Induction of outward current by orexin-B in mouse peritoneal macrophages

Mitsuyuki Ichinose[a,*], Masatoshi Asai[b], Masashi Sawada[a], Kazuo Sasaki[c], Yutaka Oomura[d]

[a] *Department of Physiology, Shimane Medical University, Izumo 693-8501, Japan*
[b] *Department of Physics, Shimane Medical University, Izumo 693-8501, Japan*
[c] *Division of Bio-information Engineering, Faculty of Engineering, Toyama University, Toyama 930-8555, Japan*
[d] *Institute of Bio-active Science, Nippon Zoki Pharmaceutical Co. Ltd., Hyogo 673-1461, Japan*

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Abstract To define effects of novel feeding regulating peptides, orexins, in immunocompetent cells, ion channel activity in mouse peritoneal macrophages was analyzed by the perforated patch-clamp method. Orexin-B (OX-B) induced an outward current at smaller holding potentials than K^+ equilibrium potentials. Reversal potentials of OX-B induced current were dependent on external K^+ concentrations but not on external Cl^- concentration. Orexin-A is less effective than OX-B. Quinine blocked the outward current and tetraethylammonium partially suppressed the current. These results suggest that OX-B can modulate macrophage functions through the activation of Ca^{2+}-dependent K^{2+} channels.

Key words: Orexin; Patch-clamp; Outward current; Mouse macrophage

1. Introduction

Two homologous peptides, orexin (OX) A and OX-B, were discovered as appetite stimulating peptides in the course of identifying the endogenous ligands to the orphan receptors OX1R and OX2R. 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 [1]. OX-A is a 33 residue peptide with two intramolecular disulfide bonds, while OX-B is a linear 28 residue peptide. The amino acid sequences of the two peptides are similar, especially in their C-terminal regions. OX-A and OX-B are coded in tandem in the same mRNA flanked by double basic amino acid residues. The same mRNA from rat hypothalamus specific mRNA clones was identified as a homeostatic regulator named hypocretin by other investigators [2].

Macrophages play key roles in the 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 [3]. In the present study, electrophysiological analysis was used to determine if the novel feeding regulating peptides, orexins, can modulate macrophage function because another feeding regulating peptide, leptin, has been reported to be an activator of hemopoietic cells and macrophages [4,5]. Examining the modulation of membrane currents by such peptides helps to define new physiological functions of feeding regulating peptides for immunocompetent cells. We found that OX-B activated charybdotoxin (ChTX)-insensitive Ca^{2+}-dependent K^+ currents on the macrophages and leptin activated the K^+ outward current and that OX-A also induced outward current less effectively than OX-B but secretin did not have such an effect. The present study suggested a new function of OX-B as an immunoregulator for macrophages.

2. Materials and methods

2.1. Chemicals

OX-A (mouse), OX-B (mouse), secretin (porcine) and ChTX were purchased from Peptide Institute, Inc., Osaka, Japan; recombinant mouse leptin was from Pepro Tech EC Ltd., London, UK; TEA was from Wako, Osaka, Japan; quinine hydrochloride was from Nacalai Tesque, Kyoto, Japan; ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was from Sigma, St. Louis, MO, USA.

2.2. Cells

Elicited macrophages were harvested from BALB/C Jcl mice (Clea Japan, Tokyo) of either sex (10–30 weeks old) by intraperitoneal injection of 5 ml of 3% thioglycollate broth (Nissui Pharmaceuticals, Tokyo) by the method of Gallily and Feldman [6]. The cells were plated on 15 mm cover glasses in 35 mm culture dishes (Falcon 1008, Becton Dickinson, CA) and cultured in RPMI 1640 (Nissui) containing 10% fetal bovine serum (Boehringer, Mannheim, Germany) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin G) at 37°C.

2.3. Electrophysiology

Electrophysiological experiments were performed with a List EPC-7 patch-clamp amplifier. Whole cell recording was achieved by the perforated-patch method as described previously [7,8]. The resistance of pipettes was 3–6 MΩ (when the pipettes were filled with an internal pipette solution containing (in mM) 145 K-aspartate, 1 $MgCl_2$, 0.1 EGTA and 10 HEPES at pH 7.8 adjusted with KOH). The tips of the pipettes were refilled with internal pipette solution containing 300 μg/ml nystatin. The liquid junction potential between the pipette and the bath solution was +12.8 mV. Cover glasses with adherent cells were mounted in a chamber (0.3 ml) and perfused at a rate of 1 ml/min with normal external solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES adjusted to pH 7.5 with NaOH. The peptides and channel blockers were applied by puff pipettes which were filled with each substance in the external bath solution and placed 30–50 μm from the recording cells. In order to change external K^+ and Cl^- concentrations, NaCl was replaced with KCl and Na-gluconate, respectively.

3. Results and discussion

Bath-applied OX-B (10^{-5} M) induced an outward current in 117 out of 196 examined cells, i.e. 59.7%, as shown in Fig. 1a. Furthermore, outward current containing outward spikes

*Corresponding author. Fax: (81) (853) 20-2115.
E-mail: michinos@shimane-med.ac.jp

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or oscillatory outward fluctuations were induced in 27 out of the 117 OX-B-responding cells, i.e. 23.1%, as shown in the first trace of Fig. 2b. Because of low appearance of the oscillatory current, only the sustained outward currents were analyzed in the present study. By the application of complement component C5a, two outward currents, i.e. a slowly rising sustained outward current and a spike-like transient outward current, were observed in peritoneal macrophages [9]. Repetitive application gradually reduced the amplitude of the outward currents even when OX-B was applied for a longer duration, as shown in Fig. 1c,d. To compare the effect of OX-B to that of leptin, which is another feeding regulating hormone produced by adipocytes [10,11], leptin was applied to an OX-B-responsive cell. As shown in Fig. 1b, leptin also induced an outward current in the same cells.

The effect of OX-B concentration was examined. As shown in Fig. 2a, 10^{-6} M did not stimulate an outward current while 2×10^{-6} and 5×10^{-6} M OX-B activated outward currents smaller than that induced by 10^{-5} M OX-B. After normalizing the amplitudes of the first OX-B response at 10^{-5} M as 100%, relative amplitudes of the following responses at concentrations of 10^{-6}, 2×10^{-6} and 5×10^{-6} M were $4.7\pm2.8\%$ (mean ± S.E.M., $n=9$), $36.4\pm6.7\%$ ($n=7$) and $50.2\pm5.6\%$ ($n=5$), respectively, as shown in Fig. 2d. No outward current was observed with 10^{-6} M OX-B in six out of the nine recordings in which 10^{-5} M OX-B activated an obvious outward current, as shown in Fig. 2a.

To clarify the ionic mechanism of the outward current, the dependence of reversal potentials on external ion concentrations was examined. In the normal solution of 5 mM K^+, OX-B induced an outward current and a little outward current at holding potentials of −50 and −78 mV, respectively (Fig. 3b). The mean reversal potential at 5 mM K^+ solution was -84.4 ± 2.2 mV (mean ± S.D., $n=13$), suggesting that the OX-B-induced outward current was mainly carried by K^+. At 2 mM external K^+ solution, OX-B induced an outward current and a slight outward current at −70 and −99 mV, respectively, as shown in Fig. 3a. The mean reversal potential at 2 mM external K^+ solution was -104.4 ± 4.2 mV (mean ± S.D., $n=11$). Similarly, the mean reversal potential at 20 mM K^+ solution was -50.3 ± 1.5 mV (mean ± S.D., $n=13$). The relationship between the mean reversal potentials and external K^+ concentrations is illustrated in Fig. 3d. An increase in external K^+ concentrations from 5 to 20 mM raised the reversal potential from −84.4 to −50.3 mV. This increase of 34.1 mV is close to the 35.5 mV which is predicted for a reversal potential only dependent on external K^+ concentration. To confirm the dependence on K^+ but not Cl^-, the external concentration of Cl^- was reduced to half of the normal solution (75 mM) by exchanging chloride ions with gluconate ions. In this solution, the reversal potential was -86.3 ± 2.0 mV (mean ± S.D., $n=9$), as indicated by the closed circle in Fig. 3d, close to the reversal potential (−84.4 mV) of the normal solution. Furthermore, the current-voltage (I-V) relationship was examined to characterize the outward current. I-V curves before OX-B application and during the peak of the OX-B response are shown in Fig. 3e. A net I-V relationship of the outward current is shown in Fig. 3f. The same outward rectification was observed in seven other cells.

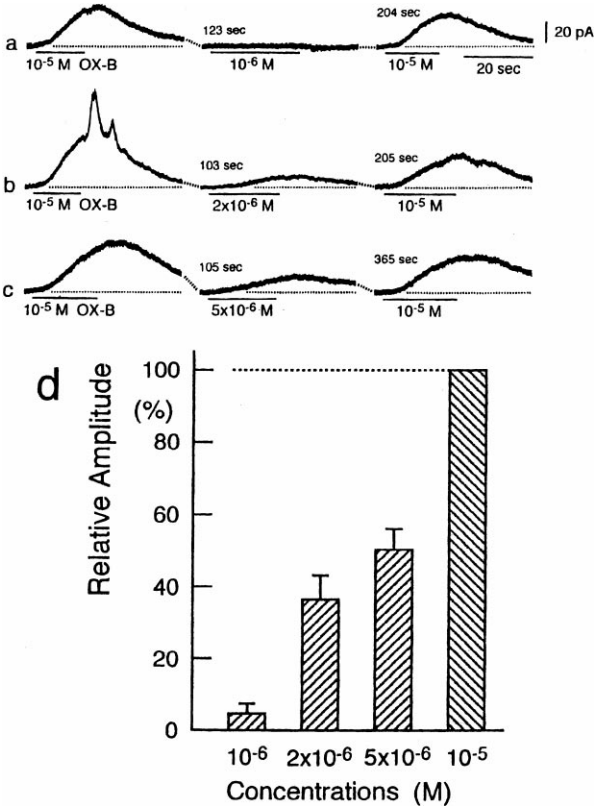

Fig. 2. Dose dependence of outward currents to OX-B. OX-B at concentrations of 10^{-6} M, 2×10^{-6} M and 5×10^{-6} M was applied between the first and the second applications of 10^{-5} M OX-B in (a), (b) and (c), respectively. The numbers on the second and the third traces in (a), (b) and (c) are application times after the onset of the first application of 10^{-5} M OX-B. All recordings were obtained at a holding potential of −30 mV. d: Summarized data of dose dependence on outward currents compared to the response by 10^{-5} M OX-B. Amplitudes of 10^{-5} M responses were normalized to 100%. Data are mean ± S.E.M. Note that oscillatory outward spikes were sometimes observed like the first trace of (b).

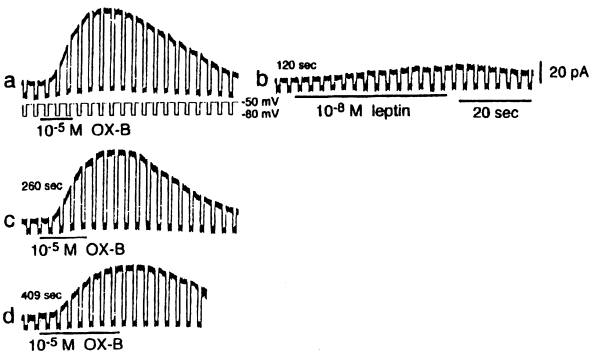

Fig. 1. Effects of OX-B and leptin on membrane currents of mouse peritoneal macrophages. Repetitive applications of OX-B (10^{-5} M), indicated by the bar under each trace, induced outward currents at a holding potential of −50 mV (a, c and d). The cell was voltage-clamped at −50 mV (2 s duration) and −80 mV (1 s duration) alternately in each trace. Voltage monitor is only illustrated in the lower trace of (a). Leptin (10^{-8} M) also induced a slowly developing outward current at −50 mV (b). The applications in traces of (b), (c) and (d) were achieved at 120, 260 and 409 s after the beginning of the first application, respectively. The peak amplitudes of (b), (c) and (d) were 18%, 94% and 69% of the peak amplitude of the first response, respectively.

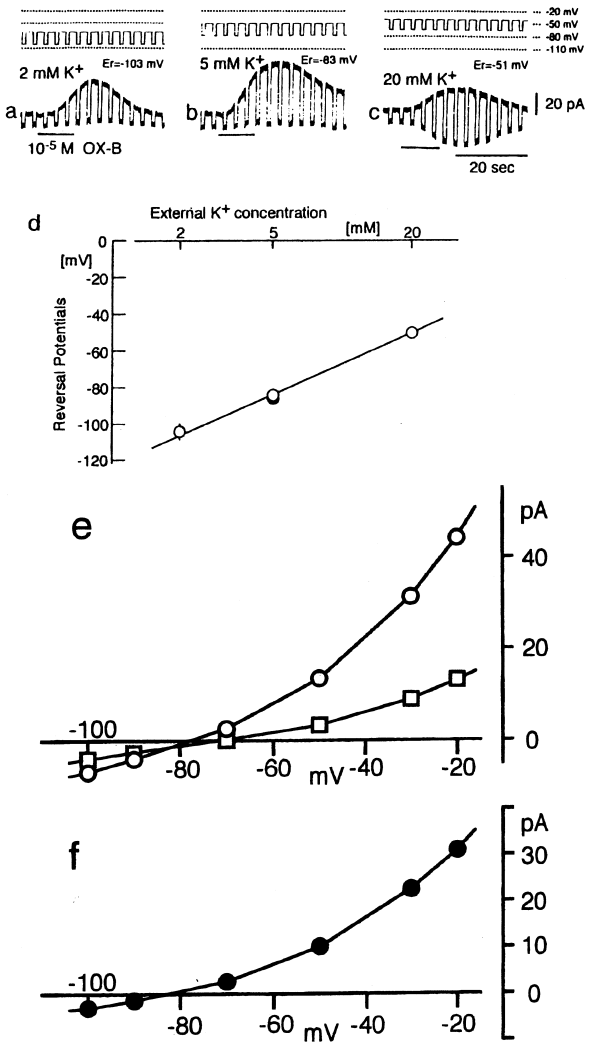

Fig. 3. Reversal potentials of the outward current by OX-B in different external concentrations of K^+ and Cl^-, and I-V relationship of the current. a–c: Representative responses to OX-B at various holding potentials in several external K^+ concentrations ($[K^+]_o$). a: $[K^+]_o$ was 2 mM. Holding potentials were −70 and −99 mV. Presumed reversal potential was −103 mV. b: $[K^+]_o$ was 5 mM. Holding potentials were −50 and −78 mV. Presumed reversal potential was −83 mV. c: $[K^+]_o$ was 20 mM. Holding potentials were −40 and −65 mV. Presumed reversal potential was −51 mV. d: Relationship between reversal potentials vs. external $[K^+]_o$ and $[Cl^-]_o$. Open circles are means obtained by changing $[K^+]_o$ and the closed circle is the mean obtained in half normal $[Cl^-]_o$. Data are means ± S.D. Only S.D. at 2 mM K^+ solution was larger than the circle indicating the mean value. e: I-V curves before OX-B application (open squares) and during the peak of OX-B-induced outward current (open circles). f: I-V relationship of a net OX-B-induced current was derived from the difference between the open squares and the open circles in (e).

Next, we examined effects of OX-B relative peptides. OX-A was isolated as a homologous peptide to OX-B [1]. However, OX-A had less effect than OX-B. The mean response amplitude by OX-A was 26.0 ± 5.6% (mean ± S.E.M., $n = 13$) of the mean OX-B-induced response. Furthermore, the mRNA of orexins was also isolated independently by De Lecea et al. [2]. They named the peptides hypocretins because they isolated those peptides based on the sequence similarity to secretin. However, secretin did not induced any outward current in seven OX-B-sensitive macrophages in the present study. Because OX-A induced an outward current but secretin did not induce any current in the present study, the outward current observed in peritoneal macrophages seems to be specific to orexins. The orexin receptor OX1R is 100 times higher in affinity to OX-A than OX-B while OX2R has the same affinity to OX-A and OX-B in orexin cDNA-transfected CHO cells [1]. In contrast, OX-B more effectively induced the outward current than OX-A in the present study. Therefore, the presence and characteristics of the orexin receptors in macrophages need to be clarified.

To characterize the K^+ channel of the outward current, the effects of three Ca^{2+}-dependent K^+ channel blockers, quinine, tetraethylammonium (TEA) and ChTX, were examined. Quinine effectively suppressed the outward current in seven cells, as shown in Fig. 4a. TEA inhibited the OX-B-induced outward current in seven cells, as shown in Fig. 4b. ChTX did not have any effect in 18 cells, as shown in Fig. 4c. To confirm the Ca^{2+} dependence in the outward current, Ca^{2+}-free solution containing 1 mM EGTA was applied in the same way as K^+ channel blockers. Though the patch membrane was broken every time a little after the application of Ca^{2+}-free solution, suppression of the outward current was observed in six cells before the break of electrical recordings, as shown in Fig. 4d. These data suggest that OX-B activated the ChTX-insensitive Ca^{2+}-dependent K^+ channel in macrophages. We have reported that neuromedin C activated the ChTX-insensitive Ca^{2+}-dependent K^+ channel in macrophages [12]. Though we did not analyze the oscillatory outward current induced by OX-B, complement C5a and platelet activating factor (PAF) activated the same oscillatory outward current which was ChTX-sensitive Ca^{2+}-dependent K^+ channels in macrophages [9,13].

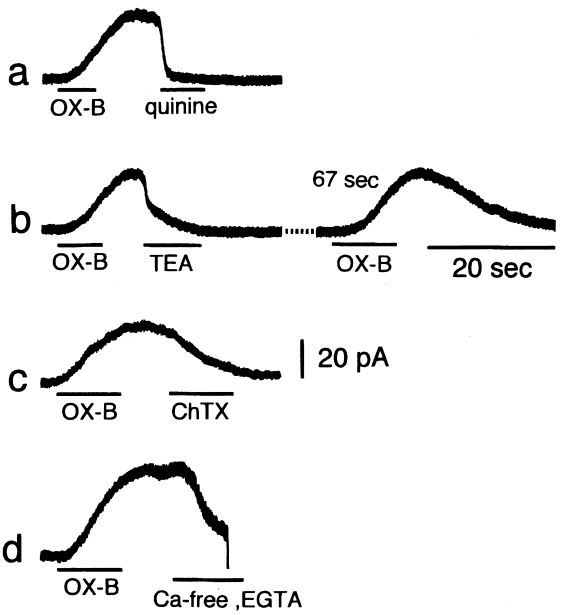

Fig. 4. Effects of potassium channel blockers and external Ca^{2+} dependence. a: Quinine (1 mM) markedly suppressed the OX-B-induced outward current. b: TEA (20 mM) slightly inhibited the current. Control decaying time course of the response is shown in the second OX-B application. The second application was achieved at 67 s after the first OX-B application. c: ChTX did not inhibit the response. d: Application of Ca^{2+}-free solution containing 1 mM EGTA reduced the outward current and then the electrical recording was broken 8 s after the solution change indicated by the sudden downward deflection.

Activation of ion channels in macrophages by bioactive molecules has been reported. Chemotactic factors (C5a, C3 and FMLP) induce oscillatory hyperpolarization in human monocytes [14] and two types of outward current in mouse peritoneal macrophages [9]. PAF induces an oscillatory outward current [13]. The neuropeptide neuromedin C, a stimulator of phagocytosis in peritoneal macrophages [15], activates an outward current [12]. Furthermore, significant physiological functions of ion channels in macrophages have been suggested. Ca^{2+}-activated K^+ channels may be related to chemiluminescence and LTB4 release from alveolar Mϕ [16], and production of tumor necrosis factor from alveolar Mϕ [17]. The inward rectifying K^+ channels may set the membrane potential to its resting level after hyperpolarizing stimuli, and the outward rectifying K^+ channels may function to restore resting membrane potentials to negative values after depolarization [18]. In addition, Cl^- and K^+ conductance may play a role in volume regulation in macrophages [18]. These data suggest that ion channel activation may be a key signal for phagocytosis and other physiological functions such as proliferation and differentiation of macrophages. Therefore, the present result suggests that OX-B can modulate some physiological function of mouse peritoneal macrophages.

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