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Oxytocin (OXT)-stimulated inhibition of Kir7.1 activity is through PIP_2-dependent Ca^{2+} response of the oxytocin receptor in the retinal pigment epithelium in vitro

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ABSTRACT

Oxytocin (OXT) is a neuropeptide that activates the oxytocin receptor (OXTR), a rhodopsin family G-protein coupled receptor. Our localization of OXTR to the retinal pigment epithelium (RPE), in close proximity to OXT in the adjacent photoreceptor neurons, leads us to propose that OXT plays an important role in RPE-retinal communication. An increase of RPE $[Ca^{2+}]_i$ in response to OXT stimulation implies that the RPE may utilize oxytocinergic signaling as a mechanism by which it accomplishes some of its many roles. In this study, we used an established human RPE cell line, a HEK293 heterologous OXTR expression system, and pharmacological inhibitors of Ca^{2+} signaling to demonstrate that OXTR utilizes capacitative Ca^{2+} entry (CCE) mechanisms to sustain an increase in cytoplasmic Ca^{2+}. These findings demonstrate how multiple functional outcomes of OXT-OXTR signaling could be integrated via a single pathway. In addition, the activated OXTR was able to inhibit the Kir7.1 channel, an important mediator of sub retinal waste transport and K^+ homeostasis.

1. Introduction

Oxytocin (OXT) is a cyclic nonapeptide produced in the paraventricular and supraoptic nuclei of the hypothalamus [1]. Although best known for its association with parturition and lactation, OXT also has numerous central and peripheral effects, including, but not limited to, the modulation of sexual and social behavior, influence over metabolic activity in adipose tissues, and skeletal muscle maintenance [1,2].

The RPE is a monolayer of polarized cells that serve as a physical and protective blood-retina barrier and act as a facilitator of phototransduction in the photoreceptors [3]. The RPE also mediates the bidirectional transport of nutrients between the choroid and photoreceptors, maintains the ionic composition of the subretinal fluid, and facilitates phagocytosis of photoreceptor outer segments that are shed on a daily basis. It is not fully understood how the RPE and photoreceptors coordinate their function. What is known, however, is that autocrine and paracrine signaling in the RPE involves G protein-coupled receptors (GPCR), including the dopaminergic, adrenergic, P_{2Y}-purinergic, and serotoninergic receptors [4–9].

We have shown that OXT is a potential mediator of retinal physiology given its presence in the cone photoreceptor extracellular matrix [10]. Moreover, the oxytocin receptor (OXTR) is expressed in the retinal pigment epithelium (RPE) where we have shown that OXT can induce an increase in $[Ca^{2+}]_i$, leading to our hypothesis that oxytocinergic signaling may serve as a means for communication between cone photoreceptors and the RPE [10]. OXTR is a GPCR and like the aforementioned receptors, it activates a phospholipase C (PLC)-mediated signaling pathway, thereby stimulating PIP_2 hydrolysis and resulting in a rise in $[Ca^{2+}]_i$ [11].

Facing the photoreceptor outer segments on the apical membrane of

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the RPE cell is the inwardly rectifying potassium (Kir7.1) channel [3,12]. As its name suggests, Kir7.1 exhibits a large inward K^+ current at hyperpolarized membrane potentials. However, at physiological membrane potentials, the channel facilitates the efflux of intracellular K^+ [13,14]. This, combined with its co-localization with Na^+-K^+-ATPase, makes Kir7.1 integral to the maintenance of the K^+ transport needed for transepithelial fluid transport [15–17]. Kir7.1 function is also directly mediated by PIP_2, supporting the possible regulation of Kir7.1 by OXTR through PLC-activated PIP_2 hydrolysis [18]. Understanding how Kir7.1 is regulated is clinically important, as disrupted Kir7.1 function is a known cause of the retinal diseases of Snowflake Vitreoretinal Degeneration and Lebers Congenital Amaurosis – Type 16 (LCA16) [19,20]. A direct impact of Kir7.1 on retinal function and vision can be clearly seen following RNA interference (RNA_i) knockdown of Kir7.1 in mice, resulting in a characteristic and abnormal electroretinogram (ERG) [21].

In the present study we sought to delineate the mechanism by which OXT elicits an increase in $[Ca^{2+}]_i$ and how this may affect Kir 7.1 function in the RPE by using cultured hfRPE cells. We also studied a human embryonic kidney (HEK293) cell line with heterologous expression of human OXTR to study the effects of OXTR on the Kir7.1 channel without the complex interactions inherent in the intact RPE cell. Lastly, we used adult mouse RPE cells to demonstrate the link between Kir7.1 channel function and OXT-OXTR signaling.

2. Materials and methods

2.1. Reagents

All chemical reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO), Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA), or Gibco (Grand Island, NY), unless otherwise specified.

2.2. Solutions

HEPES Ringers (HR) extracellular bath solution was prepared using (mM) 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and adjusted to pH 7.4 with NaOH. K^+ inhibition solutions require the same composition with 115 mM NaCl and the addition of 20 mM $BaCl_2$ or $CsCl_2$. Ca^{2+}-free extracellular bath solution was prepared using (mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 10 HEPES, 2 EGTA-KOH, and adjusted to pH 7.4 with NaOH. Final concentrations of 0.01, 0.1, 1, 6, 10, or 100 μM OXT were prepared in either HR or Ca^{2+}-free extracellular solutions. Extracellular solution containing the Ca^{2+} channel blocker nifedipine was prepared by diluting to 10 μM final concentration in HR. A final concentration of 60 μM 2-APB (Tocris Bioscience, Bristol, UK), an IP_3R inhibitor, was prepared in HR.

2.3. Cell culture

Passages 1–3 cryopreserved Primary Clonetics™ Human RPE cells (hfRPE) (LONZA, Walkersville, WA) were cultured using a previously published protocol [10].

HEK293 cells were obtained from ATCC (Manassas, VA). To generate HEK-OXTR line, cells were transfected with a pcDNA6/HisC plasmid-containing human OXTR via nucleofection (4-D Nucleofector, LONZA) as per the manufacturer's instructions. Cells were cultured in a 60 mm culture dish in complete growth medium (DMEM + 10% Fetal Bovine Serum + 1% Pen-Strep). Twenty-four hours after transfection, culture media was supplemented with 10 μg/mL blasticidin (Thermo Fisher Scientific) to select for cells expressing the OXTR-containing plasmid. Individual surviving cell colonies were selected and grown in culture media containing blasticidin in 24-well plates. OXTR expression was verified by indirect immunofluorescence and Ca^{2+} imaging. OXTR positive cells were cryo-preserved and subcultured for experimental usage.

hfRPE culture media was prepared using MEM alpha base medium, 1% N-2 supplement, 1% glutamine, 1% pen-strep, 1% MEM non-essential amino acids, taurine, hydrocortisone, and 3, 3′, 5-triiodothryonine. HEK cell culture complete growth media was prepared using 10% FBS and 1% PenStrep diluted in DMEM.

2.4. Ca^{2+} imaging

hfRPE cells were grown on coverslips and incubated in 5 μM FURA-2 penta-acetoxymethyl ester (AM) in hfRPE culture media + 0% FBS for 30 min in a dark environment. Selective Oxytocin antagonist, desGly-NH_2-d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT, (OTA) was generously provided by Dr. Maurice Manning (University of Toledo, OH). 100 μM OTA was included in FURA incubation solution as well as perfusion solution. HEK-OXTR cells grown on coverslips were incubated with 5 μM Fura-2 AM in serum-free DMEM under the same conditions. Following incubation with FURA-2 AM, coverslips were rinsed × 3 in HR solution and transferred to the recording chamber (Warner Instruments, Hamden, CT) on the microscope stage (Nikon FN1, Nikon Instruments Inc., New York, NY). hfRPE cells were continuously perfused with HR or Ca^{2+}-free solution containing tested compounds (OXT, nifedipine, 2-APB, OTA) and were exchanged using a gravity-feed 8-valve solution exchange system with a ValveLink Pinch Valve (Automate Scientific, Berkeley, CA) controlled through ValveLink8.2 (Automate Scientific, Berkeley, CA). HEK-OXTR cells were continuously perfused with HR, and HR solution containing OXT or ATP was exchanged using the same system.

Images were acquired every 10 s using a 20× water immersion objective (NA = 0.5) and Lambda LS lamp (Shutter Instruments, Novato, CA). The 300 ms shutter speed and 340 and 380 nm excitation wavelengths were controlled by the Lambda 10–2 controller (Sutter Instruments), and emission was set to 518 nm. Image frames from the CoolSnap HQ Photonics camera (Nikon) were digitized and stored for off-line analysis. Background and calibration images were similarly acquired and used to obtain absolute changes in fluorescence values. All distinct, visible cells in a visual field had regions of interest defined using NIS-elements software thresholding intensity feature to identify cells by intensity at 380 nM excitation and the amplitude of the R (340/380) was measured.

The calcium concentration was calculated using the equation: $[Ca] = K_d * (R - R_{min})/(R_{max} - R)(F_{max}{}^{380}/F_{min}{}^{380})$, assuming the K_d to be 225 nM in the cytosolic environment [22]. Calibration values were determined using 10 μM ionomycin to permeabilize cells to Ca^{2+} and exposing them to [0] Ca^{2+} solution or HR to obtain min and max values, respectively. Values were determined separately for hRPE and HEK-OXTR cells.

2.5. Live-cell fluorescence imaging

Plasmids encoding the PH domain of phospholipase Cd1 fused to GFP (PH-GFP kindly provided by T. Balla, NIH) or GFP fused to C1 domains from protein kinase C (PKC-GFP kindly provided by T. Meyer, Stanford) were used for live-cell fluorescence imaging [23,24]. After 24 h of plasmid transfection into HEK-OXTR cells using TransIT-LT1 (Mirus Bio, Madison, WI), cells were dissociated and plated onto laminin-coated coverslips (12 mm #1; Thermo Fischer Scientific).

Imaging was performed between 48 and 72 h post-transfection while cells were perfused with HR alone and OXT dissolved in HR. Using 470 nm excitation and 525 nm emission, images were acquired every 10 s. 10 μM OXT was used to stimulate cells. The images were analyzed off-line using scans of either membrane or cytoplasmic 'regions of interest' (ROI).

2.6. Animal handling and RPE isolation

Mouse RPE was isolated from 6 to 8 wk old C57BL6 mice (The