Discrepancy between TRPA1 Activation by Reversible and Irreversible Electrophiles Suggests Involvement of Cytosolic Cofactors Other Than Polyphosphates

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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 TPRA1, 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.

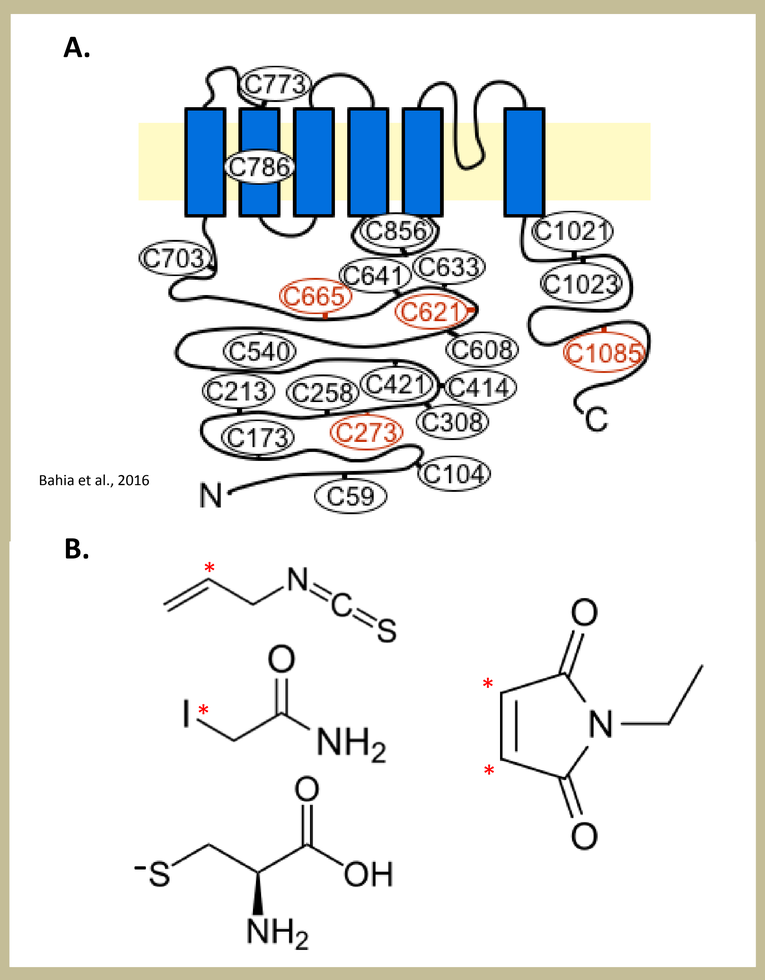

Fig.1: **A. Image of a TRPA1 subunit. The image highlights each cysteine residue identified on the channel including four highly reactive cysteines (red). B. Chemical structures of TRPA1 agonist AITC (top), IA (middle left) and NEM (middle right). The electrophilic bond is labeled by the red asterisk. NEM has two electrophilic sites, which allows for increased nucleophilic affinity. The structure of reactive cysteine is located a the bottom.**

Objectives

I. To identify TRPA1 activation via irreversible and reversible electrophilic agonists by performing
   a. Live cell Ca^{2+} imaging
   b. Whole-cell and perforated patch clamp

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

Results

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

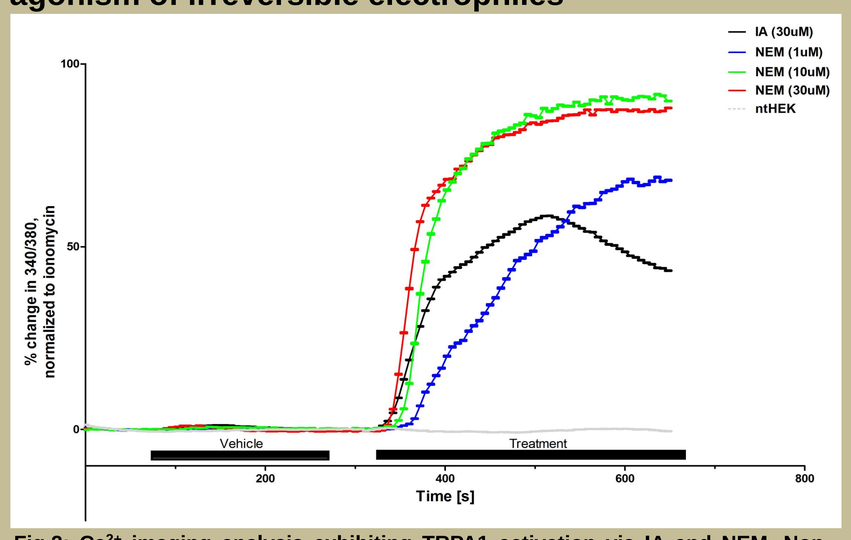

Fig.2: **Ca^{2+} imaging analysis exhibiting TRPA1 activation via IA and NEM. Non-transfected HEK 293 cells show no response to IA and NEM.**

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

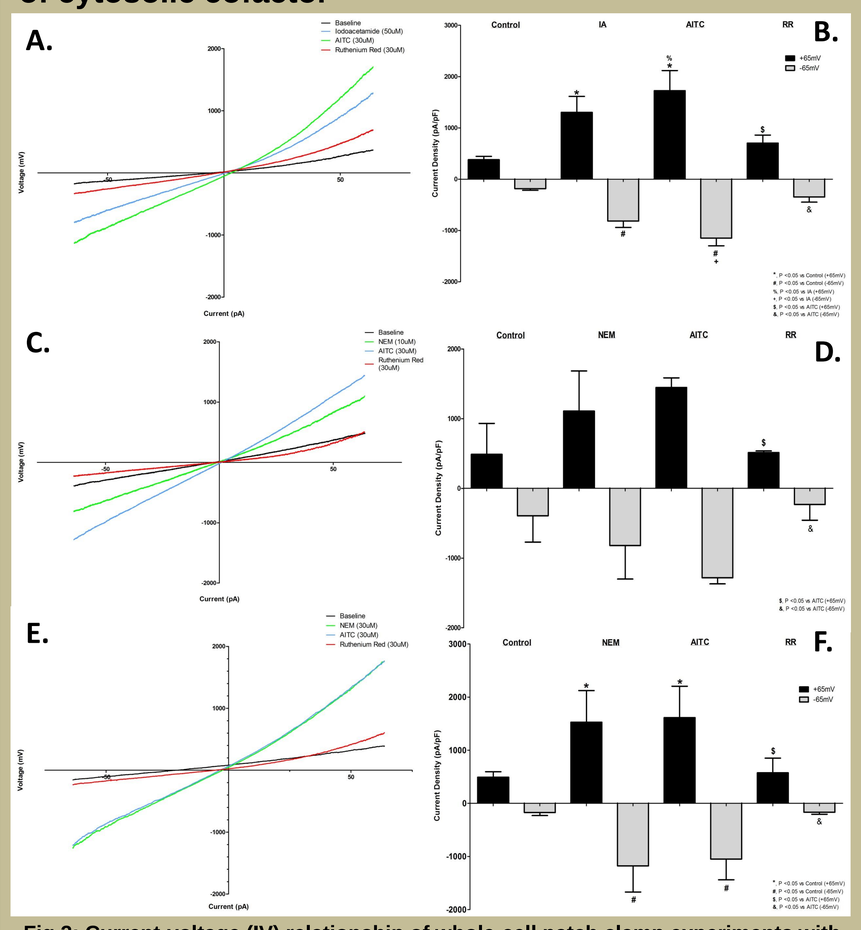

Fig.3: **Current-voltage (IV) relationship of whole-cell patch clamp experiments with 50uM IA (A) and 10uM NEM (C). Current density relationship of whole-cell patch clamp experiments with 50uM IA (B) and 10uM NEM (D). Analysis of perforated patch clamp experiments with 30uM NEM displayed through IV (E) and current density (F) relationships.**

III. Inside-out single channel patch clamp analysis further suggests cytosolic cofactor dialysis

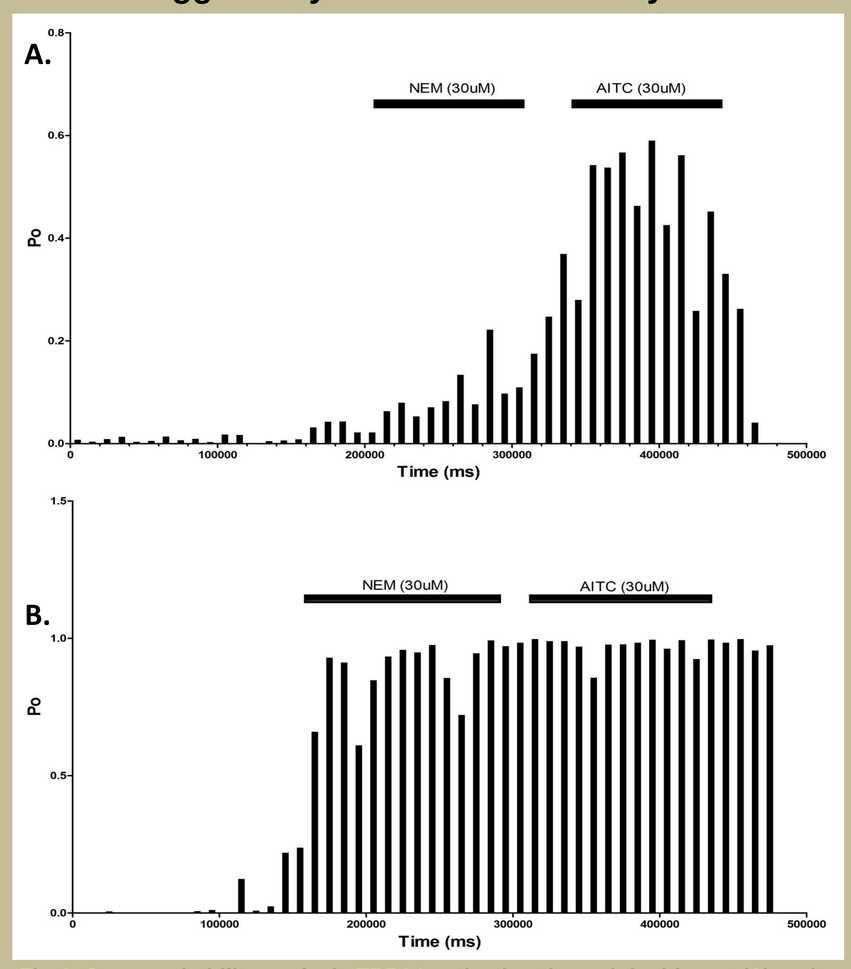

Fig.4: **Open probability analysis TRPA1 activation through inside-out (A) and cell-attached (B) single channel patch clamp.**

Conclusion

- Discrepancy between Ca^{2+} imaging (Fig.2) and whole-cell/inside-out 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 the 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 Ca^{2+} in HEK 293 cells through Fura-2AM. Fura-2AM is a membrane-permeable calcium indicator that is fluorescent when bound to calcium. Fura-2AM fluorescence can be measured at 340nm and 380nm wavelengths (emission), which provides a ratiometric calculation for intracellular calcium concentrations. Fura-2AM was incubated in DMEM with coverslips of non-transfected (ntHEK) and wildtype (WT) TRPA1. HEPES buffer was perfused over the coverslips during imaging.

Whole cell patch clamp- Whole cell patch clamp is used to measure currents of ntHEK and WT TRPA1 HEK 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), IA (50µM) and a TRP channel pore blocker (Ruthenium Red [RR, 30µM]) was applied to the cells for treatment.

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

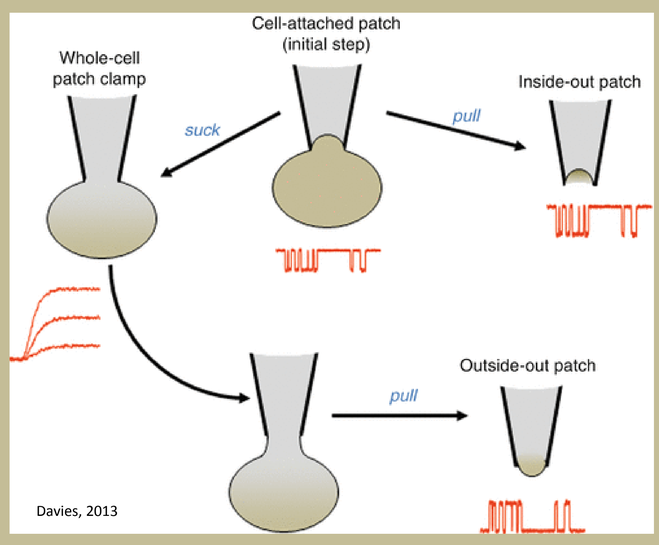

Fig.5: **Image of patch clamp techniques. The whole cell, cell-attached, and inside-out patch clamp methods were used to measure membrane currents of TRPA1 transfected and non-transfected HEK 293 cells.**

Acknowledgements

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Citations

Bahia, P. K., Parks, T. A., Stanford, K. R., Mitchell, D. A., Varma, S., Stevens, S. M., & Taylor-Clark, T. E. (2016). The exceptionally high reactivity of Cys 621 is critical for electrophilic activation of the sensory nerve ion channel TRPA1. The Journal of General Physiology, 147(6), 451–465. http://doi.org/10.1085/jgp.201611581

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