Hypo-osmosensitive TRP channels in the airway epithelium: a multilabel immunohistochemical and high resolution confocal calcium imaging study

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The transient receptor potential (TRP) channel superfamily consists of at least 7 subfamilies that are widely expressed in virtually all mammalian cell types, mediating responses to a variety of physical and chemical stimuli. Especially during the last decade, extensive research efforts have resulted in a gradual identification of the biological significance of more and more members of this heterogeneous family. Two different TRP channels, TRPV4 (TRP vanilloid 4) and TRPM3 (TRP melastatin 3), have been characterized as membrane proteins that mediate calcium (Ca^{2+}) entry in cells upon extracellular application of hypo-osmotic solutions. TRPV4 is widely expressed in mammalian tissues, including lung, heart, kidney, and brain, and has in addition to hypo-osmotic stimuli been shown to respond to mechanical stimuli, arachidonic acid metabolites and phorbol derivatives. Expression of TRPV4 has been reported in human and mouse tracheal epithelial cells, and in human bronchial epithelial cell cultures. TRPM3 appears to be also responsive to sphingosine derivatives, is expressed in epithelial cells of e.g. the choroid plexus and kidney, but has not yet been localized to the airway epithelium. The present study aimed at investigating the expression and functionality of the osmosensitive TRPV4 and TRPM3 channels in the epithelium of mouse intrapulmonary airways, by combination of high resolution confocal live cell Ca^{2+} imaging and multilabel immunohistochemistry, respectively in vibratome lung slices and cryosections of mouse lungs. For confocal live cell imaging experiments we used an *ex vivo* vibratome mouse lung slice model that was recently developed in our lab. In short, live mouse lungs were stabilized by instillation of agarose, 100 µm thick slices were cut using a vibratome, and loaded with the non-ratiometric Ca^{2+} indicator Fluo-4 to visualize changes in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. It was shown that the majority of epithelial cells in mouse intrapulmonary airways are ciliated cells and secretory Clara cells, and to a lesser extent neuroendocrine cells grouped in neuroepithelial bodies (NEBs). The model allows the real time visualization and physiological activation of individual airway epithelial cells in their natural environment to various stimuli, by high resolution imaging on an Ultra*VIEW* dual spinning disk confocal microscope (PerkinElmer), equipped with a three line (488, 568 and 647 nm) argon-krypton laser. The latter was also used for imaging the immunohistochemical results. For immunostaining, antibodies raised against TRPV4 (Rb Pc, Alomone) and TRPM3 (Rb Pc, Affinity Bioreagents) were combined with markers for Clara cells (urine protein 1) and NEBs (protein gene-product 9.5; PGP9.5) on mouse lung cryosections. Immunostaining showed strong immunoreactivity (IR) for both TRPV4 (Fig. 1) and TRPM3 on the majority of mouse airway epithelial cells. Multilabelling experiments showed co-localization between TRPV4 and TRPM3, confirmed that both ciliated cells and Clara cells express these TRP channels, but also clearly revealed that NEB cells are negative (Fig. 1). Confocal live cell imaging of mouse lung slices loaded with Fluo-4 taught us that short term (30-60s) administration of hypo-osmotic solutions (230-200-150 mOsm/kg) results in a reversible

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graded $[Ca^{2+}]_i$ rise in all Clara and ciliated cells (Fig. 2). The present study showed that all airway epithelial cells except for the endocrine cells in NEBs appear to abundantly express functional TRPV4 and TRPM3 channels that are activated by extracellular hypo-osmotic stimulation. Both TRP channels may allow the cells to adjust to changes in extracellular osmolarity and therefore potentially play a central role in airway epithelial homeostasis by modulating epithelial barrier function. The presented approach, using real time confocal live cell imaging in a whole lung slice model, opens interesting new perspectives for unraveling functional aspects of many cell and tissue types in control lungs and disease models.
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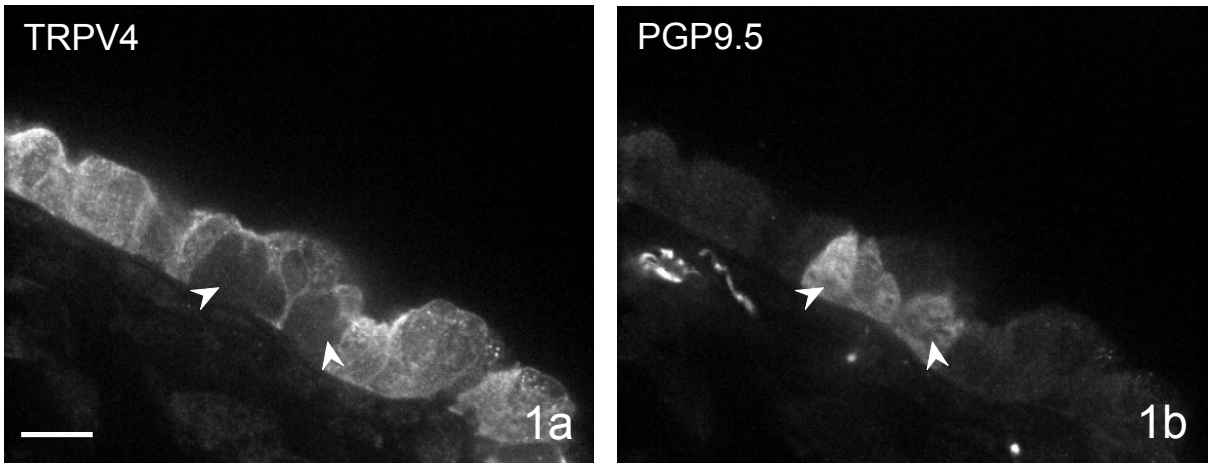

Figure 1. Double immunostaining of mouse intrapulmonary airway epithelium for TRPV4 (Cy3 labeling) and PGP9.5 (FITC labeling). a. An intense TRPV4 IR is found on almost all cells of the airway epithelium, except for a small group of cells that seem to be TRPV4 negative (*arrowheads*). b. PGP9.5 IR, as a marker for pulmonary neuroepithelial bodies (NEBs), reveals that the cluster of TRPV4-negative cells (*arrowheads*) represents a NEB. *Scalebar= 10 μm.*

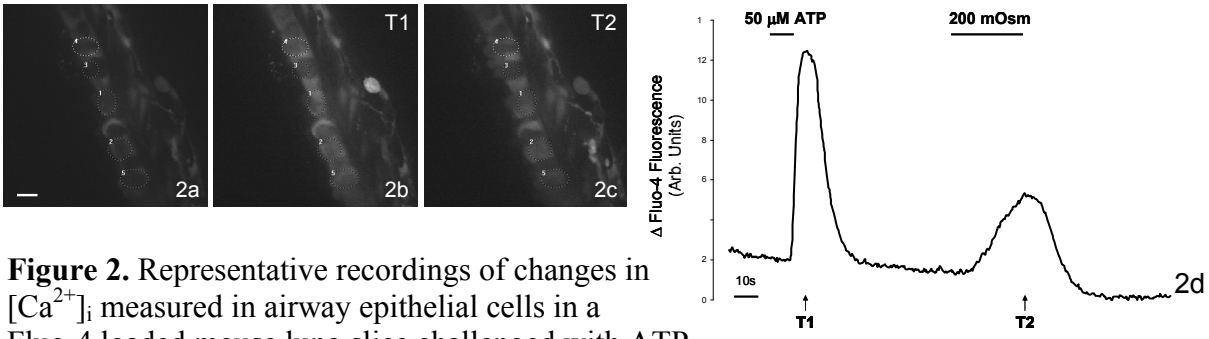

Figure 2. Representative recordings of changes in $[Ca^{2+}]_i$ measured in airway epithelial cells in a Fluo-4 loaded mouse lung slice challenged with ATP (50 μM, 10s) followed by a hypo-osmotic solution (200 mOsm/kg, 30s). a. Image of the Fluo-4 fluorescence before administration of the experimental solutions. *Scalebar= 10 μm*. b-c. Time lapse images of Fluo-4 fluorescence in the airway epithelium, indicated in the graph as T1 and T2. d. Graph plotting the time course of the average Fluo-4 fluorescence intensity of five airway epithelial cells corresponding with ROI 1-5. The airway epithelial cells respond to both application of ATP (positive control showing that cells were properly loaded) and the hypo-osmotic solution with an increase in Fluo-4 fluorescence intensity.

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