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Developing a Model System to Establish Electrophysiological Protocols Necessary for the Deorphanization of Vomeronasal Sensory Receptors (VNSRs)

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Abstract

Chemical signaling mediates many complex behavioral interactions such as mating and the establishment of hierarchy for a variety of animal species. A key form of chemical signaling in rodents is facilitated by the production and detection of intraspecific pheromones known as major urinary proteins (MUPs). Pheromones such as MUPs convey specific information which is innately recognized by members of a species. In rodents, MUPs are detected by receptors on specialized sensory neurons in the vomeronasal organ (VNO) known as vomeronasal sensory receptors (VNSRs). Once a MUP binds to a VNSR, the sensory neuron initiates neural circuits extending to other sections of the brain such as the amygdala and surrounding limbic structures leading to a behavioral response. Sensory neurons expressing different VNSRs will activate different neural circuits, thus allowing different MUPs to evoke specific behaviors. The exact behavioral response to a certain MUP is predictable for all members of the species and indicates common neural circuitry associated with MUP communication. This uniformity of neural circuitry in mice allows for MUP communication to serve as a reliable model system for studying how chemical stimuli code for behavioral outputs. This study aims to determine which MUPs activate a given VNSR in order to elucidate the neural circuits responsible for specific behavioral reactions. To achieve this understanding, patch clamp analysis will be used to deorphanize VNSRs by monitoring electrophysiological changes in cells expressing VNSRs upon exposure to specific MUPs. If the MUP being introduced is able to bind the specific VNSRs expressed by the cells, a measurable change in the cells voltage will occur. Currently, a model system using CHO cells transfected to express kir2.2 channels is being established to yield preliminary methodologies that will be used to complete this study.

1. Introduction

Chemical signaling is a major form of communication common to many animal species. Complex behavioral interactions such as mating and the establishment of social hierarchy are mediated through this robust form of communication[2]. A crucial form of chemical signaling found in rodents is facilitated by the production and detection of intraspecific pheromones. Each specific pheromone, or distinct blend of pheromones, encodes a specific message that is innately recognized by members within a species[9]. In rodents, the detection of pheromones elicits an appropriate behavioral response provoked by the selective binding of pheromones to receptors present on sensory neurons within the vomeronasal organ (VNO), known as vomeronasal sensory receptors (VNSRs)[3, 8].

Major urinary proteins (MUPs) are a class of pheromones common to mice and serve a critical role in mediating a variety of behaviors. The mouse genome encodes 21 MUPs with the sequence of some only varying by a single amino acid[5]. Once a MUP binds to a VNSR, ion channels within the sensory neuron membrane allow for an influx of positively charged ions which activate the cell[4]. Specifically, the binding of a MUP to a VNSR initiates the phospholipase C pathway leading to the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), yielding inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Membrane bound TRPC2 channels are activated by DAG, and once opened, facilitate the influx of a depolarizing calcium current in to the sensory neuron[6].

Once activated, sensory neurons initiate a chain reaction of intercellular communication which extends throughout the brain forming neural circuits. These circuits begin in the VNO and project to the accessory olfactory bulb before extending to other sections of the brain such as the amygdala and surrounding limbic structures[1]. The specific behavioral responses provoked by MUP detection are determined by the particular circuit activated and the brain regions the circuit projects to. While many neurological pathways have been elucidated, those associated with MUP detection have yet to be fully identified.

Because a specific MUP will initiate the same behavioral response in all mice without prior exposure, the neural circuitry responsible for the behavior must be common for all individuals. This uniformity in neural circuitry allows for MUP communication to serve as a reliable model to study how chemical stimuli mediate behavior. By pairing the known behavior evoked by a particular MUP with the specific VNSR(s) it activates, the neural circuitry responsible for translating pheromones into behavioral outcomes may begin to be traced throughout the brain[3].

This study aims to identify which MUPs bind to specific VNSRs in order to begin the process of tracing the various circuits that become activated following MUP detection. By elucidating such circuitry, a better understanding of the neurological mechanisms facilitating the translation of external stimuli into behavioral reactions will be achieved. To reach this understanding, patch clamp analysis will be used to deorphanize VNSRs by monitoring electrophysiological changes in cells expressing VNSRs upon exposure to specific MUPs. As evidenced by a spike in membrane voltage, a successful binding event between the MUP being introduced and the specific VNSR expressed by the cell will be recorded. This change in voltage indicates the activation of a sensory neuron which naturally serves as the beginning step in circuit initiation. With this information, future studies focused on labeling sensory neurons that express particular VNSRs of interest would allow for the delineation of the information flow that results in behavioral outputs following MUP detection. Here we report on the construction of a model system that serves as a preliminary set of methodologies that will facilitate future research in this line of study. Through the use of kir2.2 expressing CHO cells, a protocol for conducting whole cell patch clamp recordings has been established. Additionally, the development of a profusion system used to introduce and remove ligands from actively recorded cells is currently in progress.

2. Methods

2.1 Transfection and cell culture:

To serve as a model system allowing for protocol establishment and verification of proper patch clamp functioning, CHO cells were cotransfected to express kir2.2 channels and GFP using lipofectamine. Cells were transfected in 6 well plates once 80% confluency was reached and were left to incubate at 37 °C for 48h. Following transfection, cells were resuspended in serum free media, plated on 9x22mm glass coverslips, and left to incubate for an additional 2 to 3 hours to allow cells to adhere. Coverslips were briefly rinsed with Tyrode solution to remove non-adhered cells before patch clamp analysis was conducted.

2.2 Electrophysiology:

Electrophysiological recordings were achieved using an Axopatch200 integrating patch clamp (Axon Instruments) and processed using Clampex 2004. GFP tagged cells were used for whole cell voltage clamp recording using glass micropipettes with resistances of 3-4mΩ. Upon establishment of whole cell configuration, a stepwise voltage imposition ranging from -120mV to 0mV in 10mV increments was conducted while potassium current was recorded. The observed potassium current into CHO cells expressing kir2.2 channels during the stepwise voltage imposition was characteristic of proper channel functioning and verified correct use and functionality of the patch clamp.

Using this model system, CHO cells will first undergo lipofectamine transfection to express individual VNSRs using a CDNA library obtained from mouse VNO. Successfully transfected cells, as determined by GFP signal, will be patched onto using glass micropipettes with a resistance of 3-4mΩ. Following successful establishment of whole cell configuration, purified MUPs will then be sequentially profused over the cell during membrane voltage recording. As evidenced by a spike in membrane voltage, a successful binding event between the MUP being introduced and the specific VNSR expressed on the cell will be recorded by the patch clamp. A washout phase will follow the introduction of each purified MUP to remove any MUPs bound to VNSRs, absolving any spike in voltage and returning the cells back to resting potential.

3. Results

CHO cells expressing kir2.2 channels exhibited the appropriate electrophysiological response to induced membrane potential (fig. 1). Hyperpolarization of the cell membrane generated an inward current of potassium as predicted. As membrane potential depolarized, a decrease in inward current was observed. Based on the observed data, the correct use of patch clamp protocols and cell transfection was verified.

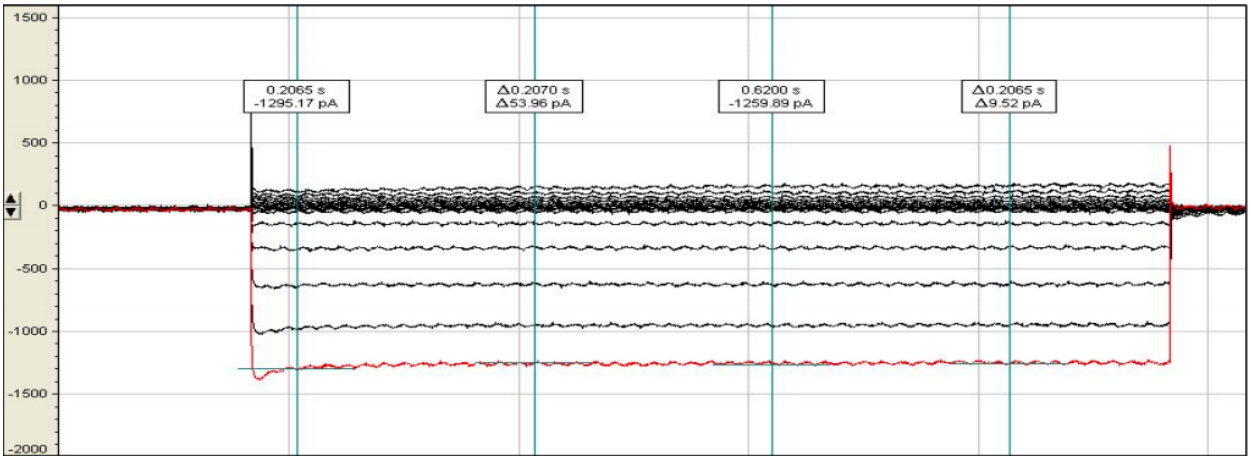

Figure 1. Whole cell recording of CHO cell expressing inward rectifying potassium channels (kir 2.2). Shown on the Y axis is a measure of potassium current (picoamperes) evoked in response to applied membrane potential ranging from -120mv (red line) to 0mv in 10mv increments.

4. Discussion

Performing stepwise voltage impositions that induce a range of membrane potentials is a common protocol allowing for the analysis of cellular ion channels based on distinct variations in observed current. Membrane potentials above and below the equilibrium potential for potassium generate predictable current patterns in cells expressing kir channels[7]. As a result of its well-researched status, the kir2.2 channel serves as a robust platform by which reliable patch clamp protocols may developed. By conducting a voltage imposition on CHO cells expressing kir2.2 channels, the proper use and function of the patch clamp was verified by the characteristic inward current recorded (fig. 1)

The construction of this model system has confirmed the efficacy of several important methodologies to be used in future investigations in this lab. The successful establishment of whole cell configuration represents a major step in developing a functional procedure for deorphanizing VNSRs using patch clamp electrophysiology. Additionally, the development of a reliable protocol for the transfection and expression of kir2.2 channels is promising with regards to the future expression VNSRs in CHO cells. However, it should be noted that the expression of VNSRs entails additional complexity compared to kir2.2 channels as VNSR functionality is dependent on a variety of mechanistically imperative components that are absent in simple ion channels. For this reason, a considerable effort to successfully isolate and clone VNSRs and their associated transduction components is currently being conducted by colleagues working on this project.

To further develop this model system, profusion and washout phases must be conducted and proven successful in ligand introduction followed by ligand removal during active cell recording. Allowing for the verification of successful profusion system functioning, a previously constructed profusion system will be used to introduce a potassium channel inhibitor to cells expressing kir2.2 channels during voltage clamp recording. Upon successful whole cell configuration, cells will be voltage clamped at -80mV to establish and maintain a driving force for potassium into the cell. Once inward current is observed, the potassium channel inhibitor (BaCl solution) will be profused over the recorded cell, inhibiting the potassium channels and attenuating inward current. Immediately following channel inhibition, a washout phase will be conducted using Tyrode solution to remove BaCl from the cells allowing for the reestablishment

of inward rectifying current. Successful functioning of the profusion system will complete the preliminary methodologies that will be used to facilitate the investigation of MUP binding selectivity and VNSR activation.

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6. References cited

1. Chamero P, Leinders-Zufall T, Zufall F. (2012) From genes to social communication: molecular sensing by the vomeronasal organ. Trends in Neuroscience. 35: 597-606.

2. Ihara S, Yoshikawa K, Touhara K. (2013) Chemosensory signals and their receptors in the olfactory neural system. Neuroscience. 254: 45-60.

3. Isogai Y, Si S, Pont-Lezica L, Tan T, Kapoor V, Murthy VN, Dulac C. (2012) Molecular organization of vomeronasal chemoreception. Nature. 478:241-245.

4. Liman E. (2003) Regulation by voltage and adenine nucleotides of a Ca^{2+} - activated cation channel from hamster vomeronasal sensory neurons. J Physiol. 583.3: 777-787.

5. Logan DW, Marton TF, Stovers L. (2008) Species specificity in major urinary proteins by parallel evolution. PLos ONE. 3: 1-11

6. Lucas P, Ukhanov K, Leinders-Zufall T, Zufall F. (2003) A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in TRPC2 mutant mice: mechanism of pheromone transduction. Neuron. 40: 551- 561.

7. Lu T, Nguyen B, Zhang X, Yang J (1999) Architecture of a K channel inner pore revealed by stoichiometric covalent modification. Cell. 22: 571-580

8. Sheehan MJ, et al. (2016) Selection in coding and regulatory variation maintains individuality in major urinary protein scent marks in wild mice. PLoS Genet. 12: 1-33.

9. Stowers L, Marton TF. (2005) What is a pheromone? Mammalian pheromones reconsidered. Neuron. 46, 699-702.