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A role for PKD1 and PKD3 activation in modulation of calcium oscillations induced by orexin receptor 1 stimulation

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The neuropeptides orexin-A/hypocretin-1 (Ox-A) and orexin-B/hypocretin-2 play an important role in the control of energy metabolism via either of two G-protein-coupled receptors, orexin receptor 1 (Ox1R) and 2. Despite its significant physiological functions, signaling via orexin receptors is still poorly characterized. The aim of this study was to improve our understanding of early signaling events triggered by the binding of Ox-A to Ox1R. Using phosphospecific antibodies, we observed that early kinase activation by Ox-A in a HEK293 cell line stably expressing Ox1R (HEKOx1R) included ERK1/2, PKCδ, and PKD1. Elevation of intracellular Ca^{2+} is a well-characterized response to Ox1R activation. Comparison of Ox-A-induced calcium elevation and PKD1 activation demonstrated that both responses are detectable soon after stimulation and increase in a dose-dependent manner, but inhibition of protein kinase C, when low Ox-A concentrations are used, affects them differently. PKD family of protein kinases has 3 members: PKD1, 2, and 3, which are all expressed in HEKOx1R cells. In response to stimulation of the cells with 1 nM Ox-A, both PKD1 and PKD3 are activated and increased in the plasma membrane, pointing at a possible role for these kinases in that cell compartment. Overexpression of either kinase-dead PKD1 or kinase-dead PKD3 disrupts Ox-A-induced calcium oscillations demonstrating the functional role of these kinases in modulating physiological responses to Ox-A.

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1. Introduction

Orexins/hypocretins, orexin-A (Ox-A) and -B, are hypothalamic neuropeptides [1,2] involved in multiple physiological functions including the regulation of feeding, wakefulness, breathing, reproduction, autonomic functions, and energy homeostasis (reviewed in references [3,4]). They activate two distinct G-protein-coupled receptors (GPCR), orexin receptor 1 (Ox1R) and 2 [2]. One well-characterized cellular response to binding of Ox-A to $G_{q/11}$-coupled Ox1R is an elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) [2,5–7]. More recently, we also characterized Ox-A-induced calcium oscillations in HEK293 cells stably expressing Ox1R (HEKOx1R) [8].

Stimulation of Ox1R by Ox-A has been shown to activate protein kinases: mitogen-activated protein (MAP) kinases [9–12], protein kinase C (PKC) [11,13], and Akt [14]. Activation of phospholipases by GPCR produces lipid second messengers, which are potent activators of novel PKC (reviewed in [15]) and protein kinase D (PKD) [16–18]. PKD1 has been shown to modulate ion channel trafficking [19] and activity [20–22].

In this study, we identified and characterized PKD1 and PKD3 responses to activation of Ox1R by Ox-A in HEKOx1R cells. We found that they are dose-dependent following a similar dose–response curve as Ox-A-induced calcium rise. Interfering with either PKD1 or PKD3 activity affects Ox-A-induced calcium oscillations, demonstrating the functional relevance of their activation in the physiological responses to Ox-A.

2. Materials and methods

2.1. Materials

P-(dipropylsulfamoyl)benzoic acid (probenecid), digitonin, and GF109203X (GF-X) and monoclonal anti-actin antibody were from Sigma-Aldrich (Helsinki, Finland). Fura-2-acetoxymethyl ester (fura-2 AM) was from Molecular Probes (Eugene, OR, USA). Human

Abbreviations: Ox-A, orexin-A; GPCR, G-protein-coupled receptor; Ox1R, orexin receptor 1; $[Ca^{2+}]_i$, intracellular calcium concentration; PKC, protein kinase C; PKD, protein kinase D; HEKOx1R, HEK293 cells stably transfected with Ox1R; GF-X, GF109203X; fura-2 AM, fura-2-acetoxymethyl ester; ERK1/2, extracellular signal-regulated kinase 1 and 2; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco's modified Eagle's cell culture medium; HBM, HEPES-buffered Na^+ medium; RIPA, radioimmunoprecipitation assay buffer; TRPC, canonical transient receptor potential channel; DAG, diacylglycerol

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orexin-A (Ox-A) was from Neuropeptide (Strasbourg, France). FuGENE6™ was from Roche Diagnostics (Espoo, Finland). Phospho-PKC Antibody Sampler Kit, PKD1/PKCμ, Phospho-PKD1/PKCμ Ser744/748 and PKD1 Ser916 antibodies were from Cell Signaling (Danvers, MA, USA). Anti-active ERK1/2 was from Promega (Madison, WI, USA). Full-length A.v. polyclonal anti-GFP antibody and JL8 monoclonal anti-GFP antibody were from Clontech (Mountain View, CA, USA) and anti-Ox1R antibody from Alpha Diagnostic International (San Antonio, TX, USA). Dynabeads Protein G was from Invitrogen (Paisley, UK).

2.2. Cell culture

The generation of HEKOx1R cells has been described earlier [8,23]. The cells were grown in standard Dulbecco's modified Eagle's cell culture medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin–streptomycin (Invitrogen), and 0.05 mg/ml hygromycin (Invitrogen), at 37 °C in 5% CO_2 in an air-ventilated humidified incubator in 260-ml culture flasks (Nunc A/S, Roskilde, Denmark) or in 800-ml culture flasks for Ca^{2+} measurements in suspension.

2.3. Media for Ca^{2+} measurements

The HEPES-buffered Na^+ medium (HBM) consisted of the following (in mM): 137 NaCl, 5 KCl, 1 $CaCl_2$, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 10 glucose, 1 probenecid, 20 HEPES, and 1 $MgCl_2$; and the pH was adjusted to 7.4 with NaOH.

2.4. Detection and identification of PKD1, 2, and 3 mRNA in HEKOx1R cells

Primers were designed using eprimer3 (EMBOSS, CSC, Finland):

PKD1 5′-GCCAGCTTCGTAATGAGG-3′/5′-CCTGCCCTTTTCACTTGA-3′

PKD2 5′-CGCTCTTCCAGAACAACACG-3′/5′-ACGAAGTAGGTGGCATTGG-3′

PKD3 5′-CATGCCTGTTACTCCTCAAGC-3′/5′-AACTGGCCTGAACCAAGC-3′

Total RNA was reverse-transcribed using Revertaid (Fermentas, Helsinki, Finland). cDNA was amplified using Dynazymes (Finnzymes, Espoo, Finland), 95 °C for 5 minutes, 94 °C/72 °C/55 °C 20 seconds each, 30 cycles, followed by 5 minutes at 72 °C. PCR products were purified and sequenced using ABI Prism system. Sequences were identified using Blast [24].

2.5. Transfection of kinase-dead PKD constructs

PKD1K612W-EGFP (PKD1kd) was a gift of A. Hausser [25], and EGFP-PKD3K605A (PKD3kd) from O. Rey [26]. For transfection, 200,000 HEKOx1R cells were seeded in 35-mm Petri dishes (Nunc A/S, Roskilde, Denmark) containing a coverslip (25-mm, Merck Eurolab, Espoo, Finland) in 2 ml of DMEM. After 18–24 hours, cells were transfected with 6 μl of FuGENE 6 (Roche Diagnostics, Espoo, Finland) and 1 μg of DNA.

2.6. Ca^{2+} imaging of single cells and cell suspension

For single-cell Ca^{2+} imaging experiments, the cells were loaded with 4 mM fura-2 AM at 37 °C in HBM for approximately 30 minutes. The coverslip was attached to the bottom of a thermostated (37 °C) perfusion chamber. Expression of the enhanced green fluorescent protein (EGFP)-tagged constructs was detected with 450–480 nm UV light and 520 nm barrier filter. The cells were excited at the wavelengths 340/380 nm under the control of an InCyt2™ system (Intracellular Imaging, Cincinnati, OH, USA). The emitted fluorescence was measured through a 430-nm dichroic mirror and a 510-nm barrier filter with a Cohu CCD camera. A new ratio image (340/380 nm) was collected every second. To investigate the effect of kinase-dead PKD constructs on peak heights induced by Ox-A, the cells were challenged by a short pulses of increasing concentrations (1, 10, and 100 nM) of Ox-A followed by 100 μM oxotremorine. Because EGFP fluorescence in transfected cells could interfere with the detected ratio (340/380 nm), response to oxotremorine was used as a control to normalize Ox-A responses. The results are expressed as percent of the peak height of the control response. Cells with no detectable response to 1 nM Ox-A were discarded in the analysis. Oscillation experiments were performed by stimulating the cells with 1 nM Ox-A for a prolonged period of time. The number of peaks appearing during the stimulation was calculated and divided by the duration of the stimulation. The oscillation frequencies are expressed as peaks per second (Hz). The data from single cell Ca^{2+} imaging experiments were imported into Microcal Origin™ 6.0, and further analysis was performed. Ca^{2+} measurements in cell suspension were performed as described earlier [23].

2.7. Data processing

The differences in the responses between two groups were evaluated by the unpaired Student's *t*-test. $p<0.05$ (*) was considered significant. Data are expressed as means ± SE.

2.8. Screen for active protein kinase

Cells were plated in 35-mm plates and grown to near confluency, then treated with 5 nM Ox-A in HBM at 37 °C at indicated times, buffer was removed, dish was transferred on ice, and cells were lysed in radioimmunoprecipitation assay buffer (RIPA). Protein concentration was determined using BCA™ Protein Assay Kit (Thermo scientific, Rockford, IL, USA). About 5–10 μg of protein was run on 7.5% or 10% acrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with antibodies according to the manufacturer's instructions. Positive bands were detected with ECL+ and scanned on STORM (GE, Uppsala, Sweden).

2.9. PKD3 microscopy and immunoprecipitation

HEKOx1R cells were transfected with EGFP-PKD3 [26], selected with 400 μg/ml geneticin (Invitrogen, Paisley, UK), and then plated on coverslips in 12-well plates. At near confluency, medium was replaced with HBM with 1 or 50 nM Ox-A. At selected time, cells were fixed in formalin, coverslips were mounted on glass slides with anti-fade and DAPI stain. Fluorescent cells were observed with fluorescence microscope, Olympus IX71. Images were captured using Olympus DP controller software. For immunoprecipitation, cells were plated to 60-mm plates, treated in HBM for 5 minutes, then lysed, and immunoprecipitated with full-length A.v. polyclonal anti-GFP antibody cross-linked to Dynabeads protein G. Western blots of immunoprecipitated proteins were probed with anti-phospho-PKD1/PKCμSer744/748 and JL8 monoclonal anti-GFP antibody.

2.10. Cell fractionation

HEKOx1R or HEKOx1R-EGFP-PKD3 cells were treated with Ox-A in HBM. Cytosolic and membrane fractions were isolated according to Brott et al. [27].

3. Results

To identify protein kinases activated by Ox-A stimulation of Ox1R, we treated near-confluent HEKOx1R cells with 5 nM Ox-A in HBM and lysed them at different time points in RIPA buffer. Total protein lysates were tested by Western blotting and detection with antibodies against selected phosphorylated PKC subtypes, PKD1, and extracellular signal-

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