Introduction

Transient Receptor Potential Ankyrin 1 (TRPA1) is a non-selective cation channel that is expressed in a subset of nociceptive afferent sensory nerves. When activated, TRPA1 can evoke nociceptive responses (e.g., pain and defensive reflexes), as well as neurogenic inflammation in the peripheral nerve endings. TRPA1 can be activated by a host of endogenous (inflammatory mediators) and exogenous (pollutants, food) agonists. TRPA1 agonists are electrophilic compounds that bind to reactive cysteine residues on the N and C-termini of the channel, which results in channel activation. These electrophiles can bind to reactive cysteines (nucleophiles) on TRPA1 through reversible and irreversible reactions. In order to maintain TRPA1 activity and prevent run down of the channel, polyphosphates need to be present in the cell. It is well known that electrophiles activate TRPA1, but the characterization of channel activity through irreversible and reversible agonists has yet to be identified and differentiated. In this project, I used calcium imaging and various patch clamp techniques to identify TRPA1 activation via a reversible (allyl isothiocyanate [AITC]) and irreversible (iodoacetamide [IA], N-ethylmaleimide [NEM]) electrophiles.

Objectives

I. To identify TRPA1 activation via irreversible and reversible electrophilic agonists by performing
   a. Live cell Ca²⁺ imaging
   b. Whole-cell and perforated patch clamp

II. To characterize TRPA1 activation by perforating
   a. Inside-out and cell-attached patch clamp

Results

I. Rapid TRPA1 activation via IA and NEM indicates full agonism of irreversible electrophiles

II. Reduced TRPA1 activation via irreversible electrophile in whole-cell patch clamp infers dia lysis of cytosolic cofactor

Conclusion

• Discrepancy between Ca²⁺ imaging (Fig.2) and whole-cell/outside single channel patch clamp experiments
• Whole-cell and inside-out patch clamp methods showed a reduction the kinetics and magnitude of TRPA1 activation (Fig. 3A-D, Fig.4A)
• Polyphosphates (prevents run down of channel), however data suggest that another cofactor may be needed
• Techniques cause cell membrane disruption, so dialysis of cytosolic cofactors that can effect irreversible electrophilic binding
• Gramicidin perforated patch clamp and cell-attached single channel patch clamp techniques were used to combat dialysis
• Perforated patch results revealed no difference between NEM and AITC-evoked TRPA1 activation (Fig.3E-F)
• Cell-attached patch clamp experiments yielded no difference between NEM and AITC-evoked TRPA1 activation as well (Fig.4B).

This suggests that there is another unidentified cofactor that is essential for full TRPA1 activation.

Methods

Calcium imaging - Calcium imaging is used to measure changes in intracellular Ca²⁺ in HEK 293 cells through Fura-2/3. Fura-2/3 is a membrane-permeable calcium indicator that is fluorescent when bound to calcium. Fura-2/3 fluorescence can be measured at 340nm and 380nm wavelengths (emission), which provide a ratiometric calculation for intracellular calcium concentrations. Fura-2/3 was incubated in DMEM with coverslips of non-transfected (iNHEK) and wildtype (WT) TRPA1. iNHEK was perfused over the coverslip using imaging buffer.

Whole cell patch clamp - Whole cell patch clamp is used to measure currents of HEK WT/WT TRPA1 293 cells. Membrane currents were recorded and analyzed using an amplifier and acquisition software. Patch pipettes were made from borosilicate glass and fire-polished. The cells were voltage-clamped at 0 mV and 500 millisecond voltage ramps from -70 to +70 were applied every second. The pipettes (resistance between 3-5 MΩ) were filled with a HEPES buffer with Na-triphosphate (to prevent rundown of TRPA1). The cells on the coverslip were perfused with HEPES buffer. AITC (30µM) and TRPA1 activation via a reversible and irreversible electrophile in whole-cell patch clamp was perfused through the coverslip using imaging buffer.

Perforated patch clamp - Whole cell patch clamp is used to measure currents of WT TRPA1 293 cells. The patch pipettes resistance is the same as whole cell patch clamp experiments. The pipettes were filled with HEPES buffer. The cells on the coverslip were perfused with HEPES buffer. Perfusion was achieved using 25-50 µg/ml gramicidin. The experiment protocol is the same as whole cell patch clamp technique. AITC (30µM), NEM (30µM) and a TRAP channel pore blocker (Ruthenium Red, 30µM) was applied to the cells for treatment.

Inside-out/Cell-attached single channel patch clamp - Single channel patch clamp is used to measure currents of iNHEK and WT TRPA1 293 cells. Membrane currents were recorded and analyzed using an amplifier and acquisition software. The pipettes (resistance between 3-5 MΩ) were filled with a HEPES buffer with Na-triphosphate (to prevent rundown of TRPA1). The patch was held at 0 mV, then the presence of TRPA1 channels was confirmed by conductances observed in a series of voltage steps: 100 milliseconds from -60 mV to +60 mV in 20 mV steps. Excised patches were held at -40mV. NEM and AITC were applied to the cells and the currents evoked by the agonists were recorded.

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Citations

