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Stimulation of phagocytosis in mouse peritoneal macrophages by orexin-B and orexin-A

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ABSTRACT

To define the effects of feeding and sleep regulating peptides, orexins, in immunocompetent cells, the effects of orexin-A and orexin-B on phagocytosis in mouse peritoneal macrophages were examined. Orexin-B induced an enhancement of phagocytosis in a dose-dependent manner. Orexin-A is less effective than orexin-B. Even in Ca^{2+}-free solutions, phagocytosis was enhanced by orexin-B. The potassium channel blocker quinine inhibited the enhanced phagocytosis by orexin-B; 4-aminopyridine and tetraethylammonium suppressed phagocytosis less effectively. These results suggest that orexins can enhance the phagocytosis of macrophages mediated by potassium channels.

Two homologous peptides, orexin-A and orexin-B (also called hypocretin-1 and -2) were originally discovered as appetite stimulating peptides in the course of identifying the endogenous ligands to orphan receptors (23). These have been identified in genomic research as two of a large number of cDNA sequences for G protein-coupled cell surface receptors without known endogenous ligands, *i.e.*, 'orphan' receptors. Orexin-A is a 33-residue peptide with two intramolecular disulfide bonds, while orexin-B is a linear 28-residue peptide. The amino acid sequences of the two peptides is similar, especially in their C-terminal region. The peptides are localized in the lateral hypothalamus (5), a feeding center, and stimulate food intake when administered into the cerebral ventricles in rats (23). Later studies have demonstrated that canine narcolepsy is caused by a mutation in orexin receptor 2 gene and that orexin knock out mice display episodes of narcoleptic attacks (22), suggesting an important role of the orexin-orexin receptor 2 system in the regulation of sleep and wakefulness. However, little is known about the function of orexins in the immune system.

Macrophages play key roles in host defense and the initiation of humoral and cellular immune responses, *i.e.*, phagocytosis, subsequent degradation of foreign or invading pathogens, antigen presentation to T-cells, and generation of immunoregulatory compounds such as interleukin-1 and tumor necrosis factor (25). It is known that there is a functional relationship between the nervous and the immune systems (3, 26). In fact, macrophage function and ion channel activity is modulated by neuropeptides (2, 4, 13). We previously reported that orexin-A and orexin-B activated a calcium-dependent potassium current (11). To extend our previous observations, we examined effects of orexin on the phagocytic activity of mouse peritoneal macrophages. The present study suggests that orexins, which are known as feeding and sleep-wakefulness regulators, are also immunoregulators of macrophages.

MATERIALS AND METHODS

Preparation of cells. Macrophages were prepared by the method of Gallily and Feldman (8). The peritoneal cells were harvested by intraperitoneal lavage with Hanks' salt solution from BALB/cA Jcl mice

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(Nihon Clea, Tokyo) of either sex (10–25 weeks old) 5 days after intraperitoneal injection of 4 ml thioglycollate medium, and were washed three times by centrifugation. The cells were plated onto dishes (Falcon 3001; Becton Dickinson, Oxnard, CA) at a density of 5×10^5 cells per dish and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin G in a CO_2 incubator at 37°C. Macrophages were allowed to adhere for 2 h in the CO_2 incubator, washed twice with cold Dulbecco's phosphate buffer solution (PBS), and cultured in RPMI 1640 medium.

Assay for phagocytosis. To measure phagocytic activity, ingestion of fluorescein isothiocyanate (FITC)-labeled latex particles was analysed by flow cytometry, as described previously (14). Latex bead (2 μm in diameter, no. 18338; Polysciences, Warrington, PA) solutions were sonicated for 3 min. Macrophage monolayers were incubated with latex beads at a density of 10^7 beads per dish for 30–50 min in normal external solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5. The monolayers were washed six times with cold PBS to eliminate non-ingested and freely floating beads. The cells in each dish were then incubated in 1 ml PBS containing 0.25% trypsin for 3 h at 37°C to detach the cells from dish. The suspended cells in each dish were transferred to plastic tubes (Falcon 2008). Flow cytometric analyses were performed by using a FACStar (Becton Dickinson Immunocytometry System, Mountain View, CA) to measure the number of beads per cell and total number of beads ingested into cells. The percentage of phagocytic cells (PP) was defined as the percentage of macrophages that ingested one or more particles. The phagocytic index (PI) was defined as the average number of particles ingested per macrophage and was calculated by dividing the total number of ingested beads by the total number of macrophages (10,000 cells). Because phagocytic activities indicated by PP and PI were dependent on phagocytic incubation time, ratio of beads/cells and days of culture (14), experimental data were collected from the same trial when relative potencies of phagocytosis were compared to one another.

Materials. Orexin-A (mouse) and orexin-B (mouse) were purchased from Peptide Institute, Inc., Osaka, Japan; 4-aminopyridine (4-AP) and tetraethylammonium (TEA) from Wako, Osaka; quinine hydrochloride from Nacalai Tesque, Kyoto; ethylene glycol bis(b-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA) from Sigma, St. Louis, MO. Trypsin was from Difco Lab., Detroit, MI; penicillin G was from Sigma; streptomycin was from Meiji Seika, Tokyo; fetal bovine serum was from Boehringer Mannheim GmbH, Mannheim; thioglycollate, PBS, Hanks and RPMI 1640 medium were from Nissui Pharmacal, Tokyo.

RESULTS

Flow cytometric analysis of phagocytosis

The effect of orexin on macrophage phagocytosis was analysed by flow cytometry. In Fig. 1, the left column through the right column are density plots of phagocytic cells showing forward scattering (FS) vs. side scattering (SS), density plots of phagocytic cells showing relative fluorescence intensity (FITC) vs. FS, and histograms exhibiting FITC intensity vs. distribution of cell numbers. In the center column, the left cluster within the square line represents the population that does not ingest any beads and the right cluster represents the bead-ingesting macrophages. Comparing the upper and the bottom density plots in the middle column, the area and density of the non-ingesting population were decreased and those of the bead-ingesting population were increased. As shown in the histogram of FITC vs. cell numbers, orexin-B decreased the number of non-ingesting cells and the number of cells which ingested one or more beads was increased, indicating that orexin from 10^{-8} to 10^{-6} M enhanced the phagocytosis of latex particles in mouse peritoneal macrophages.

Enhancement of phagocytosis by orexins

As shown in Fig. 2, orexin-B enhanced phagocytosis of latex particles in a dose-dependent manner up to 10^{-6} M. The threshold concentration of orexin-B may be lower than 10^{-9} M. The enhancement of phagocytosis at 10^{-6} M was reflected in a 2.4-fold increase in PP and a 3.2-fold increase in PI, compared with unstimulated control phagocytosis. The relative potencies of orexin-A and orexin-B were examined, as shown in Fig. 3. Orexin-A was less effective than orexin-B between concentrations of 10^{-8} and 5×10^{-7} M. Because orexin-B was more effective in stimulating phagocytic enhancement than orexin-A, the characteristics of phagocytosis were analysed using orexin-B in the remaining studies.

Dependency of extracellular Ca^{2+}

The effect of extracellular Ca^{2+} concentration on

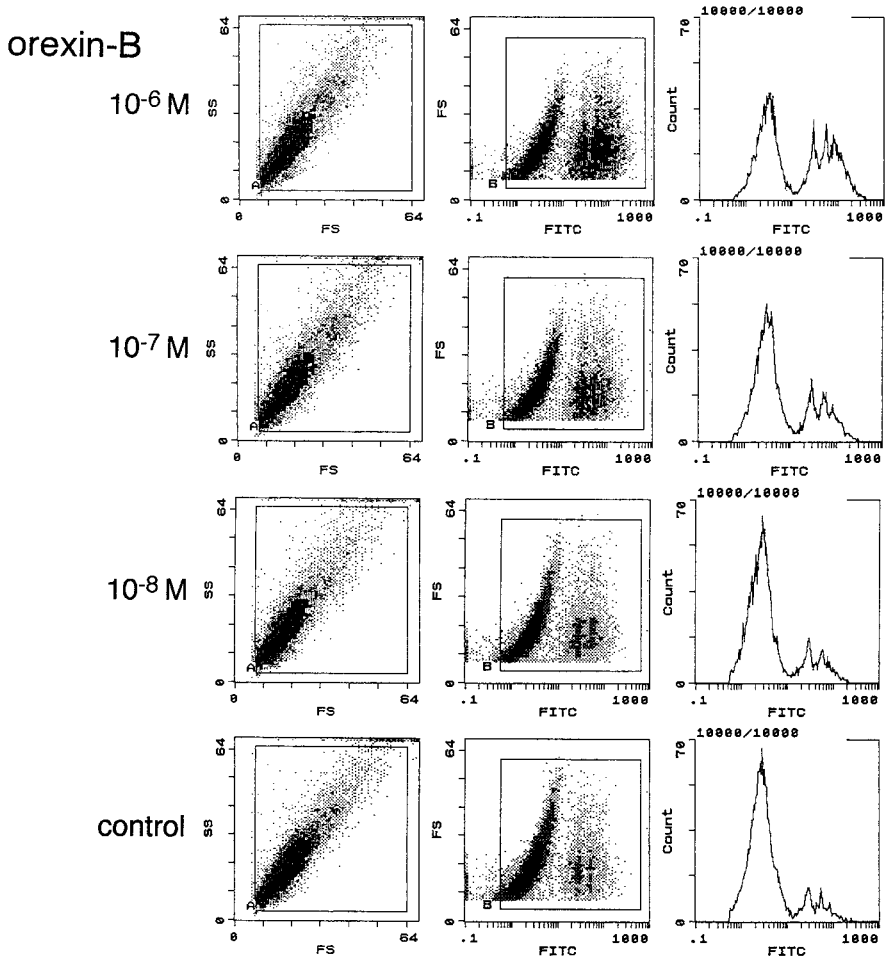

Fig. 1 Effects of orexin on flow cytometric profiles. Left column, A cell-density plot based on forward scattering (FS) versus side scattering (SS). Each dot shows a cell having measured parameters in the axis of abscissa and ordinate. Center column, A cell-density plot based on relative fluorescence (FITC) intensity and forward scattering (FS). Right column, Histogram of cells based upon FITC intensities from the rectangular square in the center column. Non-ingested, 1 bead-ingested and 2 beads-ingested cell populations are the first, second and third peaks from the left, respectively. Lowest row, Density plots and histogram in the control solution. The first, second and third rows show plots and histogram in the presence of 10^{-6}, 10^{-7} and 10^{-8} M orexin-B, respectively. Note that the height of non-ingested cells decreased and those of beads-ingested cells increased depending on the orexin concentrations, indicating that orexin stimulates phagocytosis of fluorescent-beads.

phagocytosis was examined, as shown in Fig. 4. When extracellular calcium was eliminated by adding 1 mM EGTA to Ca^{2+}-free solution, unstimulated phagocytosis was reduced to 85.4% (PP) and 75.9% (PI) of control phagocytosis in normal external solution. However, the enhancement of PP by orexin-B in normal solution and Ca^{2+}-free solution was 190.4 and 197.4%, respectively. Similarly, the enhancement of PI by orexin-B in normal and Ca^{2+}-free solution was 243.4 and 269.1%, respectively. These data indicate that phagocytic activity is reduced in the divalent cation-deficient solution, but the magnitude of the enhancement is not suppressed by the elimination of external Ca^{2+} cation.

Effect of potassium channel blockers on phagocytosis

Because potassium channel blockers suppressed an orexin-induced potassium current (11), the effects of potassium channel blockers on the enhancement of phagocytosis by orexin-B was examined, as shown in Fig. 5. Quinine (0.5 mM) suppressed the phagocytic activity of macrophages from 34 to 26% for PI and from 0.97 to 0.67 for PI (open circles in Fig. 5). In addition, quinine significantly suppressed the enhancement of phagocytosis induced by orexin-B close to the phagocytic level suppressed by quinine (0.5 mM) in the absence of orexin-B, that is, from 49 to 30% for PP and from 1.62 to 0.83 for

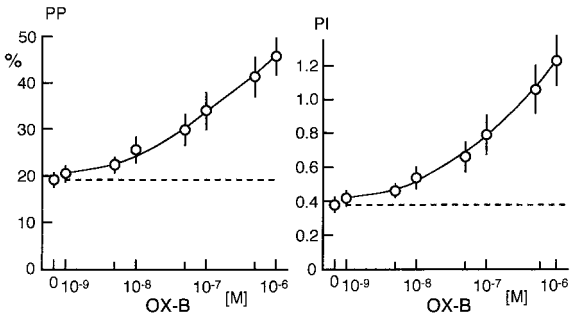

Fig. 2 Concentration-response curves showing the enhancement of phagocytosis in peritoneal macrophages by orexin-B (OX-B). Data are mean ± standard error of the mean (S.E., n = 8). The left and the right graphs show the enhancement of phagocytosis, indicated by the percentage of phagocytic cells (PP) and the phagocytic index (PI), respectively.

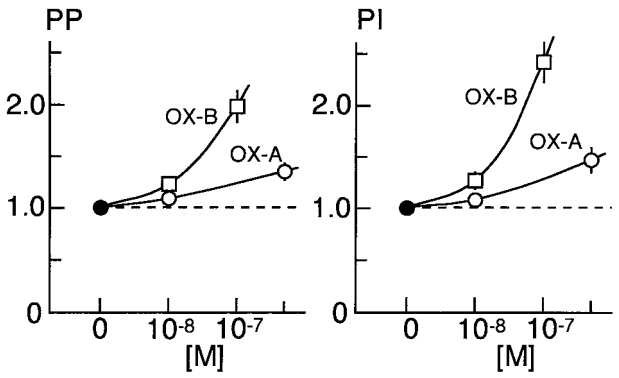

Fig. 3 Orexin-A (33-amino acid peptide, OX-A) and orexin-B (28-amino acid peptide, OX-B) differ in their efficacy in inducing enhancement of phagocytosis. The left and right graphs (open circles, orexin-A; open squares, orexin-B) show relative PP and PI values compared to control values (closed circles), which are normalized to 1.0. Data are means ± S.E. (n = 5).

PI (closed circles in Fig. 5). Another potassium channel blocker, 5 mM 4-AP suppressed the control phagocytosis from 26.0% of PP and 0.63 of PI to 22.7% of PP and 0.49 of PI (data not shown). The same concentration of 4-AP suppressed the orexin-enhanced phagocytosis from 40.5% for PP and 1.09 for PI to 28.6% for PP and 0.66 for PI. On the other hand, 20 mM TEA did not suppress the control phagocytosis in the absence of orexin, and slightly suppressed the orexin-enhanced phagocytosis to 92.7% for PP and 89.0% for PI (data not shown).

DISCUSSION

Orexins are localized in neurons within the lateral hypothalamus and are involved in different aspects of feeding behavior, stimulating appetite and food consumption (23). Therefore, orexins are recognized as potent orexigenic peptides. In addition to effects on feeding, centrally administered orexins have been demonstrated to have sympathetic and cardiovascular actions (24), play a key role in the regulation of the pathophysiology of narcolepsy (22), and activate the hypothalamo-pituitary-adrenal axis (18). Therefore, orexins are important regulators in the control of feeding, sleep-wakefulness, neuroendocrine homeostasis and autonomic regulation (6, 27). We previously reported that orexins could modulate peritoneal macrophage functions through the activation of calcium-dependent potassium channels (11). In the present study, we showed that orexins activate phagocytosis of mouse peritoneal macrophages, which is one of the main functions of macrophages. The present data indicate that the peptides may also have roles in the immune systems.

Bidirectional communication between the immune and neuroendocrine systems was proposed. In that communication, the systems share a similar group of receptors for cytokines and neuropeptides (3, 26). Though receptors to orexins in macrophages have not been reported, it may be suggested by the present study that macrophage responded to orexin-B via a specific orexin receptor. Because macrophage function is modulated by neuropeptides (2, 4, 13), receptors to the neuropeptide are also suggested. We showed that adrenocorticotropic hormone, ACTH receptor was expressed in the cultured macrophages (15).

Orexins, which regulate feeding and sleeping behavior, are exclusively localized in neurons within the lateral hypothalamus and neural fibers of the brain (5, 23). Function of the peptide in peripheral tissues is not yet defined. Because the presence of orexin was also detected in testis, gut and adrenal medulla by the later studies (17, 19, 23), function of macrophages such as phagocytosis may be activated by orexin in these tissues based on the present study. In another possibility, exudated macrophages from the circulatory system and/or brain macrophages, microglia in inflammatory region of the brain may be activated by the peptides.

PP and PI of unstimulated phagocytosis in Ca^{2+}-free solutions were reduced to 85% and 76% of the control PP and PI in normal solution, as shown in Fig. 4. Therefore approximately 15% of the phagocytosis in the normal solution is dependent on extracellular divalent cations. However, the magnitudes (197 and 269%) of the enhancement in PP and PI

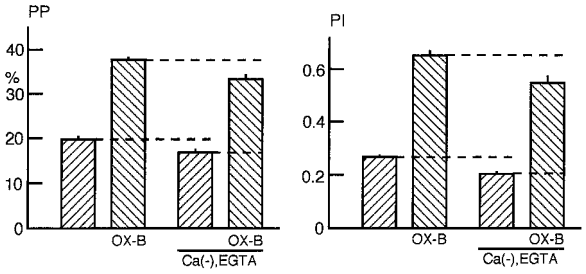

Fig. 4 Effects of extracellular Ca^{2+} on the enhancement of phagocytosis induced by orexin-B (1.5×10^{-7} M). Data are means ± S.E. (n = 4). The right two columns in the PP and PI graphs are data obtained in Ca^{2+}-free solution containing 1 mM EGTA.

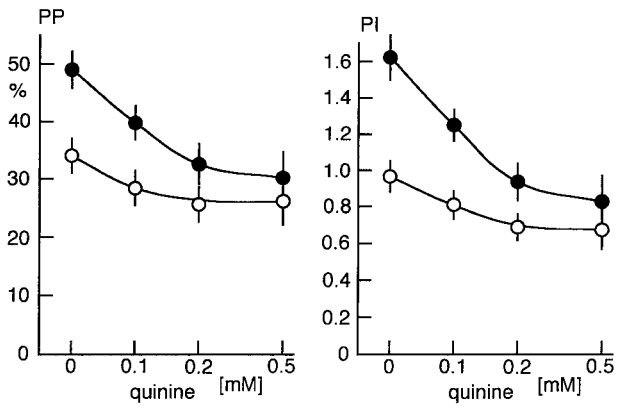

Fig. 5 Effects of quinine on phagocytosis in control solution (open circles) and in 1.5×10^{-7} M orexin-B containing solution (closed circles). Data are mean ± S.E. (n = 6). Note that quinine suppressed the magnitudes of phagocytosis in both solutions, where the enhanced phagocytosis induced by orexin-B was significantly reduced compared to that of the control solution.

by orexin-B in the Ca^{2+}-free solution were the same as those (190 and 243%) in normal solution. These results suggest that phagocytic activity in the present study consists of extracellular calcium cation dependent- and independent-phagocytosis, and that orexin-B mainly enhanced the Ca^{2+}-independent phagocytosis. Though several types of phagocytic receptors have been reported (1, 9), little is known about which type of phagocytosis involved Ca^{2+}-dependent processes. Extracellular calcium promotes a binding of latex particles to pulmonary macrophages through a trypsin-sensitive receptor (21). Fc-receptor mediated phagocytosis is slightly suppressed and nonspecific phagocytosis is markedly suppressed in Ca^{2+}-free solution (10). On the other hand, scavenger receptor might be involved in Ca^{2+}-independent phagocytosis, because a monoclonal antibody to scavenger receptor inhibits Ca^{2+}-independent macrophage adhesion (7). So the phagocytic enhancement by orexin might be mediated by the scavenger receptor or by the same characteristic receptor, but not by the Fc-receptor.

We previously reported that an outward potassium current induced by orexin-B was effectively suppressed by quinine and partially inhibited by TEA (11). So, orexins activate calcium-dependent potassium channels. Consistent with previous electrophysiological measurements, phagocytic enhancement by orexin was inhibited by the channel blockers. Therefore phagocytic enhancement may be correlated to potassium channel activation by the peptides. Other correlations between channel activation and phagocytic enhancement have been reported. Platelet activating factor and the neuropeptide, neuromedin C, stimulate phagocytosis in peritoneal macrophages (4, 14) and activate an outward potassium current (12, 13). Furthermore, the physiological function of ion channels in macrophages has been examined by using the same potassium channel blockers. Ca^{2+}-activated K^+ channels may be related to chemiluminescence and leukotrienes B_4 (LTB_4) release from alveolar macrophages (16), and production of TNF by alveolar macrophages (20). Consistent with the effects of channel blockers, phagocytic efficacy sensitivities to orexin-A and orexin-B were the same as the previous study. Namely, orexin-B induced larger outward current than orexin-A (11) and phagocytosis was enhanced more effectively by orexin-B than orexin-A in the present study, as shown in Fig. 3. The present results suggest that orexins can modulate some physiological functions of mouse peritoneal macrophages, which may be mediated by the activation of potassium channels. Therefore, ion channel activation may be related to phagocytosis and other physiological functions such as proliferation and differentiation of the macrophages.

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