, 2003.

BINGHAM S, DAVEY PT, BABBS AJ, IRVING EA, SAMMONS MJ, WYLES M, JEFFREY P, CUTLER L, RIBA I, JOHNS A, PORTER RA, UPTON N, HUNTER AJ, PARSONS AA: Orexin-A, an hypothalamic peptide with analgesic properties. *Pain* **92**: 81-90, 2001.

DAS EVCIMEN N, KING GL: The role of protein kinase C activation and the vascular complications of diabetes. *Pharmacol Res* **55**: 498-510, 2007.

de LECEA L, KILDUFF TS, PEYRON C, GAO X, FOYE PE, DANIELSON PE, FUKUHARA C, BATTENBERG EL, GAUTVIK VT, BARTLETT FS 2ND, FRANKEL WN, VAN DEN POL AN, BLOOM FE, GAUTVIK KM, SUTCLIFFE JG: The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA* **95**: 322-327, 1998.

DONNELLY-ROBERTS D, McGARAUGHTY S, SHIEH CC, HONORE P, JARVIS MF: Painful purinergic receptors. *J Pharmacol Exp Ther* **324**: 409-415, 2008.

ERIKSSON KS, SERGEEVA O, BROWN RE, HAAS HL. Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. *J Neurosci* **21**: 9273-9279, 2001.

HARPER AA, LAWSON SN: Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. *J Physiol* **359**: 31-46, 1985.

HOLLAND P, GOADSBY PJ: The hypothalamic orexinergic system: pain and primary headaches. *Headache* **47**: 951-962, 2007.

HOLMQVIST T, JOHANSSON L, OSTMAN M, AMMOUN S, AKERMAN KE, KUKKONEN JP: OX1 orexin receptors couple to adenylyl cyclase regulation via multiple mechanisms. *J Biol Chem* **280**: 6570-659, 2005.

ISHIBASHI M, TAKANO S, YANAGIDA H, TAKATSUNA M, NAKAJIMA K, OOMURA Y, WAYNER MJ, SASAKI K: Effects of orexins/hypocretins on neuronal activity in the paraventricular nucleus of the thalamus in rats in vitro. *Peptides* **26**: 471-481, 2005.

JOHANSSON L, EKHOLM ME, KUKKONEN JP: Regulation of OX1 orexin/hypocretin receptor-coupling to phospholipase C by Ca^{2+} influx. *Br J Pharmacol* **150**: 97-104, 2007.

KOSTYUK PG, VESELOVSKY NS, TSYANDRYENKO AY: Ionic currents in the somataic membrane of rat dorsal root ganglion neurons. I. Sodium currents. *Neuroscience* **6**: 2423-2430, 1981.

LEE Y, LEE CH, OH U: Painful channels in sensory neurons. *Mol Cells* **20**: 315-324, 2005.

LU SG, GOLD MS: Inflammation-induced increase in evoked calcium transients in subpopulations of rat dorsal root ganglion neurons. *Neuroscience* **153**: 279-288, 2008.

MATSUKI T, SAKURAI T: Orexins and orexin receptors: from molecules to integrative physiology. *Results Probl Cell Differ* **46**: 27-55, 2008.

NARITA M, NAGUMO Y, MIYATAKE M, IKEGAMI D, KURAHASHI K, SUZUKI T: Implication of protein kinase C in the orexin-induced elevation of extracellular dopamine levels and its rewarding effect. *Eur J Neurosci* **25**: 1537-1545, 2007.

NORENBERG W, ILLES P: Neuronal P2X receptors: localisation and functional properties. *Naunyn Schmiedebergs Arch Pharmacol* **362**: 324-539, 2000.

SAKURAI T, AMEMIYA A, ISHII M, MATSUZAKI I, CHEMELLI RM, TANAKA H, WILLIAMS SC, RICHARDSON JA, KOZLOWSKI GP, WILSON S, ARCH JR, BUCKINGHAM RE, HAYNES AC, CARR SA, ANNAN RS, MCNULTY DE, LIU WS, TERRETT JA, ELSHOURBAGY NA, BERGSMA DJ, YANAGISAWA M: Orexins and orexin receptors: a family of hypothalamic neuropeptides and G proteincoupled receptors that regulate feeding behavior. *Cell* **92**: 573-585, 1998.

SHIBAHARA M, SAKURAI T, NAMBU T, TAKENOUCHI T, IWAASA H, EGASHIRA SI, IHARA M, GOTO K: Structure, tissue distribution, and pharmacological characterization of Xenopus orexins. *Peptides* **20**: 1169-1176, 1999.

SMART D, JERMAN JC, BROUGH SJ, RUSHTON SL, MURDOCK PR, JEWITT F, ELSHOURBAGY NA, ELLIS CE, MIDDLEMISS DN, BROWN F: Characterization of recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR. *Br J Pharmacol* **128**: 1-3, 1999

SMART D, SABIDO-DAVID C, BROUGH SJ, JEWITT F, JOHNS A, PORTER RA, JERMAN JC: SB-334867-A: the first selective orexin-1 receptor antagonist. *Br J Pharmacol* **132**: 1179-82, 2001.

URAMURA K, FUNAHASHI H, MUROYA S, SHIODA S, TAKIGAWA M, YADA T: Orexin-a activates phospholipase C- and protein kinase C-mediated Ca^{2+} signaling in dopamine neurons of the ventral tegmental area. *Neuroreport* **12**: 1885-1889, 2001.

van DEN POL AN, GAO XB, OBRIETAN K, KILDUFF TS, BELOUSOV AB: Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. *J Neurosci* **18**: 7962-7971, 1998.

van DEN POL AN: Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. *J Neurosci* **19**: 3171-3182, 1999.

VAN DEN TOP M, NOLAN MF, LEE K, RICHARDSON PJ, BUIJS RM, DAVIES CH, SPANSWICK D: Orexins induce increased excitability and synchronisation of rat sympathetic preganglionic neurones. *J Physiol* **549**: 809-821, 2003.

VELÁZQUEZ KT, MOHAMMAD H, SWEITZER SM. Protein kinase C in pain: involvement of multiple isoforms. *Pharmacol Res* **55**: 578-589, 2007.

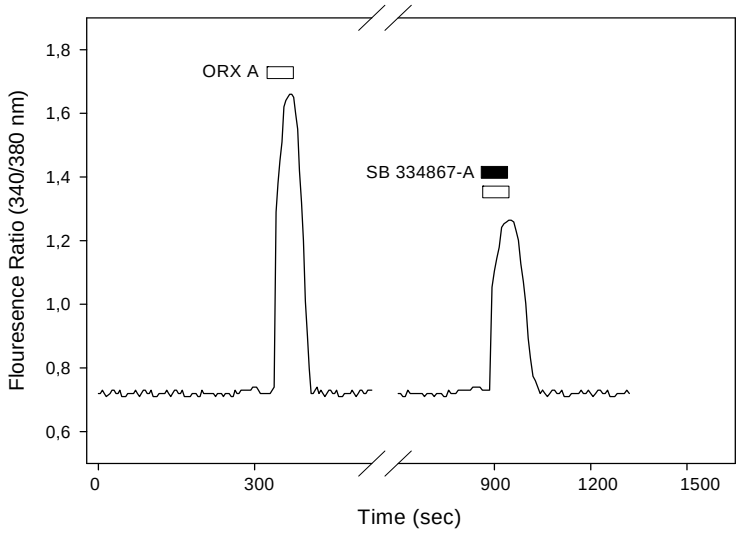
VYKLICKY L, KNOTKOVA-URBANCOVA H: Can sensory neurones in culture serve as a model of nociception? *Physiol Res* **45**: 1-9, 1996.

WOOD JN, AKOPIAN AN, BAKER M, DING Y, GEOGHEGAN F, NASSAR M, MALIK-HALL M, OKUSE K, POON L, RAVENALL S, SUKUMARAN M, SOUSLOVA V: Sodium channels in primary sensory neurons: relationship to pain states. *Novartis Found Symp* **241**: 159-172, 2002.

YAN JA, GE L, HUANG W, SONG B, CHEN XW, YU ZP: Orexin affects dorsal root ganglion neurons: a mechanism for regulating the spinal nociceptive processing. *Physiol Res* **57**: 797-800, 2008.

Figures

A



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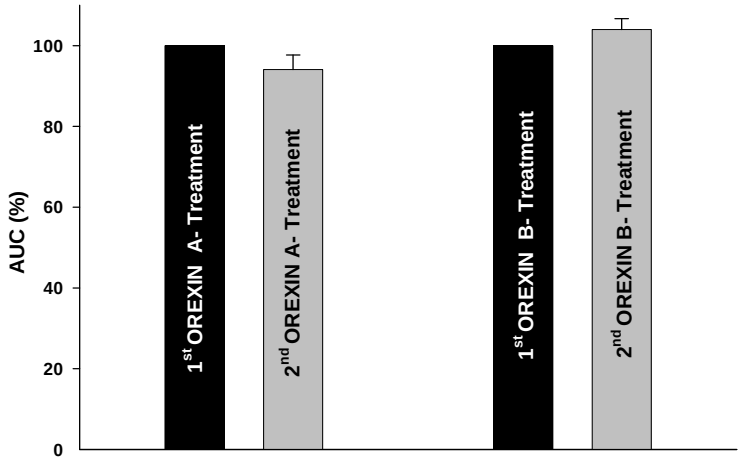

Fig. 1. A: Time-based plot for inhibition of orexin A-induced $[Ca^{2+}]_i$ by orexin-1 receptor antagonist SB-334867-A. The representative trace showing change in Ca^{2+} levels plotted against time in response to application of orexin-A alone and in the presence of SB-334867-A. Open bars indicates 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.

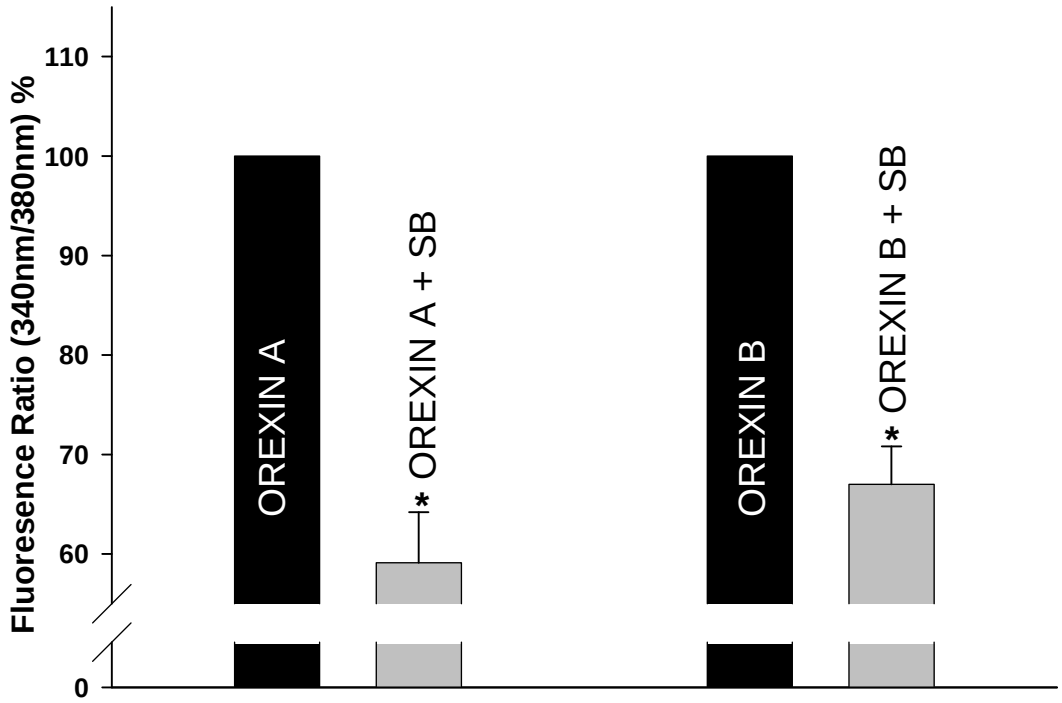

Fig. 2. Summary of the effects of the orexin-1 receptor antagonist, SB-334867-A, on Ca^{2+} signals evoked by orexin-A and -B. Cells were stimulated by 200 nM orexin-A and –B, and after the induction of $[Ca^{2+}]_i$ response the SB-334867-A was applied. $[Ca^{2+}]_i$ responses are presented as the mean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

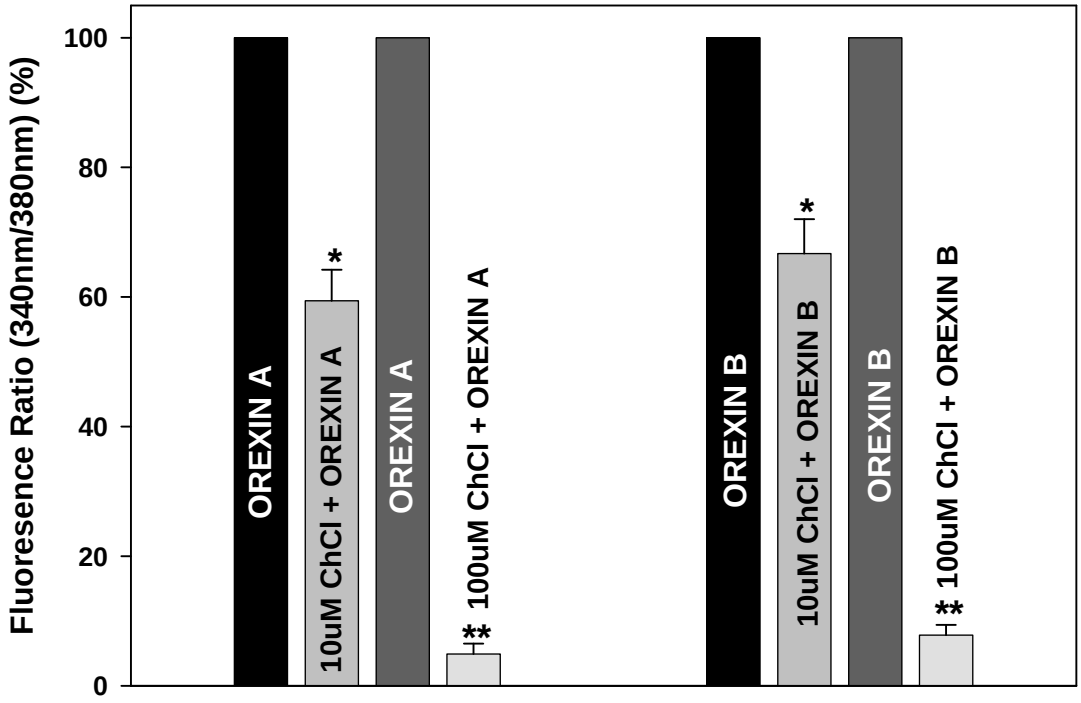

Fig. 3. Summary of the effects of PKC inhibitor chelerythrine chloride (ChCl) on orexin-A and –B induced fluorescence calcium responses in cultured rat DRG neurons. Fluorescence calcium responses are graphed as a percentage of mean fura-2 fluorescence ratio increase at the peak level compared to the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

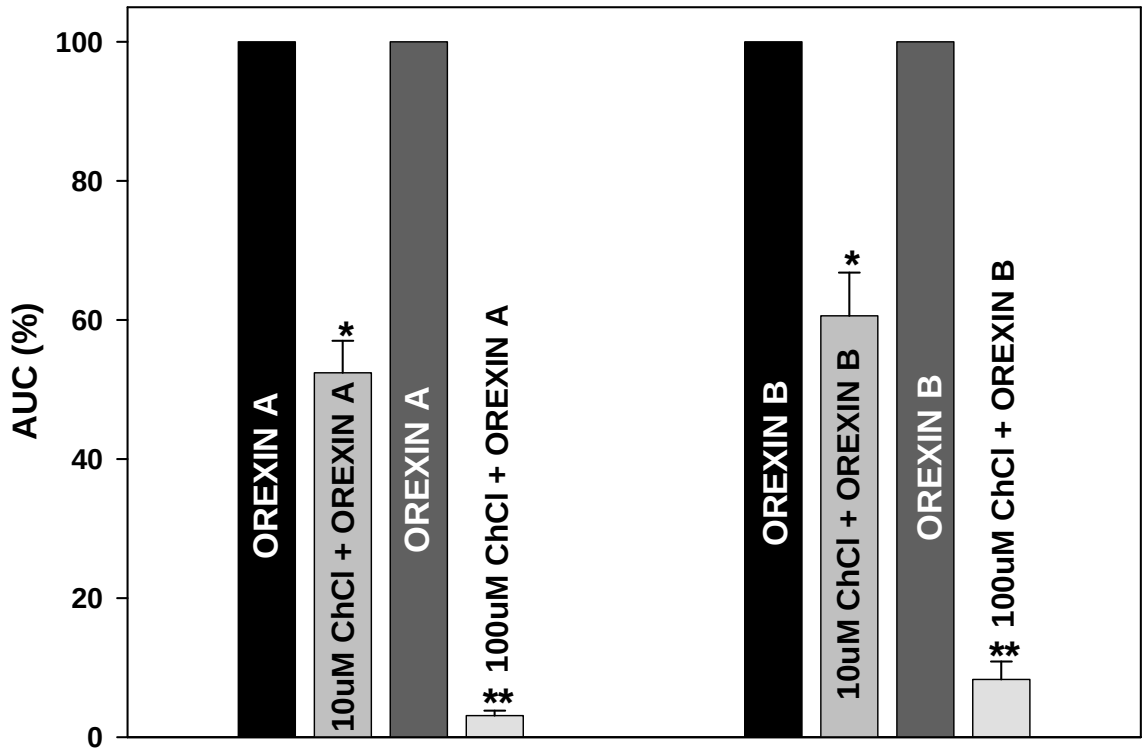

Fig. 4. Inhibition of orexin-A responses in by the chelerythrine chloride (ChCl) in terms of AUC. Data points were derived by calculating the difference between $[Ca^{2+}]_i$ AUC at each ChCl concentration as percentages of the responses to 200 nM orexin-A and -B. Results are expressed as mean ± SEM.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