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Orexin A-induced extracellular calcium influx in prefrontal cortex neurons involves L-type calcium channels

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Orexins, novel excitatory neuropeptides from the lateral hypothalamus, have been strongly implicated in the regulation of sleep and wakefulness. In this study, we explored the effects and mechanisms of orexin A on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of freshly dissociated neurons from layers V and VI in prefrontal cortex (PFC). Changes in $[Ca^{2+}]_i$ were measured with fluo-4/AM using confocal laser scanning microscopy. The results revealed that application of orexin A (0.1 ~1 μM) induced increase of $[Ca^{2+}]_i$ in a dose-dependent manner. This elevation of $[Ca^{2+}]_i$ was completely blocked by pretreatment with selective orexin receptor 1 antagonist SB 334867. While depletion of intracellular Ca^{2+} stores by the endoplasmic reticulum inhibitor thapsigargin (2 μM), $[Ca^{2+}]_i$ in PFC neurons showed no increase in response to orexin A. Under extracellular Ca^{2+}-free condition, orexin A failed to induce any changes of Ca^{2+} fluorescence intensity in these acutely dissociated cells. Our data further demonstrated that the orexin A-induced increase of $[Ca^{2+}]_i$ was completely abolished by the inhibition of intracellular protein kinase C or phospholipase C activities using specific inhibitors, BIS II (1 μM) and D609 (10 μM), respectively. Selective blockade of L-type Ca^{2+} channels by nifedipine (5 μM) significantly suppressed the elevation of $[Ca^{2+}]_i$ induced by orexin A. Therefore, these findings suggest that exposure to orexin A could induce increase of $[Ca^{2+}]_i$ in neurons from deep layers of PFC, which depends on extracellular Ca^{2+} influx via L-type Ca^{2+} channels through activation of intracellular PLC-PKC signaling pathway by binding orexin receptor 1.

Key words: Sleep/wakefulness, Orexin A, Prefrontal cortex, Protein kinase C, Phospholipase C, L-type calcium channels.

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Orexins (hypocretins) localized specially in neurons within the lateral hypothalamus are recently novel hypothalamic peptides discovered in 1998 by two groups respectively (7, 25). Orexin system includes two separate peptides orexin A and orexin B (also known as hypocretin-1 and hypocretin-2) proteolytically derived from the same precursor protein. The actions of orexins are mainly transduced by two orphan G-protein coupled receptors called orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R) (25). It has been well established that orexins play an important role in the promotion of wakefulness (5,26), which may be mainly fulfilled by the excitatory action on multiple subcortical arousal systems, as well as the final target of arousal systems-cerebral cortex (2, 17, 29, 36). VAN DEN POL *et al.* have found that a few of cultured cortical cells tested show elevation of intracellular free calcium ($[Ca^{2+}]_i$) in response to orexin A (34), which depend on extracellular Ca^{2+} entry presented by our previous study (37). Interestingly, electrophysiological experiments indicate that the region selectivity of responses to orexins really exist in the cerebral cortex (2), whereas orexin A directly activates the neurons in layer 6b of the primary somatosensory cortex, but not the neurons in layers 2-5 or 6a. Also, our previous electrophysiological results have illuminated that orexin A can excite the freshly dissociated neurons from layers V and VI of prefrontal cortex (PFC) (36), which is the cortical area most implicated in arousal level (40). As we cannot rule out the possibility that the similar selectivity of calcium response to orexin A exists in cortical regions, likely due to only a small percentage of responsive cultured cortical neurons found (34, 37), it is intriguing to test whether neurons from layers V and VI of PFC show the same $[Ca^{2+}]_i$ elevation as those from cultured cortical neurons in response to orexin A.

Additionally, there have been studies of extracellular Ca^{2+} influx through a variety of membrane channels activated by orexins (14, 15, 19, 24, 33), including voltage-gated calcium channels (14, 15, 33) and transient receptor potential canonical (TRPC) channels recently reported (19, 24). However, which types of Ca^{2+} channels should be responsible for such $[Ca^{2+}]_i$ elevation found in cortical neurons have not been determined. Therefore, the purpose of the present study was to examine the Ca^{2+} mobilizing actions and mechanisms of orexin A on acutely dissociated neurons from layers V and VI in PFC by confocal laser scanning microscopy using fluo-4/AM as an indicator.

Material and Methods

Animals.– Postnatal Wistar rats were obtained from the Center of Animal Laboratory of the Third Military Medical University. All experiments were carried out in accordance with China Animal Welfare Legislation. The animals and experimental procedures used were approved by the Third Military Medical University Committee on Ethics in the Care and Use of Laboratory Animals.

Acute dissociation of PFC neurons.– Postnatal 8-to 10-day-old Wistars rats were acutely anesthetized with ether and decapitated. Brains then were quickly removed and blocked. Slices (400 μm) were cut with a vibratome tissue sectioner (DTK-600, D.S.K, Japan) in ice-cold oxygenated (95%O_2, 5%CO_2) artificial cerebrospinal fluid (ACSF) containing (in

mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$ and 10 glucose, pH adjusted to 7.3-7.4 with NaOH. After incubated for 1 hr at room temperature in oxygenated ACSF, individual slice was digested with 1mg/ml trypsin at 36 ºC for 35~40 min. At the end of the enzymatic treatment, the tissue was rinsed several times and kept in the enzyme-free ACSF. According to the previous studies (29, 30, 36), neurons in layers V and VI of PFC were isolated (Fig.1A) under a dissecting microscope (SZ-ST, Olympus. CO., LTD, Japan) and triturated with a graded series of fire-polished Pasteur pipettes. Then the cell suspension was placed on a cover glass, and a few of mechanically dissociated PFC neurons were alive and adhered to the bottom of glass within 5 min.

Calcium imaging.– The cover glass with dissociated cells was loaded with fluo-4/acetoxymethylester (AM) (2 μM) and pluronic F127 (0.02%) in HEPES-buffered ACSF containing (in mM) 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 10 HEPES at 37 ºC for 30 min in darkness. Cells were rinsed for three times in fluo-4/AM -free HEPES-buffered ACSF at the room temperature (25-28 ºC). After the coverslip was mounted in an imaging chamber of an inverted microscope (TCS-NT, Leica, Germany), the dye in the selected cytoplasmic part of cells was excited by wavelength at 490 nm and the fluorescence images were captured at 516 nm in a 10s interval by an intensified CCD camera controlled by a computer. In some cases, extracellular Ca^{2+}- free condition was achieved in ACSF (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 0.1 EGTA, 10 HEPES and 10 glucose, pH 7.3-7.4.

Drugs.–All agents were applied extracellularly. Orexin A, L-glutamate, pluronic F127, thapsigargin, nifedipine, O-Tricyclo [5.2.1.$0^{2,6}$]dec-9-yl dithiocarbonate potassium salt (D609), bisindolylmaleimide II (BIS II) and Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co.(St. Louis, MO, USA). N-(2-Methyl-6-benzoxazolyl)-N'-1,5-naphthyridin-4-yl urea (SB 334867) was obtained from Tocris bioscience (Bristol, UK). Fluo-4/AM was purchased from Molecular Probes (Eugene, Oregon, USA) and tetrodotoxin (TTX) from Hebei Fisheries Research Institute (Qinghuangdao, China). Fluo-4/AM and pluronic F127 were first dissolved in DMSO, and then diluted with HEPES-buffered ACSF. The final concentration of DMSO was less than 0.1%. During these experiments except additional illustration, TTX was added to the bath solution with a final concentration of 0.1 μM to exclude the possible involvement of residual synapses in the neurons. Stock solution containing higher concentration of orexin A or glutamate were freshly diluted with HEPES-buffered ACSF to desired concentrations before each experiment and applied by micropipette into a coverslip mounted on the imaging chamber.

Statistical analysis.– Data are expressed as mean values ±SD. The normalized fluorescence intensity F(t)/F(0) in the soma of the selected neurons was used as an index of intracellular free Ca^{2+} concentration. F(t) and F(0) represented the fluorescence intensity of fluo-4 at time t and t=0, respectively. The differences between peak intensity value and baseline intensity value were carried out with ANOVA for statistical analysis. For all tests, $P<0.05$ were considered statistically significant.

Results

Confocal photomicrographs showed an example of $[Ca^{2+}]_i$ changes before (Fig. 1B) and about 300s after application of orexin A (1 μM) (Fig. 1C) in a fluo-4/AM-loaded neuron from layers V and VI of PFC (Fig. 1A). As seen in Fig. 1D, the cytosolic fluorescence intensity immediately increased after application of glutamate (1 mM) or orexin A (1 μM). The responses of PFC neurons to orexin A had longer transients with less rapid onsets and longer durations with slower recovery time than to glutamate. In contrast, no changes of $[Ca^{2+}]_i$ in dissociated PFC cells were observed after application of HEPES-buffered ACSF as a control during the experiments (Fig. 1D). The fluo-4 fluorescence intensity by glutamate (n=9 neurons, 9 of 9, P<0.05) and orexin A (n=8 neurons, 8 of 40, P<0.05) were markedly increased (Fig. 1D), while HEPES-buffered ACSF has no significant effect on the $[Ca^{2+}]_i$ of these cells (n=40 neurons, 40 of 40, P>0.05).

We further showed that the increase of $[Ca^{2+}]_i$ induced by orexin A (0.1 ~1 μM) was in a concentration-dependent manner (Fig. 2A). Application of 0.5 μM orexin A induced elevation of fluorescence intensity (n=9 neurons, 9 of 48, P<0.05) but lower than that induced by 1 μM orexin A, while the fluorescence intensity was just less detectable in the presence of 0.1

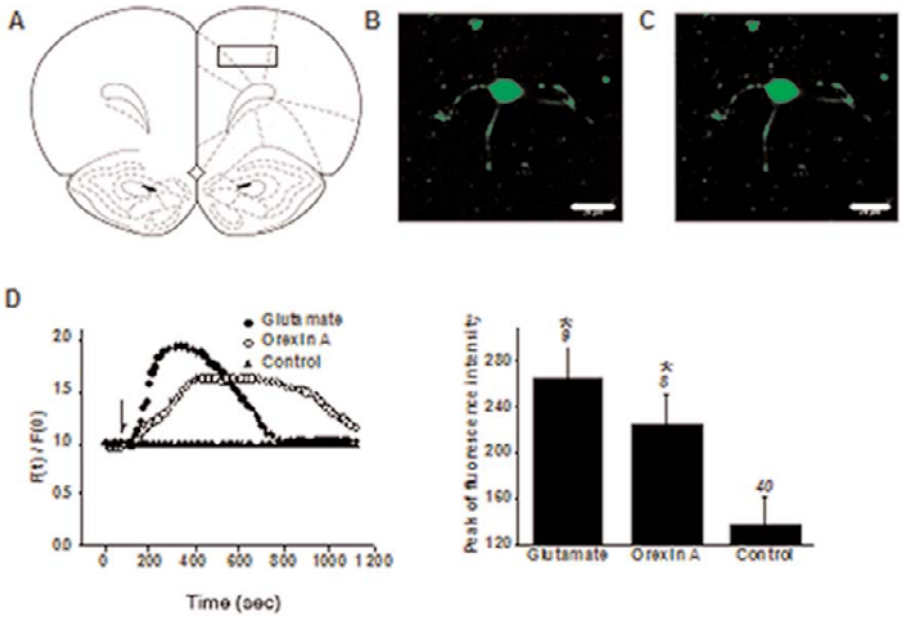

Fig. 1. *Effects of orexin A on $[Ca^{2+}]_i$ in acutely dissociated neurons from layers V and VI of PFC.* (A) Schematic representation of the place of layers V and VI in PFC. Full frame imaging of $[Ca^{2+}]_i$ in a fluo-4 loaded neuron before (B) and 300s after (C) application of orexin A (1 μM). Scale bar: 20 μm. Representative time-response traces show the changes of F(t)/F(0) in PFC neurons by glutamate (1 mM), orexin A (1 μM) or HEPE-buffered ACSF as a control (D). F(t)/F(0) is the normalized fluorescence intensity. The *numbers* indicate the number of cells recorded, and only orexin A-sensitive cells were considered after application of orexin A (1 μM). (*P< 0.05 *vs.* control).

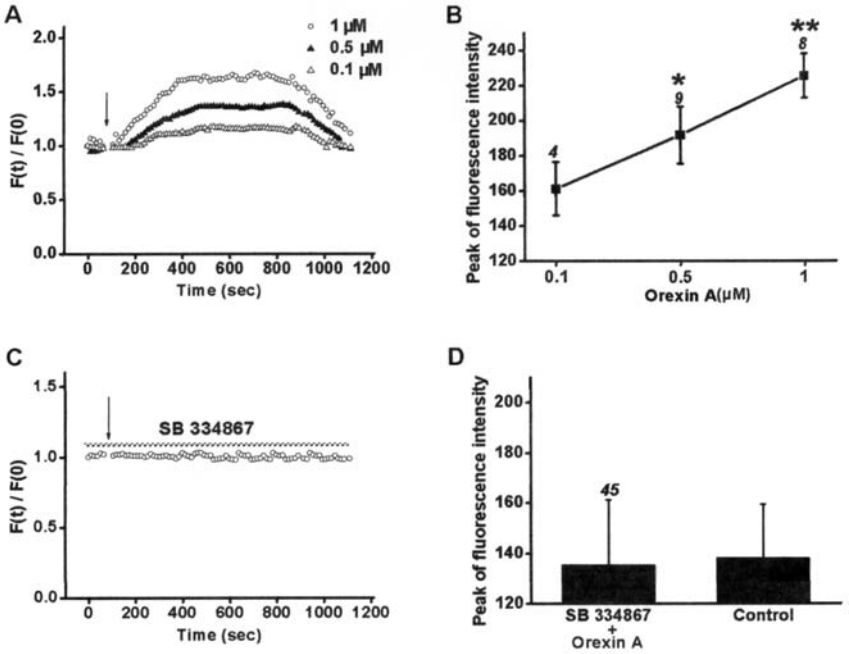

Fig. 2. *Concentration-dependent effects of orexin A on $[Ca^{2+}]_i$ of PFC neurons and the actions of selective OX1R antagonist SB 334867 on orexin A-induced $[Ca^{2+}]_i$ elevation.*

(A) Representation time-response traces of F(t)/F(0) in PFC neurons by different concentration of orexin A (1~0.1μM). (B) Concentration-response curve for orexin A on the peak of $[Ca^{2+}]_i$ fluorescence intensity in PFC neurons. The *numbers* indicate the number of orexin A-sensitive cells after application of orexin A (1~0.1μM). (*: $P<0.05$, **: $P<0.01$, *vs.* orexin A at 0.1 μM.) (C) Time course of F(t)/F(0) during application of orexin A (1 μM), in the presence of SB 334867 (10 μM). (D) Pooled data showing the peak of fluorescence intensity before (control) and after application of orexin A to dissociated PFC cells pretreated with SB 334867. Application of orexin A to PFC neurons. The *number* indicates the number of cells tested.

μM orexin A (n=4 neurons, 4/32, $P<0.05$), as shown in Fig. 2A and 2B.

OX1R and OX2R have been reported to mediate the actions of orexin A (11, 19, 21). As preliminary studies showed that a selective OX1R antagonist, SB 334867, inhibited an OX1-mediated calcium response (28). Therefore, we tested the effect of SB 334867 on the $[Ca^{2+}]_i$ of the dissociated PFC cells by orexin A. When these neurons were preincubated with SB 334867 (10μM), no elevation response of $[Ca^{2+}]_i$ was detected after application of orexin A (1 μM) (Fig. 2C). There is no significant difference of fluorescence intensity before (control) and after application of orexin A in the presence of SB 334867 (n=45 neurons, 45/45, $P>0.05$) (Fig.2D).

It has been well documented that intracellular protein kinase C (PKC) signaling pathway is involved in orexin receptors-mediated $[Ca^{2+}]_i$ mobilizing (1, 14, 22, 23, 33). We then determined whether orexin A regulates $[Ca^{2+}]_i$ of PFC neurons by modulation of PKC signaling pathway. The BIS II, a PKC inhibitory, acting on the catalytic subunit of PKC, was chosen because of their high potency and specificity for PKC (6, 31). When cells were pretreated with BIS II (1 μM), for 1 hr to

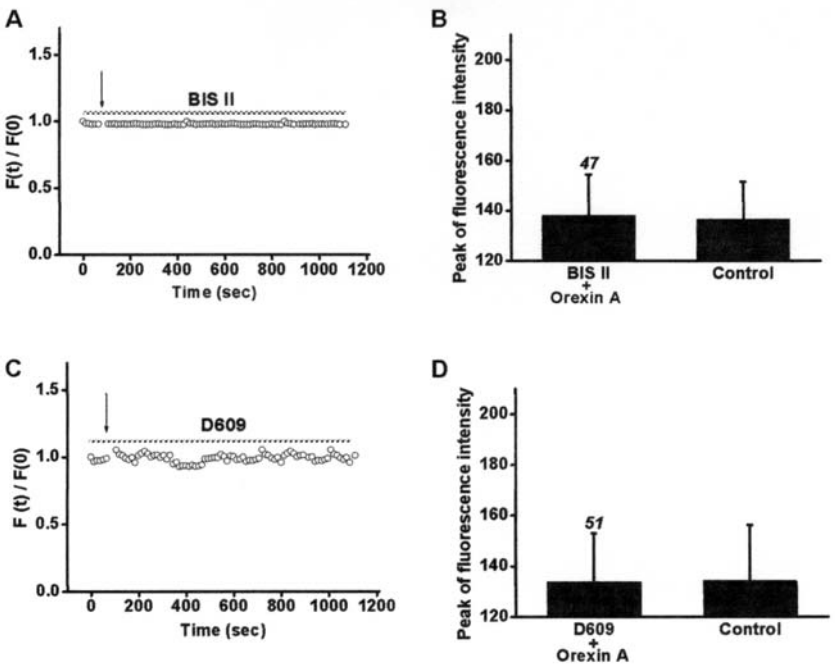

Fig. 3. *Effects of PKC inhibitor BIS II and PLC inhibitor D609 on orexin A-induced $[Ca^{2+}]_i$ elevation.* Representation time-response traces of F(t)/F(0) in PFC neurons during application of orexin A (1 μM), in the presence of BIS II (1 μM) (A) or D609 (10 μM) (B). Pooled data demonstrating the peak of fluorescence intensity before (control) and after administration of orexin A to PFC neurons preincubation with BIS II (C) or D609 (D). Application of orexin A to a PFC neuron. The *numbers* indicate the number of cells recorded.

block PKC activity, application of orexin A (1 μM) did not modify the fluorescence intensity in cells recorded (Fig. 3A). No difference of fluorescence intensity was observed before (control) and after application of 1 μM orexin A in the presence of BIS II (n=47 neurons, 47 of 47, P>0.05) (Fig. 3B). Some orexins-mediated actions in the nervous system have also been associated with an increase in PLC activity (8, 10, 12, 13, 20, 22, 27, 33, 42). Therefore, we used D609, a selective inhibitor of phosphatidylcholine specific-PLC to test whether the orexin A-induced increase of $[Ca^{2+}]_i$ in PFC neurons was also PLC-dependent. Similarly, preincubation of these cells with D609 (10 μM, 1 h) significantly attenuated orexin A-evoked $[Ca^{2+}]_i$ elevation, as illustrated in Fig. 3C and 3D (n=51 neurons, 51 of 51, P>0.05).

Compelling evidence has shown that orexins could induce elevation of $[Ca^{2+}]_i$ through extracellular Ca^{2+} entry (1, 8, 10, 11, 13, 14, 19, 20, 33, 34), releasing Ca^{2+} from intracellular store (27), or both (16). Our previous data has demonstrated that extracellular Ca^{2+} entry contributes to the elevation of $[Ca^{2+}]_i$ induced by orexin A in cultured cortical neurons (37). Whether orexin A-evoked $[Ca^{2+}]_i$ increase of PFC neurons in layers V and VI was due to the influx of extracellular Ca^{2+} as well had not been examined yet. In the current experiment, we first explored the possibility of

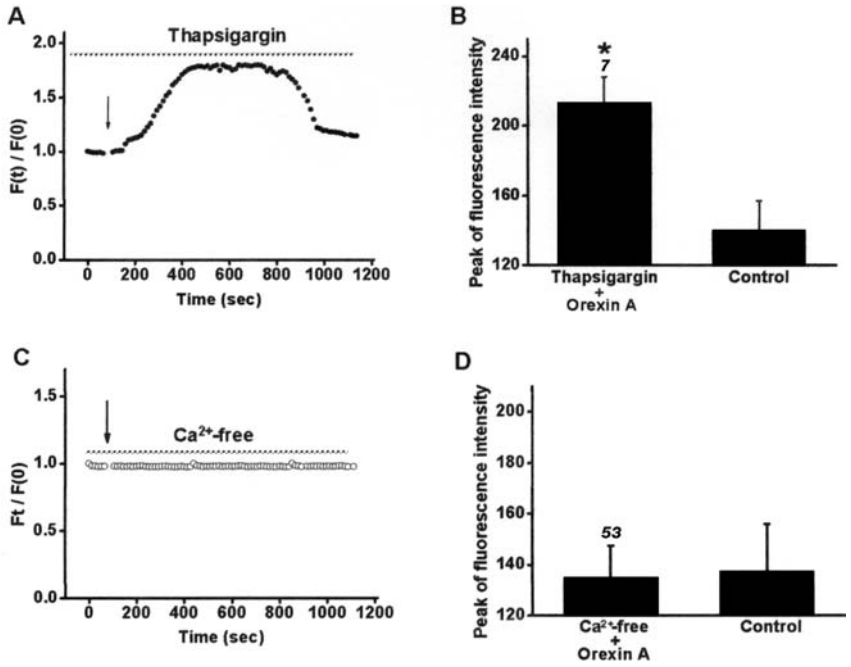

Fig. 4. *Effects of thapsigargin and Ca^{2+}-free solution on orexin A -induced $[Ca^{2+}]_i$ elevation.* Time courses of F(t)/F(0) in PFC neurons by orexin A (1 μM) in the presence of thapsigargin (2 μM) (A) or under extracellular Ca^{2+}-free solution (B). Histograms show the peak of intracellular Ca^{2+} fluorescence intensity before (control) and after application of orexin A to PFC neurons pretreated with thapsigargin (C) or in Ca^{2+}-free ACSF (D). Application of orexin A to a PFC neuron. The *numbers* indicate the number of cells recorded, and only orexin A-sensitive cells were considered after application of orexin A (1 μM) in the presence of thapsigargin. (*$P< 0.05$ *vs.* control).

Ca^{2+} releasing from intracellular store involved in such elevation of $[Ca^{2+}]_i$ in PFC neurons. In spite of pretreating these neurons with an endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (2 μM) for at least 1 h, the increase in $[Ca^{2+}]_i$ evoked by 1 μM orexin A persisted during the experiment (Fig. 4A). The difference was significant (Fig. 4B) before (control) and after application of orexin A (1 μM) in the presence of thapsigargin (n=7 neurons, 7 of 36, $P<0.05$). Next, the effects of extracellular Ca^{2+}-free solution on orexin A-induced $[Ca^{2+}]_i$ increase was further examined in PFC neurons. Our data showed that the increase of $[Ca^{2+}]_i$ evoked by orexin A (1μM) was almost completely abolished under extracellular Ca^{2+}-free condition (Fig. 4C). No significant difference of fluorescence intensity was detected before (control) and after application of orexin A (1μM) in Ca^{2+}-free solution (n=53 neurons, 53/53, $P>0.05$) (Fig.4D).

Several reports have demonstrated that such extracellular Ca^{2+} influx depends on the activation of different membrane channels (14, 19, 24, 33). To further characterize the source of the calcium influx, L-type Ca^{2+} channel blocker nifedipine was used to observe the responsiveness of PFC cells to orexin A. As shown in Fig. 5A, application of this inhibitor had no

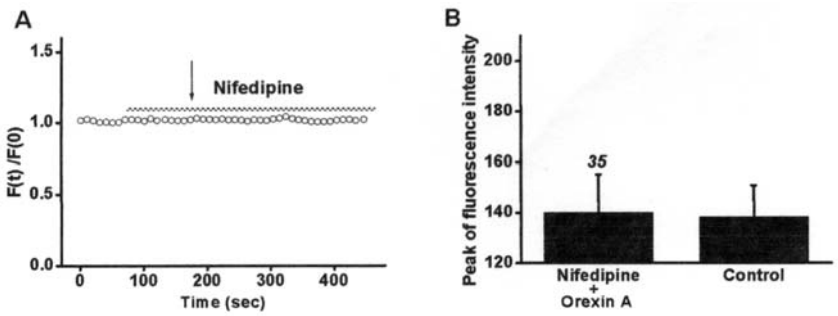

Fig. 5. *Effect of L-type Ca^{2+} channel inhibitor nifedipine on orexin A-induced $[Ca^{2+}]_i$ elevation.* (A) Representation time-course trace shows the changes of F(t)/F(0) in a PFC neuron before and after application of nifedipine (5 μM) (control) followed by administration of orexin A (1 μM). (B) Pooled data showing the peak of fluorescence before and after application of orexin A. The *number* indicates the number of all test neurons. Application of orexin A to a PFC neuron.

effect on the $[Ca^{2+}]_i$ but it could alter the orexin A-induced potentiation of $[Ca^{2+}]_i$ in PFC neurons. In the presence of nifedipine (5μM, 1~5 min), orexin A (1 μM) failed to induce any increase of fluorescence intensity in the dissociated neurons (Fig. 5A). Statistic results showed that there was no significant difference between before (control) and after application of orexin A when the L-type Ca^{2+} channels were blocked (n=35 neurons, 35/35, P>0.05) (Fig. 5B).

Discussion

The results of the current study mainly show that orexin A provokes Ca^{2+} mobilization from extracellular Ca^{2+} influx through L-type Ca^{2+} channels in freshly dissociated PFC neurons of layers V and VI, which is mediated by the activation of OX1R and intracellular PLC-PKC pathway.

Our previous data has presented that orexin A exerts an excitatory action on neurons from layers V and VI of PFC (36). While only a small portion of cultured cortical neurons display $[Ca^{2+}]_i$ elevation in response to orexin A (34,37), and the regions selectivity of responses to orexins has been identified in cortex (2), it's not sure whether these neurons from the deep layers of PFC show the similar changes in $[Ca^{2+}]_i$ after application of orexin A. In this study, we focus on the effect of orexin A on $[Ca^{2+}]_i$ of these neurons and find that orexin A causes a fast increase of $[Ca^{2+}]_i$ in dissociated PFC neurons, which is consistent with the action of orexin A on primary cultured cortical neurons from rats (37). These results suggest that in addition to a direct electrical excitation, orexin A also elevates $[Ca^{2+}]_i$ of these neurons in deep layers from PFC. This may provide another important way of orexins involved in the promotion and maintenance of the widespread cortical activation.

Similarly, compared to 25.64% of cultured cortical neurons (37), the data in this study demonstrate that about 20% of PFC neurons show an orexin A-evoked $[Ca^{2+}]_i$ elevation dose-dependently, indicating a directly excitatory effect of orexins on a small proportion of cells in layers V and VI of PFC. Moreover, we have reported that orexin A induces long-lasting increases in $[Ca^{2+}]_i$ with two different

temporal profiles ("plateau" responder and "S" responder) in the cultured cortical neurons (37), which agrees with the varying temporal profiles to orexins observed in laterodorsal tegmentum and dorsal raphe neurons (14). Nonetheless, no different response profiles have been observed in this study, and the responses of $[Ca^{2+}]_i$ elevation to orexin A in PFC neurons belong to the "plateau" responder characterized with a longer plateau period in peak value.

Here we also find that the increase of $[Ca^{2+}]_i$ induced by orexin A in PFC neurons is totally blocked by the OX1R antagonist SB 334867, indicating that the excitatory effects of orexin A on PFC neurons are mediated by OX1R, which are consistent with the previous anatomical data demonstrating that PFC neurons mainly express OX1R mRNA (32). Since very low levels of OX2R mRNA are present in PFC and SB 334867 may also inhibit OX2R-mediated calcium response (28,32), whether OX2R are involved in such elevation of $[Ca^{2+}]_i$ in PFC neurons is needs to be further clarified.

Additionally, the present data also show that the PKC and PLC inhibitors suppress the Ca^{2+} response to orexin A respectively, which suggests that intracellular PLC-PKC signaling pathway plays an important role in mediating the stimulatory effect of orexin A on $[Ca^{2+}]_i$ in PFC neurons. The contribution of PLC and PKC to the orexins-evoked $[Ca^{2+}]_i$ increase has been extensively investigated in other neurons and cells (1, 8, 10, 13, 14, 20, 22, 27, 33, 42). In particular previous studies strongly support the idea that the primary response upon stimulation of the OX1R is the activation of a Ca^{2+} influx pathway, which triggers the PLC signaling pathway (13,20). Similarly, orexin A evokes $[Ca^{2+}]_i$ signaling in the neuropeptide Y neurons from the arcuate nucleus via OX1R-PLC-PKC and IP_3 pathways (22). In all, activation of PLC-PKC signaling pathway is shown to be a common mediator for the actions of orexins on diverse cellular populations.

We also notice that the rise time before reaching peak of fluorescence intensity induced by orexin A found in this study is slower compared with that in one published report (27). This difference may result from the fact that drugs were applied by a micropipette and there would be a delay before effective concentration of drugs reached the observed neurons by gradually diffusing in this study. Another possibility is the distinct samples we used, Chinese hamster ovary cells transfected by orexins receptors and freshly isolated neurons from layers V and VI in PFC of neonatal rats.

Orexin receptors are originally discovered through screening an orphan G protein-coupled receptor library for ligand-evoked cytoplasmic Ca^{2+} transients (25). Now, orexin A is known to increase $[Ca^{2+}]_i$ through activation of extracellular Ca^{2+} influx (1, 8, 10, 11, 13, 14, 19, 20, 33, 34), intracellular stores (27), or both (16). As shown in our previous study, orexin A-induced $[Ca^{2+}]_i$ elevation is mainly from extracellular Ca^{2+} influx, independent of intracellular Ca^{2+} stores in the cultured cortical neurons (37). Likely, in this study we also find that the increase of $[Ca^{2+}]_i$ induced by orexin A disappear in extracellular Ca^{2+}-free solution, while depletion of Ca^{2+} from the intracellular Ca^{2+} store do not affect the excitatory effect of orexin A on $[Ca^{2+}]_i$ in PFC neurons. Consistent with the other reports (1, 8, 10, 11, 13, 14, 19-22, 33, 34), our results emphasize that such increase of $[Ca^{2+}]_i$ observed in dissociated PFC neurons depend on extracellular Ca^{2+} influx.

Abundant evidence has demonstrated that orexins induce extracellular Ca^{2+} entry in cells by a variety of mechanisms, including opening of voltage-gated calcium channels (4, 14, 33, 38, 39), TRPC channels (19, 24), as well as reversal of electrogenic Na^+/Ca^{2+} exchanger (3, 9, 35). However, the extracellular Ca^{2+} entry mechanisms of orexin A on PFC neurons have not been examined yet. It is well established that cortical neurons express various subtypes of Ca^{2+} channels (41). In this study, preincubation of PFC cells with a selective blocker of L-type Ca^{2+} channels, nifedipine, markedly reduce the $[Ca^{2+}]_i$ increase, indicating that activation of L-type Ca^{2+} channels are involved in the Ca^{2+} response evoked by orexin A. Indeed, the involvement of L-type Ca^{2+} channels in the orexins-induced elevation of $[Ca^{2+}]_i$ have been well documented previously in dissociated rat ventral tegmental neurons (33), as well as mouse dorsal raphe and laterodorsal tegmentum (14, 15), which is sensitive to a PKC inhibitor and/or a PLC inhibitor. In addition, it is widely known that plasma membrane Ca^{2+} channels are involved in the regulation of neuronal excitability. The enhancement of voltage-gated L-type Ca^{2+} channel currents also has been shown to mediate the function of orexins in regulating growth hormone secretion in ovine somatotropes (4,38,39) and release of cholecystokinin from intestinal neuroendocrine cell line (18).

Therefore, the present findings provide an additional data that aside from direct depolarization, binding of orexin A to the OX1R may induce increase of $[Ca^{2+}]_i$ in deep layers of PFC neurons, which is mainly from extracellular Ca^{2+} influx through L-type calcium channels by activation of intracellular PLC-PKC signaling pathway. These excitatory effects of orexin A on PFC neurons may play an important role in the maintenance of wakefulness level.

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