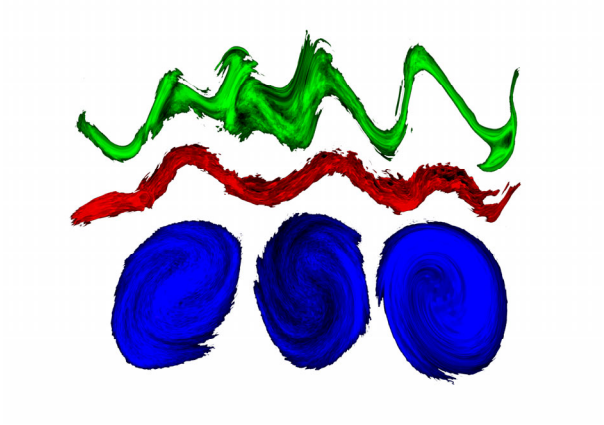
PhD Thesis:

Localization and regulation of TRPV4 channels in ciliated epithelia



IVAN LORENZO i MOLDERO

Barcelona, Juliol 2008

Laboratori de Fisiologia Molecular i Canalopaties
Departament de Ciències Experimentals i de la Salut
Universitat Pompeu Fabra

Localization and regulation of TRPV4 channels in ciliated epithelia

Memòria presentada per optar al grau de Doctor
per la Universitat Pompeu Fabra

Aquesta Tesi Doctoral ha estat realitzada per l'Ivan Lorenzo i Moldero sota la direcció del Dr. Miguel Ángel Valverde de Castro al Departament de Ciències Experimentals i de la Salut de la Universitat Pompeu Fabra.

Barcelona, Juliol 2008

Miguel Ángel Valverde de Castro

Ivan Lorenzo i Moldero

A ma Mare
i a la Charo

AGRAÏMENTS

La realització d'aquesta tesi ha sigut una àrdua tasca gens senzilla que ha requerit una costosa inversió econòmica, uns equipaments tecnològicament avantguardistes i persones ben preparades treballant per l'avenç de la ciència.

Agraeixo al Ministerio de Ciencia e Investigación per la beca concedida, encara que escassa, m'ha permès sobreviure mentre em dedicava a la realització d'aquesta tesi doctoral.

Numeroses persones han contribuït d'una o una altra manera a la realització d'aquesta tesi. Ja sigui depositant la confiança en mi, instruint-me, ajudant en la realització d'experiments, al manteniment de les instal·lacions, fent discussions constructives i xerrades alliberadores, o fins i tot amb la seva amistat. Sou molts, tants que sóc incapaç de plasmar-ho tot sobre el paper. Aquesta tesi no és només meva, és també una mica vostra.

La ben entesa em porta força al passat, a un moment on ignorava el què podia arribar a oferir. Agraeixo al meu anterior cap i ja desaparegut Senén Viliaró, la oportunitat d'introduir-me en la recerca de la biologia cel·lular.

Agraeixo al meu director de tesi, en Miguel Valverde, l'haver-me triat per portar a terme aquesta tesi, per valorar-me i per dirigir-me amb un equilibri realment sorprenent. Ben cert és que l'he disfrutada, moltes gràcies!

Agraeixo als meus companys de laboratori per ensenyar-me i compartir tantes bolades, experiments, temps i moltes vegades amistat. Vull fer menció també al laboratori d'en Michael Sanderson per tota la ciència que em van ensenyar durant la meva profitosa estància allí. Finalment, voldria destacar la gran contribució tant de la Jacquie i com la Yaniré en aquesta tesi i l'excepcional i sempre incondicional suport que m'han donat.

Portar a terme aquesta tesi ha estat tota una experiència de creixement. Del somni a la realitat. Què més es pot demanar? Poder seguir somiant amb nous projectes i contribuir per fer-los realitat.

CONTENTS

CONTENTS

I. ABSTRACT 9

II. INTRODUCTION 11

1. MUCOCILIARY TRANSPORT 13
1.1. Ciliated epithelium 13
1.2. Cilia 14
1.2.1. Body localization 14
1.2.2. Structure of motile cilia 15
1.2.3. Beat pattern 16
1.2.4. Mechanisms of ciliary motility 17
1.3. Airway Surface Liquid 18
1.3.1. Mucus 18
1.3.2. ASL component relationships 19
1.4. Mucus Transport Capability 20
1.4.1. Ciliary Beat Frequency 20
1.4.2. Metachrony 20
1.5. Associated Pathologies 21
1.5.1. Immotile Cilia Syndrome 21
1.5.2. Infertility 21
1.5.3. Asthma 22
1.5.4. Bronchiectasis 22
1.5.5. Chronic Obstructive Pulmonary Disease 23
1.5.7. Acute Respiratory Tract Infections 24

2. CILIARY BEAT FREQUENCY REGULATION 24
2.1. CBF Stimuli 25
2.2. CBF Regulation Pathways 26

3. CELL VOLUME REGULATION 27
3.1. Steady-state Cell Volume 28
3.2. Cell Volume Responses 29
3.2.1. RVD 29
3.2.2. RVI 29
3.3. Mechanisms for Cell Volume Regulation 30
3.3.1. Protein phosphorylation 30
3.3.2. Lipid metabolism 30
3.3.3. Calcium signalling 31

4. CALCIUM SIGNALLING 31
4.1. Purinergic signalling 34
4.2. Store-Operated Calcium Entry (SOCE) 35
4.3. Receptor-Operated Calcium Entry (ROCE) 36

5. TRP CHANNELS 37
5.1. TRP superfamily 38
5.2. Osmo- and mechanosensing TRPs 39
5.2.1. Mechanisms of osmo- and mechanosensation 40
5.2.2. Osmo- and mechanosensitive TRP players 41
5.3. Thermo-TRPs 43
5.4. TRP channel expression 44
5.5. TRPV4 channel 45

5.5.1. Ion channel properties 47
5.5.2. Modes of activation 47

III. OBJECTIVES 49

IV. RESULTS 53

1. ARTICLE A 55
2. ARTICLE B 73
3. BOOK CHAPTER 101

V. DISCUSSION 111

1. PREVIOUS HISTORY 113

2. PHYSIOLOGICAL ROLE OF TRPV4 IN CILIATED EPITHELIA. COUPLING TRPV4 TO CBF REGULATION 114
2.1. Cellular expression of TRPV4 115
2.2. 4α-PDD agonist 115
2.3. Temperature stimulus 116

3. TRPV4 ACTIVATION BY MECHANICAL AND OSMOTIC STIMULI 117
3.1. TRPV4 as a mechanical transducer 117
3.2. Cell Signalling Pathways 118
3.2.1. The PLA_2-AA-EET Pathway 119
3.2.2. PLC-IP_3 Pathway 119
3.2.3. Cross-talk of PLA_2 and PLC Pathways 120
3.3. Calcium oscillations 123
3.4. Autoregulation of CBF 125

4. ROCE AND SOCE 126
4.1. TRPs in ROCE and SOCE mechanisms 128
4.2. TRPV4 in ROCE 129
4.3. Pathways involved in TRPV4 activation by ATP stimuli 130

VI. CONCLUSIONS 133

VII. REFERENCES 137

$[Ca^{2+}]$: concentration of calcium ions

4α-PDD: 4α-phorbol 12,13-didecanoate

AA: arachidonic acid

AEA: endocannabinoid anandamide

AKAP: A-kinase anchoring protein

ASL: airway surface liquid

Asn: L-aspargine

Asp: L-aspartic acid

ATP: 5'-adenosine triphosphate

CaM kinase: Ca^{2+}/calmdulin-dependent kinase

CaM: calmodulin

CBF: ciliary beat frequency

CFTR: Cystic fibrosis transmembrane conductance regulator

CIF: calcium influx factor

COPD: Chronic obstructive pulmonary disease

$cPLA_2$: Ca^{2+}-dependent phospholipase-A_2

DAG: diacylglycerol

EDHF: endothelium-derived hyperpolarizating factor

EET: epoxyeicosatrienoic acid

ER: endoplasmic reticulum

Gln: L-glutamine

GTPγS: 5'-O-(3- thiotriphosphate)

IP_3: inositol-1,4,5-triphosphate

IP_3R: IP_3 receptors

$iPLA_2$: Ca^{2+}-independent phospholipase-A_2

I-V: current-voltage

MAG: monoacylglycerol

mRNA: messenger RNA

PC: phosphatidilcoline

PCL: periciliary liquid

pHi: intracellular pH

PIP_2: phosphatidylinositol 4,5-biphosphate

PIP2: phosphoinositol biphosphate

PIP3: phosphoinositol triphosphate

PKC: protein kinase C

ABBREVIATIONS

PLA_2: phospholipase-A_2

PLC: phospholipase C

PMA: phorbol 12-myristate 13-acetate

ROCE: receptor-operated calcium entry

RVD: regulatory volume decrease

RVI: regulatory volume increase

RyR: ryanodine receptors

sAC: soluble AC

SOCE: store-operated calcium entry

SR: sarcoplasmic reticulum

TG: thapsigargin

tmAC: magnesium or calcium-sensitive transmembrane adenyl cyclase

TRP: transient receptor potential

I. ABSTRACT

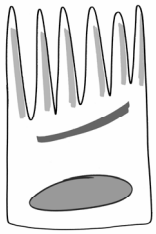
Clearance of mucus and pathogenic agents from lungs and the transport of ova and embryos in the female reproductive organs are key functions of ciliated epithelia such as those present in the airways and the oviduct. The rate of mucociliary transport is a function of ciliary beat frequency (CBF) and this, in turn, is increased by increases in intracellular calcium. Transient potential vanilloid 4 (TRPV4) cation channel mediates Ca^{2+} influx in response to mechanical and osmotic stimuli.

TRPV4 expression in ciliated epithelia from airways and oviduct is confirmed by immunofluorescence localization of the channel at the apical membrane of the polarized ciliated epithelia, where the Ca^{2+} signalling is required for CBF regulation. Ciliated tracheal cells from $TRPV4^{-/-}$ mice show no TRPV4 expression, neither increases in intracellular Ca^{2+} and CBF in response to the TRPV4-specific activator 4α-phorbol 12,13-didecanoate (4α-PDD), and reduced responses to mild temperatures (~25ºC - 38ºC), another TRPV4-activating stimulus.

TRPV4 gating by high viscous loads and hypotonicity depends on phospholipase A_2 (PLA_2) pathway activation and subsequent production of epoxyeicosatrienoic acid (EET). Under conditions of low PLA_2 activation, mechanical and hypotonic stimuli use extracellular ATP release-mediated activation of phospholipase C (PLC)-inositol triphosphate (IP_3) signalling to support TRPV4 gating. We describe that IP_3, without being an agonist itself, sensitizes TRPV4 to EET activation. Besides, the functional coupling between plasma membrane TRPV4 channels and IP_3 receptors (IP_3R) is required to initiate and maintain the cellular oscillatory Ca^{2+} signal triggered by high viscous loads and hypotonic stimuli.

One of the main CBF activators, adenosine-5'-triphosphate (ATP), triggers both Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} entry. Interestingly, TRPV4 contributes to ATP-induced increase in CBF. Furthermore, our work implicates TRPV4 channel exclusively in receptor-operated Ca^{2+} entry.

Collectively, this PhD thesis shows the role of TRPV4 channels coupling physiologically relevant mechanical, thermal, hypotonic and chemical stimuli to CBF regulation in motile ciliary epithelia



II. INTRODUCTION

1. MUCOCILIARY TRANSPORT

Many epithelial cells lining the airways and the reproductive tract contain motile projections called cilia, whose function is the transport of mucus and the particles trapped in it, a process called mucociliary transport. In the case of the airways, mucus clearance is the primary form of innate pulmonary defence brushing away foreign particles, pathogens and allergens toward the oropharynx where it is either swallowed or expectorated (Afzelius, 1995;Houtmeyers et al., 1999). Mucus clearance of the airways is performed by mucociliary transport, cephalad airflow bias (normal breathing cycles) and cough (Fink, 2007). Cough serves as a back-up system for the clearance of the mucus if the mucuciliary transport fails (Houtmeyers et al., 1999). Similarly, the fallopian tube, connecting the uterus with the ovaries, where fertilization usually occurs, transports oocytes and embryos to the uterus. This transport within the oviduct depends on both ciliary and peristaltic muscular activity (Halbert et al., 1976).

The mucociliary interface is comprised of an epithelial cell layer and airway surface liquid (ASL) (see figure 1). ASL is divided into a well-defined periciliary liquid (PCL) layer and mucus layer. The correct function of these three components is essential for an optimal mucus transport.

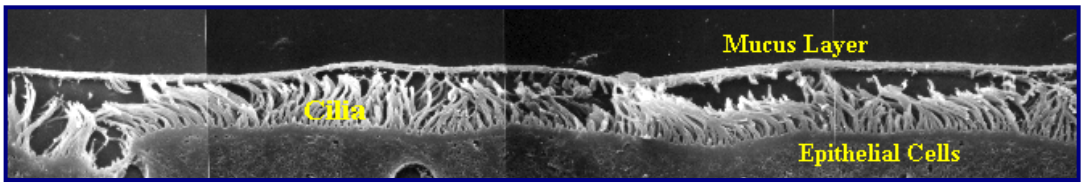


Figure 1. ***Mucociliary interface.*** A scanning electron micrograph showing the profile of the mucociliary interface. Obtained from (Sanderson, 2008).

1.1. Ciliated epithelium

Ciliated epithelia usually present two kinds of epithelial cells: ciliary and secretory cells. In the oviduct the epithelium is simple cuboidal and the nonciliated secretory cells are called peg cells. The number of ciliated cells increases from the isthmus, close to the uterus, through the ampulla until the fimbria in the infundibulum, where they are the main

cell type. Ciliated cells also line the respiratory tract, with its communicating chambers of sinuses and middle ear. Upper airways have a columnar pseudostratified epithelium with great diversity of cell types: ciliated, brush, basal, and small granule cells and for the mucus secretion there are goblet cells and submucosal glands. On the other hand, lower airways have more watery secretions because there are less goblet and ciliated cells and submucosal glands. Simple cuboidal cells become increasingly common in the epithelium of lower airways. Ciliated cells have about 200 cilia per cell. Each cilium has a length of 5-7 μm in the trachea and 2-3 μm in the seventh airway generation, and a diameter of 0,25-0,33 μm.

1.2. Cilia

Cilia or 'little feet', as they were originally described, were first discovered by the Dutch light microscopist Antoni van Leeuwenhoek in 1675. However, only in the last 40 years has an understanding of ciliary structure and function been approached. Mammalian flagella are in many respects simply long cilia; both their structure and mechanisms of motility are almost identical. However, there are differences in the ciliary beat frequency regulation between mammalian species and care should be taken when comparing species.

1.2.1. Body localization

Cilia are found in a wide diversity of organisms, including both animals and plants, where they perform a variety of specialized functions. The basic ciliary functions of fluid transport occur in two distinct forms. Either the cilia move the cell body to which they are attached through a fluid environment, or the cilia are part of a stationary epithelium and serve to produce a fluid flow across the epithelium. A variation on the transport of fluid by cilia is the transport of mucus; a visco-elastic glycoprotein gel.

Ciliated epithelia are present in nasal cavity, paranasal sinuses, eustachian tubes, pharynx, trachea, middle ear, oviducts and cervix in females, ductili efferentes in males, and ependymal lining of the ventricular system in the brain (Afzelius, 1995).

1.2.2. Structure of motile cilia

Cilia structure contains three major parts: a central core of microtubules called axoneme, an anchorage structure called basal body and an enveloping ciliary membrane (see figure 2).

The axoneme is composed of a series of specialized protein filaments called microtubules; hollow cylindrical structures. Microtubules are polymers of α- and β- tubulin that confer extremely high stability. Motile cilia have 9+2 ciliary axoneme, which comprises nine interconnected doublet microtubules surrounding and joined by cross-bridges to two centrally positioned microtubules. Each doublet is formed by microtubules, the subfiber A and B. Subfiber A is a complete microtubule possessing all 13 protofilaments whereas subfibre B has only 10 protofilaments, but shares part of the A subfiber as well. The (−) ends of the microtubules in a cilium are anchored in the basal body and are extensions of microtubules located there. Elongation of cilia occurs by addition of αβ-tubulin subunits to the distal (+) end of axonemal microtubules. Paired 5'-adenosine triphosphatase (ATPase) dynein arms are located on subfibre A and act as motor proteins. Axonemal dynein is larger and more complex than cytosolic dynein and are comprised of multimers: heavy chains, intermediate chains, and light chains. Adjacent to these dynein arms, subfibre A also has radial links or spokes. These structures joint the outer doublets with the sheath that surrounds the two central microtubules. Each radial spoke terminates in a head near the surface of the inner sheath. Nexin links, which posses elastic proprieties, appear to connect the terminal portion of the inner arm located in the subfibre A to the adjacent subfibre B. Cilia have a surrounding ciliary membrane which is an extension of the cell membrane.

Basal body or kinetosome is essentially composed of a core of microtubule elements with a series of filamentous connections. The outer doublets extend into the cell body to form part of the basal body, in contrast to the central pair. The termination of the central pair is not symmetrical. When the outer doublets are at the level of cell membrane, an additional microtubule (subfibre C) is associated with the nine peripheral doublets to produce triplets.

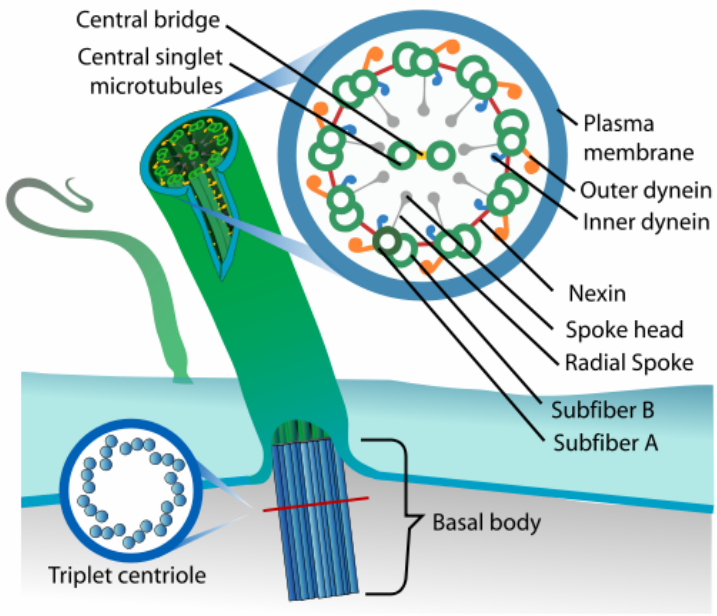


Figure 2. ***Structure of ciliary axoneme.*** Middle cross-sectional diagram of a typical cilium showing the major structures. There are three major parts: a central core of microtubules called axoneme, an anchorage structure called basal body and an enveloping ciliary membrane. The dynein arms and radial spokes with attached heads occur only at intervals along the longitudinal axis.

1.2.3. Beat pattern

The ciliary axoneme structure is highly conserved throughout evolution, but the beat pattern of ciliary beating varies considerably among flagella, single cell organisms, and mucociliary epithelium, reflecting the unique physiological function of each cliliated cell type (Tamm and Terasaki, 1994).

The beat pattern of mucus-propelling cilia is asymmetric. Ciliary movement is divisible into two distinct phases: an effective stroke and a recovery stroke. Cilia start moving with the recovery stroke from the resting position by bending sideways and backwards in an unrolling action near the cell surface (Sanderson and Sleigh, 1981;Sleigh et al., 1988). The recovery stroke is slower, amounts to three-quarters of the cycle time (Clarke, 1989). This is followed by an effective stroke during which the cilia move in a plane almost perpendicular to the cell surface. This phase ends with the cilium bent over in its resting position and with its tip pointing in the direction of propulsion, thereby minimizing

resistance to mucus flow (Sanderson and Sleigh, 1981;Sleigh, 1983). The active phase occupies one-quarter of the total cycle time. During this active phase, claws on the tip of the cilia engage in the overlying mucus and sweep it into the desired direction, but move beneath it in their recovery stroke (Clarke, 1989). In the case of the oviduct is toward the uterus, while in the airways is a cephalic direction, toward the oropharynx.

1.2.4. Mechanisms of ciliary motility

The basis for axonemal movement, as in the movement of muscle during contraction, is the sliding of protein filaments relative to one another. In cilia and flagella, the filaments are the doublet microtubules, all of which are arranged with their (+) end at the outer tip of the axoneme. Axonemal bending is an ATP-dependent movement and is produced by a mechanochemical cycle of forces that cause sliding between pairs of doublet microtubules. The active sliding occurs all along the axoneme, so that the resulting bends can be propagated without damping. The force-generating proteins responsible for this movement are the inner- and outer-arm dyneins, which bridge between the doublet microtubules. Based on the polarity and direction of sliding of the doublet microtubules, it has been proposed a model in which the dynein arms on the A tubule of one doublet “walk” along the adjacent doublet's B tubule toward its base, the (−) end. The ATP-dependent force producing active sliding is caused by successive formation and breakage of cross-bridges between the dynein arm and the B tubule. Successive binding and hydrolysis of ATP drives dynein arms into an uninterrupted release from and attach to the adjacent doublet. Outer dynein arms are responsible for adjusting ciliary beat frequency and inner dynein arms are responsible for bend formation and beating form (Brokaw, 1994). Although this general model most likely is correct, many important details such as the mechanism of force transduction by dynein are still unknown (Harvey Lodish et al., 2008).

Ciliary beating is characterized by the propagation of bends that originate from the base of the axoneme. On the other hand, the active sliding of microtubules relative to each other is a linear phenomenon. How, then, is microtubule sliding converted to bending of a cilium or flagellum? A bend is formed between a region of sliding and a region that resists sliding. Bending is regulated by controlling the regions where dynein is active along and around the axoneme. At least three distinct functions for different isoforms of dynein can now be identified: Bend initiation, maintenance of the angle of propagating bends, and generation of power to overcome viscous resistances. Only the latter is an outer arm dynein function (Brokaw, 1994). The nine outer doublets and their dynein arms are

arranged in a circle so that, when viewed from the base of the axoneme, the arms all point clockwise. Since the dynein arms walk in only one direction, toward the (−) end, and each doublet slides down only one of its two neighbouring doublets, active sliding in one half of the axoneme produces bending toward one side and active sliding in the other half produces bending toward the opposite side (Brokaw, 1982). By regulating the timing and location in which dynein arms are active, the axoneme can propagate bends in both directions from base to tip. The inner- and outer-arm dyneins contribute differently to the waveform and beat frequency of an axoneme, where the outer-arm dyneins accelerate active sliding of the outer doublets but do not contribute to bending. In contrast, the inner-arm dyneins are responsible for producing the sliding forces that are converted to bending. Phosphorylation of the inner dynein arm inactivates it, while dephosphorylation activates it to cause sliding between outer doublet microtubules. A bend is propagated when inner-arm dynein is inactivated in one region and activated in a neighbouring region (Brokaw, 1982). The radial spokes that link together all doublets through the central complex of the axoneme, tend to restrict the sliding between peripheral doublets and cause the axoneme to bend rather than slide apart. The nexin links limit the extent of relative sliding and help to maintain the integrity of cylinder doublets (Sleigh, 1983;Sleigh et al., 1988).

1.3. Airway Surface Liquid

The airway surface liquid (ASL) is produced and remains on the ciliated epithelium. The ASL is divided into a well-defined periciliary liquid (PCL) layer and a mucus layer. ASL ensures both the protection of mucociliary transport and ciliated epithelium. Surface liquid with similar characteristics has been found in other ciliated epithelia although has not been so deeply studied as the ASL.

1.3.1. Mucus

Mucus is a non-Newtonian, viscoelastic fluid composed by a complex mixture of secretory products that is found in digestive, respiratory and reproductive systems. Mucus is composed of ~1% by weight of salts and other dialyzable components, 0,5%-1% free protein and another similar proportion of highly glycosilated “gel-forming” proteins called mucins and ~95% water. When mucus is secreted onto a ciliated epithelium, occurs an ionic exchange of Na^+ by Ca^{2+} in the mucus composition that triggers a polymer-gel phase

transition, whereby the mucin polimer matrix undergoes extensive swelling and, therefore, changes from the condensed to the hydrated phase (Verdugo, 1984;Verdugo, 1991), it spreads as droplets or strings above the periciliary liquid layer to form a mucus layer (Sanderson and Sleigh, 1981;Meyer and Silberberg, 1980). Although it is still not clear how this layer is formed in vivo, nor the processes that maintain it as a physical layer (Boucher, 2003).

Besides the rheological properties, mucus possesses surface properties such adhesivety and wetability. These physical properties determine the capacity of the mucus to protect, hydrate and lubricate the underlyning epithelium. Although the most widely studied function of the mucus in the airways is the mucociliary transport, there are a variety of other functions as important as clearance of mucus, such as liquid reservoir, airway hydration, regulation of the thickness of the periciliary layer, bacterial and foreign particles adhesion and clearance, and acts as a filtration and diffusion barrier (Boucher, 2003;Houtmeyers et al., 1999;Tarran et al., 2001).

1.3.2. ASL component relationships

During mucociliary transport, both layers, periciliary liquid and mucus, are transported at approximately equal velocities (Matsui et al., 1998). The efficiency of mucus clearance largely depends on the volume of the ASL, including both the PCL and mucus layer (Boucher, 2003). The cilium moves into the watery layer of periciliary liquid whose depth is a little less than the ciliary length. This means that the overlying mucus is penetrated by the ciliary tips in the effective stroke and not in the recovery stroke, thereby optimizing the propulsive force of the ciliary beating on mucociliary transport. The thickness of the periciliary liquid is critical for effective propulsion of mucus by the tips of the cilia and its height is regulated passively by the mucus layer as a water reservoir and actively by ion transport (Na^+ absorption and/or Cl^- secretion) trough the epithelium (Tarran et al., 2001). Regulation of periciliary fluid is thought also essential to produce an environment in which airway antimicrobial peptides and defensins are effective (Chilvers and O'Callaghan, 2000).

1.4. Mucus Transport Capability

Cilia are capable of transporting relatively heavy objects with quite high velocity. The lineal velocity in the trachea, although very variable and diminishing progressively towards the lung periphery, is around 3-10 mm/min (Houtmeyers et al., 1999). In general, the transport capability of a mucociliary system is a product of the energy transferred by a cilium to the mucus during the effective stroke multiplied by the number of cilia which beat synchronously (Gheber et al., 1998). Furthermore, cilia with faster beat frequencies have a greater influence on the surrounding PCL and thereby increase the hydrodynamic coupling between adjacent cilia. Increased hydrodynamic coupling enhances the metachronal organization of ciliary activity, and as result more cilia can be recruited and coordinated into the process of mucus transport.

1.4.1. Ciliary Beat Frequency

The density (cilia per cell and number of ciliated cells) and length of the cilia correlates with an increase in mucus transport. A key factor in the regulation of mucociliary clearance is the ciliary beat frequency (CBF) (Satir and Sleigh, 1990b) because both tip velocity and co-operative cilia activity (see metachrony) are increased by increases in CBF (Zhang and Sanderson, 2003). Relatively small increase in CBF (16%) can result in a large increase (56%) in surface liquid velocity (Seybold et al., 1990), a response that is likely to enhance mucus clearance. That is because the energy which is transferred to the mucus by a cilium is a function of the ciliary velocity during the effective stroke which is proportional to the CBF.

1.4.2. Metachrony

The coordination between neighbouring cilia is crucial to optimize the cilia energy transferred to the mucus. On normal ciliated epithelium, all of the ciliary bases have the same alignment and therefore all the effective strokes take place in approximately the same direction, thereby cooperating to propel the overlying mucus to the desired direction (Sleigh, 1983;Sleigh et al., 1988). When a cilium is motile, it will exert a force on the surrounding fluid. Consequently, an active cilium influences the beat form of adjacent cilia through the intervening fluid. This hydrodynamic coupling encourages adjacent cilia to beat with a fixed phase relationship to one another to minimize interference (Machemer, 1972).

Adjacent cilia in ciliary rows in one direction will beat slightly out of phase with one another to form a line of metachrony. In most cases, the direction of the metachronal wave is not the same as the direction of the propulsion (Gheber et al., 1998). Due to the phase shifts between beating cilia, there are patches of adjacent cilia that add their propulsive effort to the propulsion of mucus (Sanderson and Sleigh, 1981). The characteristics of the metachronal wave determine the size and spacing of these patches, and therefore determine the number of cilia that actively propel mucus at a given time.

1.5. Associated Pathologies

Disruption of mucociliary transport in airways or oviduct may be caused by diseases such as cystic fibrosis, primary ciliary dyskinesia and asthma or may be secondary to pollutant exposure, tobacco smoke, hormonal changes and viral or bacterial infections (Chilvers and O'Callaghan, 2000;Lyons et al., 2006).

1.5.1. Immotile Cilia Syndrome

Immotile cilia syndrome (ICS) is an autosomal recessive disease. ICS patients have impaired ciliary activity and sperm motility. A variety of ultrastructural aberrations in the axonemal microtubular apparatus has been observed, where the most common is the absence of dynein arms that moves the bundle of microtubules (Palmblad et al., 1984). The result is male sterility due to sperm immotility, female subfertility (Lyons et al., 2006), chronic or recurrent infections of respiratory tract, sinuses and auditory ducts and bronchiectasis due to mucus plugs that become a hotbed for persistent bacterial airway infections that ultimately lead to a markedly anaerobic luminal environment (Afzelius, 1995;Afzelius, 1976;Boucher, 2002). There are motile cilia in the embryonic node responsible of the left-right body simetry, therefore in the 50% of ICS patients also have situs inversus or Kartagener syndrome (Afzelius, 2004).

1.5.2. Infertility

The oviduct (Fallopian tube) plays an essential role in the transport of both ova and embryos and in early embryogenesis. Propulsion of ova and embryos is achieved by complex interactions between muscle contractions and mucociliary activity. The oviduct

undergoes cyclical changes in morphology and ciliary activity in response to ovarian hormones (Lyons et al., 2006). Muscle contractions play a role in mixing the secretions rather than in propulsion of ova and embryos (Muglia and Motta, 2001).

Ciliary activity is more vigorous in the luteal phase of the menstrual cycle. Many pathological conditions associated with infertility and ectopic pregnancy have been shown either to destroy cilia or to reduce ciliary motion or both. Tobacco smoking, previous ectopic pregnancy, endometriosis and subsequent inflammation due to infection of *N. gonorrhoeae*, *Chlamydia trachomatis* or *Chlamydia salpingitis* cause mucociliary impairment (Lyons et al., 2006).

1.5.3. Asthma

Asthma is a disease of the respiratory system in which the airways constrict, become inflamed, and are lined with excessive amounts of mucus, often in response to one or more triggers, such as exposure to environmental stimulant, allergen, cold air, exercise or emotional stress. This narrowing causes symptoms such as wheezing, shortness of breath and chest tightness.

The disorder is a chronic or recurring inflammatory condition in which the airways develop increased responsiveness to various stimuli, characterized by bronchial hyper-responsiveness, inflammation, increased mucus production and airway obstruction. Mucociliary dysfunction has been shown to be a feature of asthma (Bateman et al., 1983). Constant irritation in asthmatics, caused by inflammation, and therefore, at least in the earlier stages, increased the mucociliary transport. On long-term basis, hyperstimulation of mucociliary transport itself or other factors associated with asthma like epithelial damage and hypersecretion might lead to a impaired mucociliary transport (Svartengren et al., 1989). Thus the mucus clearance impairment is secondary to the disease.

1.5.4. Bronchiectasis

Bronchiectasis develops when mucus plugging and infection occur together in the absence of functioning cilia (Warwick, 1983). Bronchiectasis is a chronic inflammatory or degenerative condition of bronchi or bronchioles of the lungs marked by dilatation and loss of elasticity of the walls. Therefore there is airflow obstruction and mucus accumulation that cause inflammation and more tissue damage.

Mucus clearance is variable from normal to extremely slow (Svartengren et al., 1986), although there are bronchiectatic patients with generalized impairment of mucociliary transport as a major fact in the latter development of the disease.

1.5.5. Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is not one single disease but an umbrella term used to describe chronic lung diseases that cause limitations in lung airflow. The more familiar terms 'chronic bronchitis' and 'emphysema' are no longer used, but are now included within the COPD diagnosis. The most common symptoms of COPD are breathlessness, or a 'need for air', excessive sputum production, and a chronic cough. Mucociliary dysfunction has been shown to be a feature of COPD and asthma, although contradictory opinions exist. Chronic bronchitis is characterized by mucociliary dysfunction resulting from structural and functional defects of cilia and the secretory apparatus. Chronical bronchitis is defined clinically as chronic productive cough (Wanner, 1990). It is a common disease of habitual tobacco smokers and residents of polluted cities. The initial event to develop the pathology is the chronic irritation due to inhalation of smoke and pollutants, this leads to tissue inflammation resulting in tissue damage and hypersecretion of mucus which contributes to the airways obstruction. Mucociliary transport is impaired in patients with simple as well as obstructive chronic bronchitis (Houtmeyers et al., 1999).

1.5.6. Cystic Fibrosis

Cystic fibrosis is an autosomal recessive disorder characterized by abnormalities in the epithelia of the lungs, intestine, salivary and sweet glands, liver, and reproductive organs, often as a result of inadequate hydration of their secretions (Quinton, 1999). The primary defect in cystic fibrosis is the altered activity of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which has the ability to function as both a cAMP-activated cloride channel and a channel regulator (Sheppard and Welsh, 1999;Schwiebert et al., 1999).

More than 90% of the male patients are infertile, with aspermia secondary to agenesis of vas deferens and epididymis, but spermatogenesis are normal. Female reproductive function is normal, although mucus can be dehydrated which might impair fertility (Ratjen and Doring, 2003).

Cystic fibrosis is considered as a genetic form of chronic bronchitis where mucociliary clearance is impaired. There is a depletion of the PCL volume caused by the combined dysfunctions of accelerated Na^+-dependent volume absorption and failure to secrete Cl^-. Both dysfunctions are a direct consequence of missing CFTR at the apical membrane of airway epithelial cells. PCL depletion leads to failure of mucus transport, which is associated with persistent mucin secretion and formation of adherent mucus plaques and plugs (Boucher, 2002).

1.5.7. Acute Respiratory Tract Infections

Respiratory tract infection impair mucociliary transport by means of two principal mechanisms, namely through cytotoxic effects on the airway epithelium or through production of substances by microorganisms (Salathé M. et al., 1997). Viral agents such as myxovirus as well as different types of the rhinovirus or influenza virus, bacterial agents such as *Haemophilus influenzae* or *Streptococcus pneumoniae* and various types of mycoplasma are described as influencing mucociliary transport, mostly negatively (Pavia, 1987;Lindberg, 1994).

2. CILIARY BEAT FREQUENCY REGULATION

The main task of mucociliary cells is to transport particles trapped on the mucus layer over the epithelium. To rapidly convey physiological changes in mucus viscosity and large heavy loads, the ciliated cells must beat co-ordinately at a high rate. Despite variations in fluid viscosity and particle loads, mucociliary transport efficiency is preserved. Ciliated cells are able to maintain relatively constant their CBF over a rage of viscosities and it has been proposed that this autoregulatory response of the CBF aimed to prevent the collapse of mucus transport under high viscous loads (Johnson et al., 1991). The cilia return to a low rate of beating when the task is complete because they do not operate at full capacity under normal conditions (Kerem et al., 1999).

Thus the ability to enhance beating in response to various physiological cues is a critical hallmark of mucociliary cells. Two distinct stages in ciliary beating are clearly identified: the spontaneous beating that only requires intracellular ATP, and the stimulated

stage requiring cyclic nucleotides and Ca^{2+} in addition to intracellular ATP (Ma et al., 2002).

2.1. CBF Stimuli

In vivo, the changes in CBF are mediated mainly through the stimulation of receptors by naturally released ligands. Extracellular stimuli that increases the CBF are nucleotides via purinergic receptors (Evans and Sanderson, 1999;Korngreen and Priel, 1996). ATP is one of the strongest signals to accelerate CBF via apical membrane $P2Y_2$ receptors (Salathe, 2006;Morse et al., 2001). Some neurotransmitters like serotonin via 5-HT receptors (Stephens and Prior, 1992), certain adrenergic drugs via β-receptors agonists like bradykinin or isoproterenol (Zhang et al., 2005a;Paradiso et al., 1991) and acetylcholine via cholinergic muscarinic receptors (Salathe et al., 1997). Nitric oxide (NO) (Uzlaner and Priel, 1999) and prostaglandins (Verdugo, 1980) also increase the CBF. Vasopressin stimulates CBF via V1b receptor. On the other hand, neuropeptide Y inhibits CBF via NPY2 receptor and PKC (Wong et al., 1998). All of these effects stem from the activation of G-protein coupled receptors. However, small hyaluronans through RON receptors are another endogenous CBF stimulator ligands (Forteza et al., 2001). Capsaicin, a well known agonist of transient receptor potential channel TRPV1, increases the CBF (Cortright and Szallasi, 2004;Wong et al., 1990).

Changes in pH is another proposed regulator of CBF. Variations in pHi of airway epithelia may occur in vivo in response to shifting luminal CO_2 concentration from 5% to 0,02% during full breathing cycle (Willumsen and Boucher, 1992). Then during inspiration and exacerbations of airway diseases the P_{CO_2} drops and the alkalinization of the epithelium stimulates cilia to beat faster (Sutto et al., 2004).

Mechanical influences also play a very important role in CBF stimuli like cough, particles trapped and viscosity increments (Sanderson and Dirksen, 1986;Houtmeyers et al., 1999;Spungin and Silberberg, 1984). CBF responds to temperature change with a sigmoid pattern. There is an inflexion point around 20ºC and with a maximum plateau above physiological temperature (37ºC) (Green et al., 1995;Mwimbi et al., 2003b).

2.2. CBF Regulation Pathways

The CBF must be adjusted by cellular pathways that controls dynein-microtubule interactions. The ciliary axoneme structure is highly conserved, but the mechanisms controlling the CBF are different between species, although is quite similar among mammals. Essentially, CBF changes in response to variations in the phosphorylation state of ciliary targets, intracellular [Ca^{2+}], and intracellular pH (pHi). Changes in phosphorylation occur mainly through cyclic adenosine monophosphate (cAMP)-dependent kinase (PKA) but also through cyclic guanosine monophosphate (cGMP)-dependent kinase (PKG) and PKC.

It is well established that calcium plays a crucial role in CBF regulation. Elevations of intracellular Ca^{2+} concentration are almost always associated with an increase in CBF (Lansley and Sanderson, 1999;Delmotte and Sanderson, 2006). Regulation of CBF in airway cells, as with many other Ca^{2+}-dependent cellular functions, is dependent on amplitude, frequency modulation, and duration of Ca^{2+} signalling. This means that changes in ciliary activity depend on both the differential, but not absolute changes, and the frequency of these calcium oscillations (Berridge, 1997;Evans and Sanderson, 1999;Putney, Jr., 1998). CBF regulation by calcium is rapid (within one beat cycle) and occurs over a relatively small change in calcium concentration (Lansley and Sanderson, 1999;Salathe and Bookman, 1999); whereas decreases in CBF occur more slowly and significantly lag behind decreases in intracellular Ca^{2+}, a process called hysteresis. This process explain how is possible maintain relatively stable and fast CBF between intracellular Ca^{2+} oscillations. Calcium seems to increase CBF via an initial direct action on the axoneme but also via cross talk with cAMP and cGMP pathways as well as ion channels (Weiss et al., 1992;Verdugo et al., 1983).

Different kinases participate in CBF regulation. PKA is located at the ciliary axoneme by A-kinase anchoring protein (AKAP). Increased levels of cAMP activate PKA and phosphorylates the outer arm of dynein light chain to increase its ATPase enzyme activity. This mediates a switch from slow to the fast dynein-duty cycle and thus increases the CBF (Kultgen et al., 2002). There are a variety of cAMP intracellular sources: $G_{\alpha s}$-proteins differently regulated isoforms which activate Mg^{2+} and Ca^{2+}-sensitive transmembrane adenyl cyclases (tmAC). At least some of them must be expressed in the apical membrane of the ciliated cells, but not in the ciliary membrane. Beta-adrenergic agonists activate Mg^{2+}-sensitive tmAC, while adenosine via A_{2b} receptors activates Ca^{2+}-sensitive tmAC, therefore increasing cAMP production. There is another AC, called soluble

AC (sAC) which differs enzimatically from the tmAC by its preference for Mn^{2+}, its insensitivity to G-protein activation and forkolin, and ciliary axoneme localization. The sAC is directly activated by Ca^{2+} and HCO_3^- in a pH-independent manner (Salathe, 2006). PKG activity have been measured in cilia and the protein localized in the ciliary axonemes (Wyatt et al., 2005) and have been shown to be involved in CBF regulation. As for the other kinases also involved in CBF regualtion, PKG activation changes the phosphorylation pattern of axonemal proteins, (Gertsberg et al., 2004) resulting in CBF increases while activated PKC decreases CBF in mammalian cilia and one of its target is a ciliary membrane protein (Salathe et al., 1993).

3. CELL VOLUME REGULATION

Cell volume regulation is essential for the cell viability and cells tightly regulate their volume. The volume of a given cell is an important factor not only in defining its intracellular osmolarity and its shape, but also in defining other cellular functions, such as transepithelial transport, cell migration, cell growth, cell death, intracellular metabolism, hormone release, excitability and differentiation (Lang et al., 1998;Wehner et al., 2003).

Most cells of higher organisms are only exposed to subtle tonicity changes due to tightly regulated plasma osmolarity by the renal function (Lang et al., 1998). Considerable cell volume changes occur in renal medullary cells, chondrocytes, during maturation of erythrocytes, and in pathologies like hypo- and hypernatremia. Intestinal epithelium has to bear huge changes in extracellular osmolarity; during meals is increased and is decreased after water drinking. Cell volume gain also occurs without a change in external osmolarity, as consequence of ion redistribution or increased cellular content of molecules acting as organic osmolytes. This isosmotic swelling, occurs during nutrients absorption, in hypoxia, ischemia, epilepsies, cranial trauma or hepatic encephalopathy (Vazquez-Juarez et al., 2008). In the particular case of ciliated epithelia, PCL tonicicity also affects CBF function. It has been shown that inspiration of dry air increases the tonicity of PCL and then decreases the CBF and the mucociliary clearance (Winters and Yeates, 1997a). Whereas there is slightly CBF stimulation in ciliary cell culture by hypotonic swelling and *in vivo* during inhalation of water aerosols (Winters and Yeates, 1997b;Sanderson and Dirksen, 1991). Cells have developed specific adapting mechanisms to restore the original cell volume and keeping cell function despite osmotic changes (Hoffmann and Dunham, 1995).

Changes in extracellular osmolarity (anisosmotic) or intracellular osmolarity (isosmotic) cause a vectorial flux of water through the cell plasma membrane to find the osmotic equilibrium, therefore avoiding the osmotic pressure difference between both sides of the plasma membrane. Water diffusion to restore the thermodynamic equilibrium induce cell volume changes (Wehner et al., 2003). This volume changes influence the balance between protein synthesis and breakdown: swollen cells become anabolic while shrunken cells become catabolic.

3.1. Steady-state Cell Volume

Cytoplasm is richer than extracellular fluid in charged impermeant macromolecules and must be compensated by lowering cytosolic ion concentrations. The discrepancy gives rise to colloid osmotic forces that, if unopposed, would cause cells swell and disperse their contents. Reversal of this tendency requires energy. Through the mechanisms of active transport, ion gradients are established that would cause the cell to shrink if it had no cytoplasmic proteins. The result of these opposing forces is a steady-state disequilibrium to maintain a constant cell volume (Parker, 1993). This ion transport trough the plasma membrane is due to a cellular electrochemical gradient, this is defined as the spatial variation of both electrical potential and chemical concentration through the plasma membrane. The combination of these two factors determines the thermodynamically favorable direction for ion movement across a membrane. The constant cell volume (steady-state cell volume) in isotonic solutions is performed by both the equilibrium of passive ion flux and the Na^+-K^+-ATPase pump activity. The Na^+/K^+ ATPase extrudes Na^+ in exchange for K^+, which can permeate the cell membrane through K^+ channels. K^+ exit generates a cell-negative potential difference across the cell membrane, driving the exit of anions such as Cl^-. The low cytosolic Cl^- concentrations counterbalance the excess cellular osmolarity by organic substances (Lang, 2007). This dynamic activity maintains the electrochemical gradient through the plasma membrane necessary for the cotransport with Na^+ of sugars and essential aminoacids (Lang et al., 1998;Hoffmann and Dunham, 1995;Hallows and Knauf, 2008).

3.2. Cell Volume Responses

The high permeability to water characteristic of most animal cells requires the presence of mechanisms allowing the cell to face contingencies derived from water entry or extrusion (Vazquez-Juarez et al., 2008). After osmotic perturbation cells ensue a volume regulatory phase, in which the cells tend to return to the volume they had in an isotonic medium. Regulatory volume decrease (RVD) follows sudden osmotic swelling; regulatory volume increase (RVI) follows shrinkage. Although the most obvious interpretation is that cells regulate cell volume, they could be regulating some other property, i.e., shape, ionic concentrations, or macromolecular crowding (Parker, 1993).

3.2.1. RVD

RVD is a cellular adaptive response to extrude water, therefore reducing cell volume to its original size. Typically it involves an increase in the plasma membrane permeability to some cellular ions. This activation leads to osmolytes exit, reducing the intracellular osmolarity and allowing water to be lost together with the ions. RVD involves releasing ions through activation of K^+ and anion channels, KCl-cotransport, or parallel activation of K^+/H^+ exchange, Cl^-/HCO_3^- exchange and organic osmolytes via non-specific transport systems (Wehner et al., 2003;Strange, 2004;Lang, 2007).

3.2.2. RVI

RVI is a cellular adaptive response to increase the water content and cell volume to its original size by increasing osmolytes uptake. The plasma membrane permeability is increased for inorganic osmolytes such as Cl^- and Na^+ by means of activated Na^+/H^+ and HCO_3^-/Cl^- exchangers in parallel to Na^+-K^+-$2Cl^-$ cotransporters or Na^+ channels, resulting in the entry of osmotically obligated water to restore osmotic equilibrium. Shrunken cells further accumulate organic osmolytes such as sorbitol and glycerophosphorylcholine, and monomeric amino acids by altered metabolism and myoinositol (inositol), betaine, taurine, and amino acids by Na+ coupled transport or biosynthesis (Lang, 2007;Strange, 2004).

3.3. Mechanisms for Cell Volume Regulation

Cell volume regulation is a complex phenomenon due to the molecular diversity of the different mechanisms set in different cell types (Hoffmann, 2000). Theoretically, there are three mechanistic components: a cell volume sensor, transduction signalling pathway and an effector mechanism to restore the original volume (Hallows and Knauf, 2008).

The mechanisms or molecules acting as osmollarity sensors are not fully identified. There are two major theories about cell volume detection: (a) One holds that the volume signal arises from mechanical events, like bending or stretching of the membrane or rearrangement of structures within the cell. (b) The other is that volume is sensed by virtue of changes in concentration of cell component, like water, salt or cytoplasmatic proteins (Parker, 1993). Different elements have been implicated in the sensing mechanisms: integrins, growth factor receptors, changes in macromolecular crowding, ionic strength, transmembrane mucins and plasma membrane stretch (Lang et al., 1998;Vazquez-Juarez et al., 2008;de et al., 2007).

3.3.1. Protein phosphorylation

A huge variety of protein kinases like PKC, PKA, PI3K, MAPK (JNK, ERK-1, ERK-2, p38), tirosin kinases, among others, are regulated by cell volume changes (Lang et al., 1998;Jakab et al., 2002). Usually, MAPK proteins have a role in the activation of slower effector mechanisms, due to its regulation of transcription factors that modulates the gene expression of both transport and metabolism of organic osmolytes. The other kinases, activates quickly the transport of inorganic osmolytes through the plasma membrane (Cohen, 2005a).

3.3.2. Lipid metabolism

Changes in the lipid levels, phosphatidilcoline (PC), phosphatidic acid (PA), Phosphoinositols (4,5-PIP_2, 3,4,5-PIP_3), diacylglycerol (DAG), arachidonic acid (AA), and their derivates regulates the cell response to osmotic variations (Jakab et al., 2002). These lipid messengers are involved at different levels of the cellular pathways as substrate or cofactors for enzymes: phospholipases PLA_2, PLC, PLD and protein kinases (PI3K), rho GTPase, PKC, and cotransporters or channels (Hoffmann and Dunham, 1995;Jakab et al., 2002). For example, under hypotonic solutions or following membrane

stretch, PLA_2 plays an important and in some cases essential role. It has also been proposed as a volume sensor, but also a transducing element secondary to the generation of intracellular Ca^{2+} signals (Wehner et al., 2003) (Hoffmann, 2000).

3.3.3. Calcium signalling

In many cells, mainly epithelial, RVD process requires calcium as a messenger. Classically, the source of Ca^{2+} has been attributed to its influx via non-selective cation channels activated either directly in response to the membrane stretch or following swelling-activated intracellular signalling (Hoyer et al., 1994;Hoffmann and Dunham, 1995;Sachs and Morris, 1998), although intracellular Ca^{2+} stores may also participate (Yule and Gallacher, 1988;Yule and Gallacher, 1988). The participation of intracellular stores mainly depends on autocrine/paracrine ATP release after cell volume increase that causes G protein-coupled receptors activation and IP_3-mediated calcium release from stores (McCarty and O'Neil, 1992;Wehner et al., 2003). These Ca^{2+} increments activate Ca^{2+}-dependent K^+ channels, the truly RVD effectors. Therefore, Ca^{2+}-dependent K^+ channels are particularly relevant in epithelia, where RVD is normally triggered by changes in intracellular Ca^{2+} concentration (Pasantes-Morales and Morales-Mulia S., 2000), although exceptions exist. Large (KCNMA1, also known as BK_{Ca} or Maxi-K) (Fernandez-Fernandez et al., 2002), intermediate (KCNN4, also known as IK) (Vazquez et al., 2001) and small conductance (KCNN2, also known as SK2) (Feranchak et al., 2004) K^+ channels, among others, are activated by osmotic cell swelling in epithelial cells.

4. CALCIUM SIGNALLING

Ca^{2+} signalling is one of the main and most versatile intracellular signalling systems in cells (Berridge et al., 2000). It controls many processes during development, and once cells have differentiated it governs the activity of many different cellular processes, effectively determining how we metabolize, secrete, move and think. There is also a darker side to its action because larger than normal elevations can cause cell death by apoptosis or necrosis, as occur during processes such stroke or cardiac ischaemia (Berridge, 2006). One of the challenges is to understand how these widely different Ca^{2+}-signalling systems can be set up to control so many divergent cellular processes.

At any moment in time, the level of intracellular Ca^{2+} is determined by a dynamic balance between the 'on' reactions that introduce Ca^{2+} into the cytoplasm and the 'off' reactions through which this signal is removed by the combined action of buffers, pumps and exchangers. These heterogeneous Ca^{2+}-signalling systems are assembled from an extensive Ca^{2+}-signalling toolkit of components. Each cell type express a unique set of components from Ca^{2+}-signalling toolkit, that can be further diversified through alternative splicing to create Ca^{2+}-signalling systems with different spatial and temporal properties (see Fig. 3) (Berridge et al., 2000).

Ca^{2+} mobilization consist of release of Ca^{2+} from intracellular stores, as well as increased entry of Ca^{2+} from extracellular medium through Ca^{2+}-permeable channels (Rosado, 2006). The release of Ca^{2+} coming from a subcompartment of the endoplasmic reticulum (ER) or its muscle equivalent, the sarcoplasmic reticulum (SR) is IP_3-mediated and Ca^{2+}-sensitive and depends on the activity of IP_3 receptors (IP_3R). Ryanodine receptors (RyR) are also important in different cell types, mainly muscle, but in epithelial cells IP_3R are the main players in Ca^{2+} release after activation of PLC-IP_3 signalling pathway (Hansen et al., 1995). On the other hand, there are many different plasma-membrane channels that control Ca^{2+} entry from external medium, including voltage-dependent, store-operated (SOC), receptor-operated (ROC) and stimulus-operated (e.g., mechanosensitive channels). All these channels are activated in response to stimuli that include membrane depolarization, stretch, noxious and thermal stimuli, extracellular agonists directly or through intracellular messengers, and depletion of intracellular stores (see figure. 3) (Berridge et al., 2003).

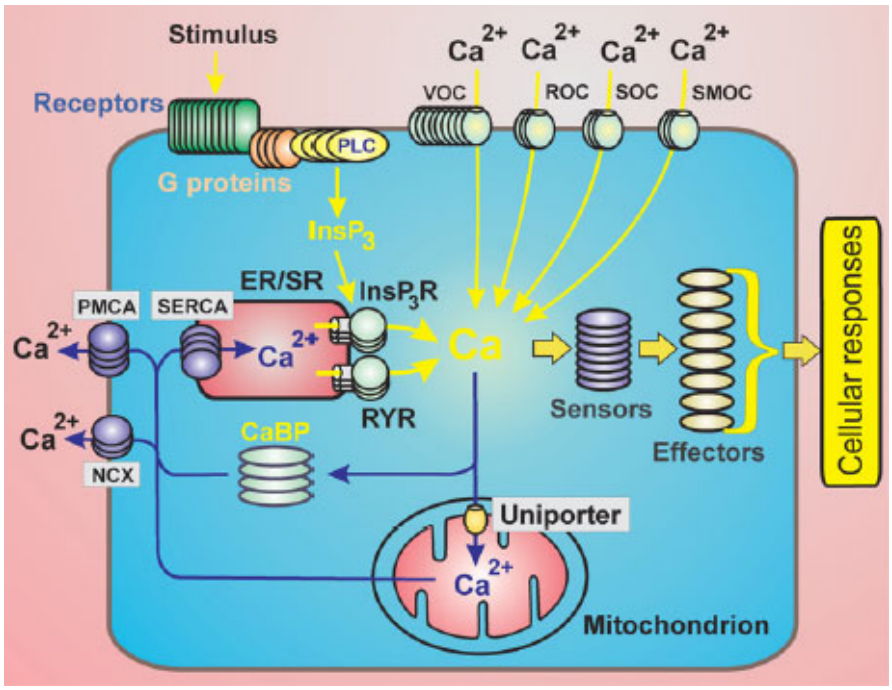


Figure 3. ***Summary of the major components that contribute to the Ca^{2+} signalsome.*** A generic Ca^{2+} signalsome is made up from components of the extensive Ca^{2+} signalling toolkit. The duplication of the individual components represents the fact that there are numerous isoforms that further enhance the diversity of the Ca^{2+} signalling systems. During its passage through the cytoplasm, Ca^{2+} resides temporarily on the Ca^{2+}-binding proteins (CaBP) acting as buffers or within the mitochondria. To carry out its signalling function, Ca^{2+} binds to sensors that then employ a range of effectors to stimulate diverse cellular processes. These different components are mixed and matched to construct Ca^{2+} modules that are then assembled to produce cell-specific signalsomes. $InsP_3R$: inositol 1,4,5-triphosphate receptor, NCX: Na^+/Ca^{2+} exchanger, PMCA: plasma membrane Ca^{2+}-ATPase; SERCA: Sarco/endoplasmatic reticulum Ca^{2+}-ATPase. From (Berridge, 2006).

4.1. Purinergic signalling

Purinergic signalling controls a wide range of cellular functions via Ca^{2+} signalling, particularly interesting in the context of this thesis is its regulation of mucociliary transport in epithelia.

Extracellular ATP may be released in response to mechanical and hypotonic stimuli as an autocrine and paracrine molecule. ATP activates two types of purinergic receptors: P2X and P2Y receptors. The P2X receptors are ligand-gated ion channels permeable to extracellular cations that play an important role in cells involved in immunological and inflammatory responses (North, 2002). The P2Y receptors are G protein-coupled receptors that are categorized into a subfamily of receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) that predominantly couple to Gq, and therefore activate phospholipase C (PLC-β), and into a family of Gi-coupled receptors ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) that inhibit adenylyl cyclase and regulate ion channels (Lazarowski et al., 2003).

As with many other agonists of G protein-coupled agonists, ATP at nanomolar concentrations mainly generates oscillatory calcium signal, while at micromolar concentrations generates a biphasic pattern (see figure 4). An initial transient rise is followed by a less pronounced but sustained elevation. The initial phase originates from the IP_3-induced release of Ca^{2+} from the intracellular stores into the cytoplasm, then the cell extrude the released Ca^{2+} with the action of plasma membrane Na^+/Ca^{2+} exchangers and Ca^{2+} pumps that take out Ca^{2+} from the cytosol and back into the stores. The sustained phase of elevated Ca^{2+} requires both (i) the continued activation of a Ca^{2+} influx pathway which is maintained until the agonists that activate PLC ceases to act and (ii) the stores are replenished (Berridge and Irvine, 1989;Berridge, 1993;Putney, Jr., 1987). The intensity of the Ca^{2+} signal: frequency of oscillations and magnitude of the biphasic response is dependent of the agonist concentration and the sensitivity of each cell.

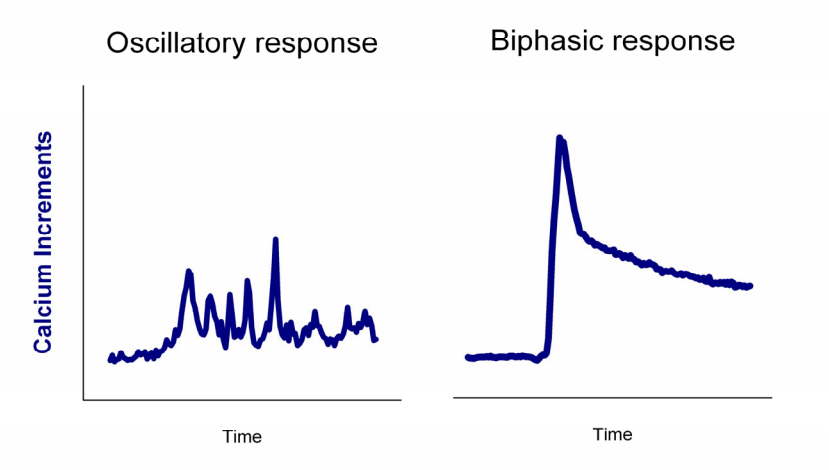


Figure 4. ***Dose-dependent calcium response patterns.*** **Left panel** shows a ciliated tracheal cell response to low ATP dose (200nM) with a clear oscillatory pattern. **Right**, typical cell calcium response to a high ATP dose (20μM) presenting a biphasic response: an initial Ca^{2+} peak from the stores followed by a sustained Ca^{2+} entry. Notice the amplitude of the oscillatory response is lower than the biphasic one. Own unpublished data.

4.2. Store-Operated Calcium Entry (SOCE)

SOCE, also refereed to as capacitative Ca^{2+} entry, is the Ca^{2+} entry triggered by the depletion of the intracellular Ca^{2+} stores independently of receptor activation. In other words, depletion of Ca^{2+} stored within the ER lumen serves as the primary trigger for a message which is returned to the plasma membrane resulting in the activation SOC channels which allow entry of external Ca^{2+}. SOCE plays, on a time scale ranging from milliseconds to many hours, important roles in cell physiology: maintaining Ca^{2+} signals that mediates physiological processes like secretion, gene transcription and cell proliferation, and allowing stores to be replenished and hence preserving ER Ca^{2+} homeostasis (Parekh and Penner, 1997;Spassova et al., 2004).

At least two types of SOCE can be distinguished electrophysiologically and now molecularly (Yuan et al., 2007). Electrophysiological analysis of channel activity triggered by store-emptying has defined one current, the long-known Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). The activation of this small, highly Ca^{2+}-selective, non-voltage gated current, appears to require substantial Ca^{2+} store-depletion (Spassova et al., 2004). The second type of channels that might function as store-operated Ca^{2+} channels (SOCs) are

the TRPC channels. Up to now, the molecular candidates to generate I_{CRAC} upon stores depletion were TRPC channels, although their biophysical properties differed substantially (Putney, Jr., 2007). Recently, the combined function of newly discovered proteins, STIM1, a Ca^{2+} sensor of the ER lumen and the Ca^{2+} permeable plasma membrane ion channel called ORAI have changed the thinking about SOCE and I_{CRAC} currents. Resolving the molecular identity of I_{CRAC}, TRPC may be considered a new kind of SOCE, different to I_{CRAC} currents.

4.3. Receptor-Operated Calcium Entry (ROCE)

ROCE, also known as non-capacitative Ca^{2+} entry, simply refers to a current that is secondary to receptor activation. This contrasts with ligand-gated ion channels which are activated by ligand binding directly to the channel subunits. ROCE is not as well studied as SOCE and is a term used too loosely, applying it in an exclusionary sense (Janssen and Kwan, 2007).

ROCE is observed in the sustained phase of the biphasic response and during oscillatory Ca^{2+} response. It is the Ca^{2+} entry occurring through non-selective cationic channels activated secondary to the stimulation of a receptor and downstream of PLC pathway (Liao et al., 2008). There are studies also reporting activation of Ca^{2+} entry via arachidonic regulated channels (ARC) at low agonist concentrations; also considered as a novel pathway for receptor-activated Ca^{2+} entry. The molecular nature of these Ca^{2+}-selective channels, biophysically distinct from I_{CRAC} channels, arachidonic acid-regulated, and independent of store depletion is still under debate (Shuttleworth et al., 2004). The Ca^{2+} entry through these channels may be important in modulating the frequency of Ca^{2+} oscillations.

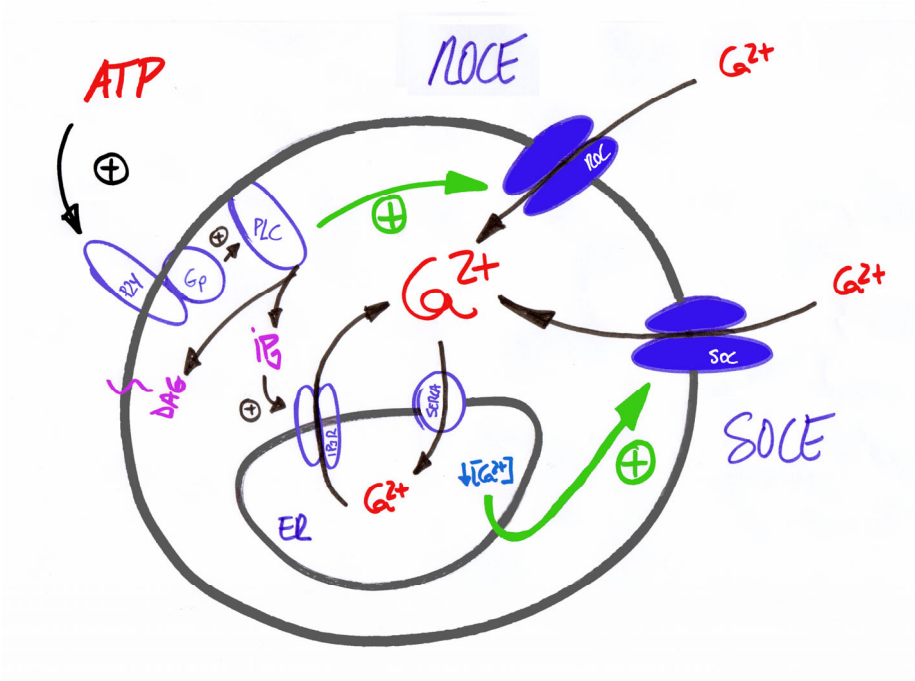


Figure 6. ***Current understanding of ROCE and SOCE pathways.*** Agonist activation of plasma membrane receptor results in the activation of membrane G protein-coupled receptor. The PLCβ associated enzime is activated to breakdown PIP_2 to formation of IP_3 and DAG metabolites. IP_3 diffuses rapidly and activates IP_3 receptors (IP_3R) to increase cytosolic Ca^{2+}, causing discharge of stored Ca^{2+} ions from ER. The activation agonist receptor-PLC-IP_3R leads to the activation of ROCE mechanism, a process of Ca^{2+} entry secondary to receptor activation. At the same time, a stimulus-depletion of Ca^{2+} stored in the ER causes Ca^{2+} unbinds from the intraluminal face of Stim-1 leading its activation. Then, activated Stim-1 conveys the signal of store depletion to Orai channels which allows the Ca^{2+} entry through the plasma membrane, a mechanism called SOCE.

5. TRP CHANNELS

The human genome encodes hundreds of channels that allow the passage of charged ions across impermeable lipid bilayers (Hille, 1992). While energy-requiring pumps labour to build charge and concentration gradients across the membrane, ion channels spent this stored energy of a battery. Small conformational changes cause channels to open, allowing over ten millions ions to flow per second through each channel. Ca^{2+} ions are particularly important in cellular homeostasis and activity, and the surface of

each cells holds thousands of channels that precisely control the timing and entry of Ca^{2+} ions (Clapham, 2003).

5.1. TRP superfamily

The transient receptor potential (TRP) superfamily comprises 33 genes in mammals encoding widespread ion channels with a daunting diversity in ion selectivities, modes of activation, and physiological functions (Montell, 2005). Although TRP channels have many roles in both neuronal and non-neuronal cells, they are of particular importance in sensory physiology. These include roles for TRP channels in each of the sensory modalities, ranging from vision to taste, smell, hearing, mechanosensation, and thermosensation (Lin and Corey, 2005). TRP also serve to allow individual cells to sense changes in the local environment, such as alterations in fluid flow, osmotic and mechanical stress (Christensen and Corey, 2007).

The TRP superfamily is composed of seven subfamilies, all of which share the common feature of six transmembrane domains with varying degrees of sequence similarity and permeability to cations. The division into subfamilies on the basis of amino acid sequence and structural similarity does not provide functional classification for TRP proteins (Huang, 2004). The structure of a TRP channel has not been solved; however, the functional channels appear to form tetrameric assemblies (Amiri et al., 2003;Hoenderop et al., 2003b;Schaefer, 2005), consistent with the structures of voltage-dependent K^+ channels (Montell, 2005). Some TRP channel subunits can form heterotetrameric channels with different gating and permeability properties (Schaefer, 2005).

The initially identified members of the TRP superfamily are referred to as the “classical” or “canonical” TRPs and fall into the TRPC subfamily. The names of the remaining subfamilies are based on the original designation of the first recognized members of each subfamily. There are two large groups (see figure 7), the **Group 1** TRPs: TRPC, TRPV (“vanilloid”), TRPM (“melastatin”), TRPN (“NOMPC”, but not in higher vertebrates), and TRPA (“with multiple ankyrin domains”), share substantial sequence identity in transmembrane domains. The TRPC, TRPM, and TRPN (except for NOMPC) proteins include a 23- to 25- aminoacid “TRP domain” C-terminal to sixth transmembrane domain. The **Group 2**, has 2 subfamilies: TRPP (“polycystin”) and TRPML (“mucolipin”), are only distantly related to the Group 1 TRPs, due to low sequence similarity and a large

extracellular loop between the first and second transmembrane domain (Montell, 2005;Lin and Corey, 2005).

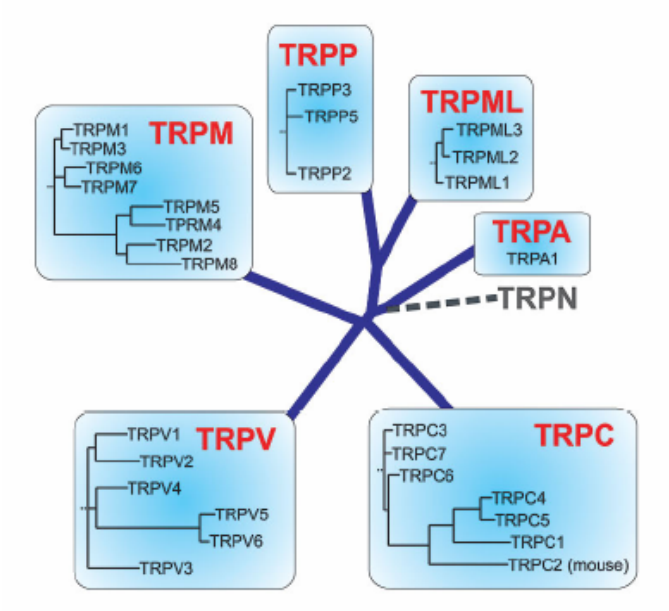


Figure 7. ***TRP channel superfamily.*** Phylogenetic relationships between members of the human TRP-channel superfamily. The multiple-aligment Phylogenetic tree illustrates the relation between the different TRP subfamilies. Note that TRPC2 is a pseudogene in primates and that TRPN channels have not been identified in mammals. Obtained from (Voets et al., 2005).

5.2. Osmo- and mechanosensing TRPs

Many cells are subjected to both static and dynamic physical forces in the body. These forces are exerted at both the macroscopic (as the gravity) and microscopic level. At the microscopic level, cell shape is determined by the dynamic balance between contractile forces of microfilaments and the compression-resistance of microtubules inside the cells (Watson, 1991). Then, stretch-activated ion channels have been identified in many cell types including epithelial cells, oocytes, fibroblasts, erythrocytes, aortic endothelium, heart cells, kidney cells, skeletal muscle cells, smooth muscle cells, and yeast (Morris, 1990). The selectivity of these channels in mammalian tissues forms two

groups: (a) those relatively nonselective for mono- and divalent cations and (b) those that have prioritized selectivity for specific ions (Watson, 1991).

We may distinguish between mechanically gated channels that act as force sensors themselves and mechanically sensitive channels that are activated by second messengers downstream of the true sensors (Christensen and Corey, 2007).

The first evidence for an involvement of TRP channels in mechanosensation came from mutations in the nematode *Caenorhabditis elegans osm-9* gene, which encodes a TRPV-like channel. Worms with mutations in this channel eliminate sensitivity to nose touch, to hyperosmolar solutions and to the attractive odorant diacetyl (Colbert et al., 1997). Interestingly, when TRPV4 is expressed in the place of OSM-9 it can rescue the sensitivity to nose touch and hyperosmolarity (Liedtke et al., 2003).

5.2.1. Mechanisms of osmo- and mechanosensation

Membrane transport proteins appear to translate mechanical forces and/or changes in osmolarity into channel opening by tree fundamental mechanisms: **(a)** Changing the forces acting within the lipid membrane causes conformational changes and gating embedded channel protein due to variations in tension in the interface between the lipid membrane and transmembrane segments of the channel (see figure 8.a). Forces may be caused by mechanical deformation of changes in lipid membrane composition. Convincing structural evidence of this mechanism has been presented for MscL, a large-conductance mechanosensitive cation channel ubiquitously expressed in bacteria (Perozo et al., 2002). **(b)** Mechanical transduction to the channel through its cytosolic tails, which may be connected to cytoskeletal elements (see figure 8.b). For example, reorganization of the cortical cytoskeleton, as cell volume perturbations elicit substantial rearrangements of the actin-based cytoskeleton (Pedersen et al., 2001). **(c)** Mechanical or osmotic stress may trigger intracellular signalling cascades. They may involve, for example, lipid metabolism or changes in protein phosphorylation/ dephosphorylation events, which may affect channel gating and/or plasma membrane insertion/retrival (see figure 8). In the case of TRPV4 gating, PLA_2 is activated by cell swelling, leading to breakdown of phospholipids into arachidonic acid and its derivate metabolites, some of which are the final, direct activators of TRPV4; PLA_2 it is conversely inhibited by cell shrinkage, leading to reduced AA release (Basavappa et al., 1998). Moreover, hypotonic cell swelling has been shown to decrease cellular PIP_2 levels, whereas hypertonic cell shrinkage to increase PIP_2 levels (Pedersen et al., 2001;Hilgemann et al., 2001).

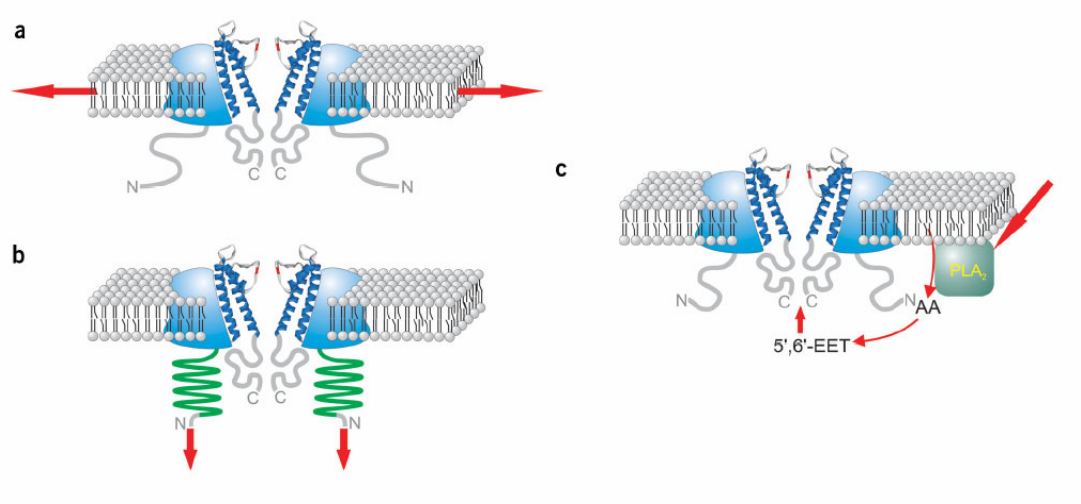


Figure 8. ***Main regulatory mechanisms of mechano- or osmosensing TRP channels.*** **(a)** Channel gating by changes in bilayer tension. **(b)** Channel gating by tethering to cytoskeletal elements, which are reorganized by mechanical or osmotic stress. **(c)** Channel gating by intracellular signalling cascades. In this exemple, for the TRPV4 osmo- and mechano-gating, secondary messenger (5'-6'-EET) is produced by an enzyme, P-450 epoxygenase, acting as a mechano-, osmo-, or volume sensor. Obtained from (Voets et al., 2005).

5.2.2. Osmo- and mechanosensitive TRP players

TRPC1 is a stretch activated channel which is directly activated by the same mechanism than bacterial channel MscL. It participates in the generation of stretch-activated currents in Xenopus laevis oocytes (Maroto et al., 2005). TRPY1, the only member of the TRP family in yeast, has been implicated in the ability of yeast vacuoles to respond to hyperosmotic stimuli, by the same direct membrane-stretch mechanism (Zhou et al., 2003).

TRPC3 and TRPC6, along with TRPV2, TRPM4 and TRPM7 are implicated in mediating myogenic tone. Their mechanism is not clear, seems that TRPC3, TRPV2 and TRPM4 are activated via G protein-coupled receptors. TRPM7 expression in plasma membrane is increased after stretching and like TRPC6 is activated directly by the stimulus. Both TRPC6 and TRPM7 act as stretch sensors without the involvement of secondary messengers (Christensen and Corey, 2007).

Regarding the hyposmotic stimulus, this is not well defined because contains both a chemical stimulus (ionic strength is decreased), and a mechanical stimulus, in that the cell swells, leading to both a stretch of the cell's cytoskeleton and an increase in membrane surface tension (Christensen and Corey, 2007). TRPV4 together with TRPM3 channel mediate calcium entry in cells upon extracellular application of hypotonic solutions and are involved in renal volume homeostasis (Grimm et al., 2003;Strotmann et al., 2000). The complementary distribution in water-permeable and water-impermeable epithelials as well as the differential activation by sphingolipids and eicosanoids suggests that the channel proteins are integrated in different cellular signalling cascades (Harteneck and Schultz, 2007). TRPV4 has also been implicated in a wide variety of mechanosensory processes including changes in mucus viscosity (Andrade et al., 2005), or in the systemic response to changes in the osmolarity of the body (Liedtke and Friedman, 2003;Mizuno et al., 2003). TRPV1 has also been involved in the hypothalamic osmosensitive response to changes in osmolarity (Ciura and Bourque, 2006) and controlling the response of bladder urothelium to strech. TRPV1$^{-/-}$ mice have defects in bladder voiding (Birder et al., 2002), a process that is mediated by TRPV1 located in urothelial cells (Birder et al., 2001).

Members of the TRPP subfamily are also thought to mediate mechanosensation in the primary cilium kidney epithelial cells in the nephron (Praetorius and Spring, 2005). When the primary cilia of the kidney cells are experimentally displaced by flow stimulus, intracellular calcium levels rise. Mutations in two TRPP channels, TRPP1 and TRPP2, also known as PKD1 and PKD2, cause polycystic kidney disease in humans. When these genes are abolished in mice, the calcium response in kidney is abolished (Nauli et al., 2003).

TRPA1 mRNA expression is restricted to the inner ear and certain peripheral sensory ganglia: dorsal root, trigeminal and nodose. TRPA1 mediates the mechanotransduction for nociception in peripheral sensory ganglia acting as pain-receptor channel (Nagata et al., 2005). TRPA1 is localized at the tips of steriocilia and is pulled open by filamentous tip links that connect adjacent steriocilia (Di et al., 2002). This channel contains N-terminal domains with large number of ankirin repeats for binding other proteins, and they are thought to be directly gated, probably by structural proteins rather than lipids (Lin and Corey, 2005).

5.3. Thermo-TRPs

The abilities to sense a range of environmental and internal temperatures are required for survival, both for maintenance of homeostasis and for avoidance of tissue-damaging noxious temperatures. Mammals can discriminate temperatures ranging from extreme cold (-10ºC) to extreme heat (about 60ºC) (Lumpkin and Caterina, 2007). Temperatures over about 43ºC and below about 15ºC evoke not only a thermal sensation, but also feeling of pain (LaMotte and Campbell, 1978). Thermo-TRPs, a subset of TRP superfamily of ion channels, which are expressed in sensory nerve endings and in skin, respond to distinct thermal thresholds and represents the first illustration of a sensory ion channel gated by a physical stimulus (Dhaka et al., 2006).

Six thermo-TRP ion channels detect the range of temperatures, four are heat-activated (TRPV1-4) and two cold-activated channels (TRPM8 and TRPA1). Each thermo-TRP has unique characteristics, highlighted by distinct temperature thresholds of activation (see figure 9). Thermo-TRP channels present sensitization or desensitization to repeated thermal stimuli (i.e., TRPV3 and TRPV2 are sensitized to repeated stimuli, whereas TRPV4 and TRPA1 are desensitized) and the ability to be modulated by distinct signalling mechanisms. Four members of the TRPV (vanilloid) subfamily can be activated by warm (32ºC-42ºC) to hot temperatures (>43ºC). TRPV1 (capsaicin receptor) is regulated by extracellular protons (pH<6) and has a gating threshold of ~43ºC (Tominaga and Caterina, 2004). TRPV1 and TRPV2 (~52ºC) have properties consistent with noxious heat sensors detecting temperatures above 43ºC (Caterina et al., 1999). Both TRPV3 (~33ºC) and TRPV4 (~25ºC-38ºC) respond to warm temperatures (Benham et al., 2003). The precise threshold for TRPV4 activation depends on the cellular context and environmental history of the channel (Nilius et al., 2004). TRPM8 and TRPA1 mediate cold and noxious cold sensation, respectively. TRPM8, the cold and menthol-sensitive receptor, is activated directly by gentle cooling (~22-30ºC) and depolarizes sensory neurons (McKemy, 2005).

These thermo-TRPs are receptors for naturally occurring sensory compounds (Peier et al., 2002;McKemy et al., 2002). Most oral sensory qualities distinct from olfation and taste (chemesthesis) are now attributed to thermo-TRP activation (Voets et al., 2005). The list of such compounds includes capsaicin (active component of hot chilli peppers) for its TRPV1 receptor, camphor has both TRPV1 and TRPV3 receptors, the synthetic icilin, menthol (derived from mint) and eucalyptol have the TRPM8 receptor and TRPA1 is the receptor of isothiocyanate compounds (components of Japanese wasabi and mustard oil),

allicin (garlic component), and cinnamaldehyde (puntgent component of cimmamon) (Dhaka et al., 2006;Bandell et al., 2007).

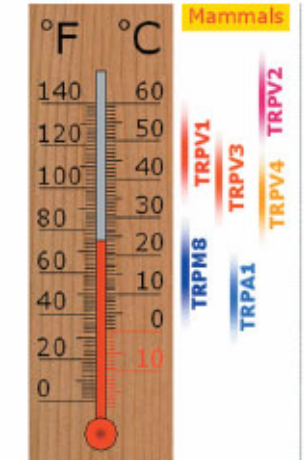


Figure 9. ***Activation range of human thermo TRPs.*** Indicative temperature range for activation of each thermo TRPs expressed in heterologous systems. Note that TRPM8 and TRPA1 are activated upon cooling, whereas all other indicated channels are heat activated. Obtained from (Voets et al., 2005).

5.4. TRP channel expression

As the TRP channels are a superfamily of ion channels that play a wide diversity of physiological functions, they are present in many tissues and almost all cell types (Clapham, 2003;Montell et al., 2002).

Several members of the TRP family have been found in epithelial tissues: airways (TRPC1, TRPC4, TRPC6 (Corteling et al., 2004); TRPV2, TRPV4 (Kowase et al., 2002;Arniges et al., 2004)), intestine and pancreas (TRPV5, TRPV6 (Nijenhuis et al., 2003)), female reproductive tract (TRPV4 and TRPP1, TRPP2 (Andrade et al., 2005;Teilmann et al., 2005)), kidney (TRPM3, TRPM6, TRPM7 (Grimm et al., 2003;Schlingmann et al., 2002); TRPV1, TRPV4, TRPV5, TRPV6 (Hoenderop et al., 2003a;Tian et al., 2005;Cohen, 2005b); TRPP1, TRPP2 (Nauli and Zhou, 2004)), prostate (TRPM8 (Tsavaler et al., 2001);TRPV5, TRPV6 (Nijenhuis et al., 2003)), inner ear hair cells (TRPA1 (Corey et al., 2004); TRPV4 (Liedtke et al., 2000); TRPN1 (Shin et al., 2005b)), cornea (TRPC4 (Yang et al., 2005)), and epidermal ciliated cells (TRPN1 (Shin et al., 2005a)).

TRPV4 have been found in many tissues with diverse physiological functions. TRPV4 messenger RNA (mRNA) has been detected in heart, endothelium, brain, liver, placenta, lung, trachea, salivary gland, testis, fat, skin, kidney and spleen (Nilius et al., 2004;Liedtke et al., 2000;Strotmann et al., 2000;Wissenbach et al., 2000;Delany et al.,

2001). In the airway, TRPV4 is not only expressed in the epithelium, but also in smooth muscle cells (Jia et al., 2004). Similarly, in blood vesels, TRPV4 is found in endothelium and in some vascular smooth muscle cells, such as those of the cerebral arteries (Earley et al., 2005). In the brain, in situ hybridization shows expression of TRPV4 mRNA in neurons of circumventricular nuclei of the hypothalamus and in ependymal cells of the choroid plexus of the lateral and fourth, but not third, ventricles, and in scattered neurons in other regions of the brain (Liedtke et al., 2000;Guler et al., 2002b;Liedtke and Friedman, 2003). Consistent with a possible role in sensory transduction, TRPV4 mRNA is present in large sensory neurons of the trigeminal ganglion and dorsal root ganglia (Delany et al., 2001;Alessandri-Haber et al., 2003). TRPV4 is also expressed in kerationcytes where it play a role in sensory transduction (Guler et al., 2002a;Chung et al., 2004). Strong expression is detected in the distal tubule of kidney epithelia, concretely is restricted to nephron segments with a constitutively or conditionally low water permeability (Tian et al., 2004).

5.5. TRPV4 channel

The TRPV subfamily of cation channels consist at least six mammalian channels homologous to the vaniloid receptor 1 (TRPV1). TRPV channels are activated by a variety of signals, including chemical and thermal stimuli, cell swelling, low intracellular Ca^{2+}, and endogenous or synthetic ligands (Ramsey et al., 2006). TRPV subfamily can be subdivided in two groups: TRPV1 to TRPV4 which display moderate Ca^{2+}-selectivity (relative permeability Ca^{2+}/Na^{+} is between 1-10), non-selective cation channels with steep temperature dependence. The second grup is formed by TRPV5 and TRPV6, which are the most highly Ca^{2+}-selective TRP superfamily channels (relative permeability Ca^{2+}/Na^{+} is above 100) with little temperature sensitivity (Nilius et al., 2004;Montell, 2005).

TRPV4 was given a number of names: Osm-9-like TRP channel 4, OTRPC4 (Strotmann et al., 2000); vanilloid receptor-related osmotically activated channel, VR-OAC (Liedtke et al., 2000); TRP12 (Wissenbach et al., 2000); and vanilloid receptor-like channel 2, VRL-2, (Delany et al., 2001). The human TRPV4 gene is localized on chromosome 12q23-q24.1 and has 15 exons. These exons code for a full-length protein with 871 amino acids.

The putative transmembrane topology is similar to that of other TRP channels with intracellular N- and C-terminal regions of variable length, six transmembrane-spanning

helices (S1-S6), and pore-forming loop between S5 and S6. TRPV4 has at least three ankyrin domains in its cytosolic N terminus, but multiple ankyrin domains appear in TRPC, TRPA, TRPN, and TRPV subfamilies (Montell, 2005). Functional TRP channels are supposed to result following the assembly of four TRP subunits, including TRPV4. Ankyrin, transmembrane, and C-tail domains have been identified as important determinants of the oligomerization process (Schaefer, 2005;Xu et al., 1997;Engelke et al., 2002;Erler et al., 2004;Arniges et al., 2006;Hellwig et al., 2005;Garcia-Sanz et al., 2004). Five splice variants of TRPV4 have been described (Arniges et al., 2006). Only two of these splice variants, TRPV4-A (the full-length and most extensively studied form of TRPV4) and TRPV4-D, are correctly processed, oligomerize in the ER, and are targeted to the plasma membrane as fully functional TRPV4 channels. The other splice variants TRPV4-B, TRPV4-C, and TRPV4-E lacking parts of ankyrin repeats are retained in the ER (Arniges et al., 2006). Mutation of Asn^{651} to Gln prevented a glycosylation site and increased both cell surface expression of TRPV4 and its functional responses (Xu et al., 2006).

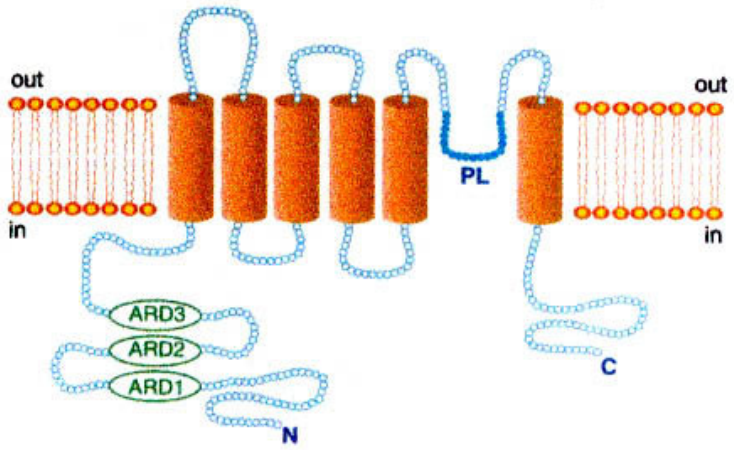


Figure 10. ***TRPV topology.*** The subfamily has six transmembrane-spanning helices (S1-S6), both N- and C-terminal regions of variable length are intracellular, and pore-forming loop between S5 and S6. TRPV4 has at least three ankyrin domains (ARD) in its cytosolic N terminus, but multiple ankyrin domains appear in TRPC, TRPA, TRPN, and TRPV subfamilies. Reproduced from (Liedtke et al., 2000).

5.5.1. Ion channel properties

Heterologously expressed and native TRPV4 forms Ca^{2+}-permeable, non-selective cation channels. Channel currents reverse at potentials just positive to 0mV. The current-voltage (I-V) relation displays outward rectification. Currents through TRPV4 also do not remain low at negative potentials, but increase with stronger membrane hyperpolarization. The outwardly rectifying shape of the I-V relation in physiological solutions results from a block by extracellular Ca^{2+} ions. Reducing extracellular [Ca^{2+}] leads to a progressive loss of rectification, until the I-V relation becomes linear in Ca^{2+} free media (Voets et al., 2002). The block by Ca^{2+} results from binding to negatively charged aminoacids (Asp^{672} and Asp^{682}) in the pore loop. These residues are also involved in determining the Ca^{2+} permeability of the channel (Voets et al., 2002). Single channel conductance is larger for outward currents (88-105 pS) than for inward currents (30-61 pS) (Strotmann et al., 2000;Watanabe et al., 2002b).

TRPV4 is cation-selective but does not discriminate well between cations. The relative permeabilities for monovalent cations are $K^+ > Cs^+ > Na^+ > Li^+$, and for divalent cations, the relative permeabilities are $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$. The relative permeability Ca^{2+}:Na^+ is between 6-10 (Watanabe et al., 2002a;Voets et al., 2002;Strotmann et al., 2000).

5.5.2. Modes of activation

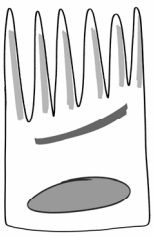
TRPV4 can be activated by a wide range of signals, include diverse physical and chemical stimuli, implicating multiple mechanisms of regulation and function. Its functional characterization has been greatly advanced by the discovery that the synthetic TRPV4 agonist, 4α-phorbol 12,13-didecanoate (4α-PDD), which acts as a robust and direct channel activator. 4α-PDD is specific for TRPV4 because it does not appear to activate other ion channels and therefore it is an interesting tool to activate TRPV4 and study TRPV4-mediating currents (Plant and Strotmann, 2007). The 4α-PDD is a PKC-inactive phorbol ester, that activates TRPV4 via its direct binding to the transmembrane domains of the channel (Watanabe et al., 2002a;Vriens et al., 2007). The PKC-activating 4α-phorbol esters, like the classic phorbol 12-myristate 13-acetate (PMA), were originally considered only to activate TRPV4 weakly at room temperature. If temperature is elevated to physiological range, these phorbol esters activates TRPV4 via PKC-independent and PKC-dependent pathways (Gao et al., 2003;Xu et al., 2003a;Watanabe et al., 2002a),

suggesting TRPV4 activation secondary to PKC phosphorylation. A compound extracted from the Chinese plant *Andrographis paniculata*, identified as bisandrographolide A, potently activate TRPV4 channels (Smith et al., 2006).

Endocannabinoid anandamide (AEA) and its lipoxygenase metabolite arachidonic acid (AA) cause a robust TRPV4 activation. This provided the first evidence for endogenous agonists of the channel (Watanabe et al., 2003b). TRPV4 is activated by AEA and AA in an indirect way involving the cytochrome P450 epoxygenase-dependent formation of epoxyeicosatrienoic acids. Application of 5',6'-epoxyeicosatrienoic acid (5',6'-EET) at submicromolar concentrations activates TRPV4 directly (Watanabe et al., 2003b).

TRPV4 can be activated by exposing cells to hypotonicity, implying that this channel might be a cellular osmosensor (Liedtke et al., 2000;Nilius et al., 2001;Strotmann et al., 2000;Wissenbach et al., 2000). TRPV4 is highly sensitive to changes in extracellular osmolarity in the physiological range. TRPV4 is activated by mechanical stimuli exposing cells to flow, shear stress and high viscous loads in renal tubular epithelial, vascular endothelial, and ciliated cells, respectively (O'Neil and Heller, 2005;Hartmannsgruber et al., 2007a;Andrade et al., 2005). Hypotonic activation of PLA_2 leads to AA production and therefore, via P450 epoxygenase, to the generation of EET and TRPV4 activation (Vriens et al., 2005;Watanabe et al., 2003b).

Like many other Ca^{2+}-permeable ion channels, the activity of TRPV4 is strongly regulated by Ca^{2+}. Most Ca^{2+}-permeable channels show some feedback regulation by Ca^{2+} to prevent deleteriously large increases in intracellular [Ca^{2+}] or to shape the time course of channel activity. TRPV4, half maximal inactivation (IC_{50}) by intracellular [Ca^{2+}] increase has been described around 600nM (Watanabe et al., 2003a). Ca^{2+} is involved both in the decaying phase and the activation phase of TRPV4 (Plant and Strotmann, 2007). Irrespective of the primary activator, hypotonic medium, mechanical stimulus, lipophilic ligands, or heat, Ca^{2+} entering through TRPV4 binds to CaM, and Ca^{2+}-CaM interacts with the C-terminal binding domain, resulting in positive feedback activation of TRPV4, increasing both the speed and amplitude of the response (Strotmann et al., 2003). On the other hand, Ca^{2+} limits the entry of more Ca^{2+} through TRPV4 by feedback inhibition, involved in the current decay (Watanabe et al., 2003a).



III. OBJECTIVES

TRPV4 is a nonselective cationic channel that plays an important role in the transduction of mechanical and osmotic stimuli into cell calcium responses. Its participation in the adaptation of ciliary beat frequency (CBF) to the changing viscosity of the medium has also recently established, placing TRPV4 as an important molecule in the regulation of the mucociliary transport in ciliated epithelia.

The main objectives of this thesis work are:

1. Study of the expression and subcellular localization of TRPV4 in ciliated epithelia.

2. Characterization of the signaling pathways participating in the TRPV4 gating in response to mechanical and osmotic stimuli.

3. Characterization of the role of TRPV4 in the generation of Ca^{2+} signals in ciliated epithelia.

4. Study of the coupling of TRPV4 to the regulation of CBF.

5. Study of the role of TRPV4 in the cell's response to ATP.

IV. RESULTS

1. ARTICLE A

"IP_3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid"

Jacqueline Fernandes*, Ivan M. Lorenzo*, Yaniré N. Andrade, Anna Garcia-Elias, Selma A. Serra, José M. Fernández-Fernández, and Miguel A. Valverde

* J. Fernandes and I.M. Lorenzo contributed equally to this paper

Laboratori de Fisiologia Molecular i Canalopaties, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, PRBB, Barcelona, Spain

Fernandes J, Lorenzo IM, Andrade YN, Garcia-Elias A, Serra SA,Fernández-Fernández JM, Valverde MA.
IP3 sensitizes TRPV4 channel to the mechano-and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid.
J Cell Biol. 2008Apr 7;181(1):143-55. Epub 2008 Mar 31.

2. ARTICLE B

"TRPV4 channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airways epithelial cells"

Ivan M. Lorenzo*, Wolfgang Liedtke†, Michael J. Sanderson‡ and Miguel A. Valverde*

* Laboratori de Fisiologia Molecular i Canalopaties, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, PRBB, Barcelona, Spain

† Center for Translational Neuroscience, Duke University Medical Center, Durham, North Carolina, USA

‡ Department of Physiology University of Massachusetts Medical School Worcester, MA, USA

BIOLOGICAL SCIENCES: Physiology (PNAS 2008 in press)

TRPV4 CHANNEL PARTICIPATES IN RECEPTOR-OPERATED CALCIUM ENTRY AND CILIARY BEAT FREQUENCY REGULATION IN MOUSE AIRWAYS EPITHELIAL CELLS.

Ivan M. Lorenzo*, Wolfgang Liedtke†, Michael J. Sanderson‡ and Miguel A. Valverde* §

*Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, PRBB, C/ Dr. Aiguader 88, Barcelona 08003, Spain; † Center for Translational Neuroscience, Duke University Medical Center, Durham, North Carolina, USA and ‡ Department of Physiology University of Massachusetts Medical School Worcester, MA, USA.

§ To whom correspondence should be addressed: Prof. Miguel A. Valverde,
Laboratory of Molecular Physiology and Channelopathies,
Universitat Pompeu Fabra,
Parc de Recerca Biomèdica de Barcelona, Room 343
C/ Dr. Aiguader 88,
Barcelona 08003, Spain.
Phone: 34 93 3160853, Fax: 34 93 3160901,
Email: miguel.valverde@upf.edu

Manuscript information: 23 pages, 5 figures ; 1 supporting figure
Word and character counts: Abstract 211 words; total 39200 characters

ABSTRACT

The rate of muco-ciliary clearance in the airways is a function of ciliary beat frequency (CBF) and this, in turn, is increased by increases in intracellular calcium. The TRPV4 cation channel mediates Ca^{2+} influx in response to mechanical and osmotic stimuli in ciliated epithelia. With the use of a TRPV4-deficient mouse we now show that TRPV4 is involved in the airways' response to physiologically relevant physical and chemical stimuli. Ciliary TRPV4 expression in tracheal epithelial cells was confirmed with immunofluorescence in $TRPV4^{+/+}$ mice. Ciliated tracheal cells from $TRPV4^{-/-}$ mice showed no increases in intracellular Ca^{2+} and CBF in response to the synthetic activator 4α-phorbol 12,13-didecanoate (4αPDD) and reduced responses to mild temperature, another TRPV4-activating stimulus. Autoregulation of CBF in response to high viscosity solutions is preserved in $TRPV4^{-/-}$ despite a reduced Ca^{2+} signal. More interestingly, TRPV4 contributed to ATP-induced increase in CBF providing a pathway for receptor-operated Ca^{2+} entry, not store-operated Ca^{2+} entry, the former mechanism lost in $TRPV4^{-/-}$ cells. Collectively, these results suggest that TRPV4, is predominantly located in the cilia of tracheal epithelial cells and plays a key role in the transduction of physical and chemical stimuli into a Ca^{2+} signal that regulates CBF and mucociliary transport. Moreover, these studies implicate the participation of TRPV4 in receptor-operated Ca^{2+} entry.

INTRODUCTION

In mammalian airways, ciliated and mucus secreting epithelial cells form a structural and functional unit functioning as conveyor belt system for particle transport. In this analogy, cilia provide the power while the mucus serves as the viscoelastic belt (Satir and Sleigh, 1990). A critical factor regulating the velocity of mucociliary transport is the ciliary beat frequency (CBF), a mechanism in which cytosolic Ca^{2+} plays a major role (Satir and Sleigh, 1990;Salathe, 2006). Increases in cytosolic Ca^{2+} are associated with increases in CBF (Ma et al., 2002;Zhang and Sanderson, 2003) although the ultimate molecular mechanism explaining the CBF regulation by Ca^{2+} remains controversial (Ma et al., 2002). Both mechanical and chemical (paracrine) stimulation of epithelial ciliated cells can lead to an increase in intracellular Ca^{2+} concentration and the consequent enhancement of CBF (Salathe, 2006). ATP-mediated activation of G-protein-coupled receptors, typically P2Y receptors, is one of the strongest signals known to increase CBF (Salathe, 2006). As with many other agonists of G-protein-coupled receptors, ATP promotes both the release of Ca^{2+} from inositol trisphosphate (IP_3)-sensitive intracellular stores and Ca^{2+} entry across the plasma membrane (Zhang and Sanderson, 2003), the latter process is more evident at micromolar concentrations of ATP where a sustained Ca^{2+} influx occurs. The stimulation of Ca^{2+} influx involves signaling from the depleted stores to plasma membrane Ca^{2+} channels (store-operated calcium entry, SOCE; also known as capacitative Ca^{2+} entry) and/or through a phospholipase C (PLC)-dependent mechanism (receptor-operated calcium entry, ROCE; also known as non-capacitative Ca^{2+} entry) (Putney, Jr., 2007).

The molecular identity of the SOCE pathway has just started to emerge due to the discovery of two key molecules, the Ca^{2+} sensor within the depleted stores and the plasma membrane store-operated channel, the STIM and ORAI proteins, respectively (reviewed by (Lewis, 2007;Putney, Jr., 2007)). However, the role for the family of transient receptor potential (TRP) cationic channels in the SOCE mechanism remains unknown (Lopez et al., 2006;Huang et al., 2006;Liao et al., 2008).

Less clear is the molecular identity of the ROCE pathway. There is a fundamental difference between ROCE and SOCE. While the latter depends on the presence of a Ca^{2+} sensor within the endoplasmic reticulum (ER), ROCE is independent of the Ca^{2+} content of the ER but involves one or several of the signaling molecules resulting from the

stimulation of PLC activity, such as diacylglycerol, IP_3 or arachidonic acid (Putney, Jr., 2007). Different TRPC channels have been shown to respond to these downstream molecules (Trebak et al., 2007), thereby implicating them in the ROCE mechanism. Outside the TRPC subfamily of channels, members of the melastatin (TRPM) (Zhang et al., 2005) and vanilloid (TRPV) subfamilies (Chuang et al., 2001;Lukacs et al., 2007;Lee et al., 2005) appear to be regulated by phosphatidylinositides, key molecules in the PLC signaling pathway, although none of these TRP channels have been formally implicated in ROCE.

The TRPV4 cation channel, a member of the TRP vanilloid subfamily, responds to a range of stimuli, including osmotic cell swelling, mechanical stress, temperature, endogenous arachidonic acid metabolites as well as phorbol esters (Nilius et al., 2004;Xu et al., 2003). As a result, 4α-phorbol 12,13-didecanoate (4αPDD) has become a valuable pharmacological tool to functionally test TRPV4 activity, as 4αPDD interacts directly with transmembrane domains 3 and 4 of TRPV4 (Vriens et al., 2007). TRPV4 can be also sensitized by co-application of different stimuli (Gao et al., 2003;Alessandri-Haber et al., 2006;Grant et al., 2007) or participation of different cell signaling pathways (Fernandes et al., 2008). TRPV4 messenger and protein have been identified in both native ciliated epithelial cells of oviducts (Andrade et al., 2005;Teilmann et al., 2005;Fernandes et al., 2008) and in cell lines derived from human ciliated airways cells (Arniges et al., 2004). In these epithelial cells, TRPV4 channel plays a key role in cell volume homeostasis, by activating Ca^{2+}-dependent K^+ channels (Fernandez-Fernandez et al., 2002;Vazquez et al., 2001), and in the regulation of CBF by providing a Ca^{2+} entry pathway in response to changes in fluid viscosity or tonicity (Andrade et al., 2005;Fernandes et al., 2008). TRPV4 spliced variants, some of which do not oligomerise and are retained intracellularly, have also been found in airways epithelial cell lines (Arniges et al., 2006) (for detailed review on TRP splicing see (Vazquez and Valverde, 2006)).

In this study, we have evaluated the coupling of TRPV4 channel activity to the regulation of CBF in tracheal ciliated cells by using TRPV4-KO mice. We report that TRPV4-deficient ($TRPV4^{-/-}$) mice dispay a significant reduction in both Ca^{2+} entry and CBF activation in response to different stimuli that can activate TRPV4. Furthermore, we have identified a novel role for TRPV4 in ATP-dependent ROCE mechanism and the subsequent increase in CBF.

RESULTS.

Expression and localization of TRPV4 in mouse ciliated tracheal cells.

Figure 1 shows ciliated tracheal cells from wild-type ($TRPV4^{+/+}$) (Fig. 1A) and KO ($TRPV4^{-/-}$) mice (Fig. 1B). TRPV4 immunofluorescence (green) was clearly identified in the cilia of $TRPV4^{+/+}$ but not in the cilia of $TRPV4^{-/-}$ cells. Double staining with anti-tubulin antibody (red), to mark the cilia axoneme, confirmed the ciliary localization of TRPV4. Additional immunofluorescence images are shown in Supplementary Fig. S1. Cytoplasmic immunoreactivity spots (more pronounced than in hamster oviductal ciliated cells (Fernandes et al., 2008)) were detected in both $TRPV4^{+/+}$ and $TRPV4^{-/-}$ cells, a clear indication of their non-specificity. Molecular identification of TRPV4 was also investigated by western blot. Figure 1C shows only a double band of the expected size in $TRPV4^{+/+}$ trachea, which also suggest that the cytoplasmic immunoreactivity spots shown in Fig. 1B were TRPV4 non-specific. Similar non-specific immunoreactivity has been previously reported in kidney sections of $TRPV4^{+/+}$ and $TRPV4^{-/-}$mice probed with an antibody raised against the same C-terminal epitope of rat TRPV4 (Gevaert et al., 2007).60

Intracellular Ca^{2+} measurements were carried out to test functional expression of TRPV4 in primary cultures of tracheal explants exposed to the relatively specific TRPV4 agonist 4αPDD (10 μM). Monitoring intracellular Ca^{2+} concentration in fura-2 loaded ciliated tracheal cells showed significant increases that commenced at different times in many $TRPV4^{+/+}$ cells (Fig. 1D), but were completely absent in $TRPV4^{-/-}$ ciliated cells (Fig. 1E). A quantitative analysis of the Ca^{2+} signal measured after 10 minutes in 4αPDD is shown in Fig. 1F. In order to check whether the TRPV4-mediated Ca^{2+} signals can also be associated with the activation of the CBF, we measured CBF in $TRPV4^{+/+}$ and $TRPV4^{-/-}$ tracheal cells using high-speed digital video microscopy. Basal CBF of ciliated tracheal cells did not differ between $TRPV4^{+/+}$ (10.7±0.4 Hz; n=37) and $TRPV4^{-/-}$ (10.3±0 Hz; n=37, $P>0.05$ vs $TRPV4^{+/+}$, measured at RT) and was not affected by removal of extracellular Ca^{2+} (Fig. 2A), in accordance with previous studies suggesting that basal Ca^{2+} CBF is not directly under the influence of Ca^{2+} (Ma et al., 2002). However, $TRPV4^{+/+}$ tracheal cells, unlike their $TRPV4^{-/-}$ counterparts, increased CBF in response to 10 μM 4αPDD. Figure 2B shows the time course of the relative changes in CBF of a $TRPV4^{+/+}$

(●) and TRPV4$^{-/-}$ (O) cells exposed to 4αPDD. Mean increases in CBF are shown in Fig. 2C.

Response of ciliated tracheal cells to physical stimuli activating TRPV4.

We tested the effect of warm temperature and high viscous solutions on the generation of Ca^{2+} signals and modulation of CBF in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ ciliated tracheal cells. Switching the temperature of the bathing solution from 24ºC to 38ºC triggered a Ca^{2+} response characterised by a peak followed by a slow decline towards the baseline (Fig 3A) while TRPV4$^{-/-}$ cells showed a reduction in both the Ca^{2+} peak and the more sustained component, without significant modification of the time constant of the signal relaxation (0.95±0.12 min for TRPV4$^{+/+}$ and 1.12±0.07 min for TRPV4$^{-/-}$, P>0.05). Accordingly, TRPV4$^{-/-}$ cells exposed to warm temperatures responded with a smaller increase in CBF (Fig. 3B).

Ciliated tracheal cells from TRPV4$^{+/+}$ mice (Fig. 3C) responded to high viscous solutions (20% dextran containing solutions) with an oscillatory Ca^{2+} signal (3.9±0.6 peaks/cell/10min) in 19 out of 71 cells (26%). On the other hand, TRPV4$^{-/-}$ ciliated cells (Fig. 3D) typically presented an initial transient peak (18 out of 85 cells, 21%) with sporadic Ca^{2+} oscillations (1.4±0.1 peaks/cell/10 min; P<0.001 *vs* TRPV4$^{+/+}$). We also tested the impact of TRPV4 disruption on CBF response to high viscous loads. Following the addition of solutions containing 5% or 20% dextran CBF declined to a new stable value within 5 min (Fig. 3E). However, despite the apparent difference in the Ca^{2+} signal pattern between the two genotypes (Fig. 3C-D), no differences were detected in the maintenance of a steady-state CBF in response to either 5% (4.8 cP) or 20% (73 cP) dextran-containing solutions (Fig. 3E).

Participation of TRPV4 in the ATP-induced Ca^{2+} signal.

We have recently reported a cross-talk between the ATP-PLC-inositol trisphosphate receptor (IP_3R) pathway and TRPV4 in order to initiate and maintain the oscillatory Ca^{2+} signal triggered by mechanical and osmotic stimuli (Fernandes et al., 2008). In the present study the role of TRPV4 in the generation of ATP-mediated Ca^{2+} signals and regulation of CBF was addressed. The Ca^{2+} signal obtained following the addition of 20 μM ATP showed clear differences between the two genotypes. While ciliated TRPV4$^{+/+}$ and

TRPV4$^{-/-}$ cells showed no statistical difference in peak increases in [Ca^{2+}], the sustained component was significantly reduced (by ~30%) in the latter (Fig. 4A-B), without significant differences in the time constant of the signal relaxation (1.45±0.2 and 0.96±0.09 min for TRPV4$^{+/+}$ and TRPV4$^{-/-}$, P>0.05). TRPV4$^{-/-}$ cells also showed a diminished increase in the CBF when exposed to 20 μM ATP (Fig. 4C). Altogether, the data suggested the contribution of TRPV4 to the sustained component of the Ca^{2+} signal and its coupling to the acceleration of CBF induced by 20 μM ATP. As with many other G protein-coupled receptor agonists, the Ca^{2+} signal generated by low ATP concentrations (200 nM) usually presents a more oscillatory pattern instead of a large transient peak followed by sustained elevation (Fig. 4D). Neither the pattern (Fig. 4D) nor the amplitude of the Ca^{2+} signal (Fig. 4E) generated by 200 nM ATP was significantly different in ciliated tracheal cells from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. Accordingly, no differences in the CBF response to 200 nM ATP were detected between the two genotypes (Fig. 4F).

The sustained component of the Ca^{2+} signal recorded with 20 μM ATP can also be evaluated using a Ca^{2+}-free protocol as follows. Figure 5 shows representative Ca^{2+} traces obtained from TRPV4$^{+/+}$ (Fig. 5A) and TRPV4$^{-/-}$ (Fig 5B) ciliated tracheal cells exposed to 20 μM ATP in the absence of extracellular Ca^{2+}, followed by replacement of external Ca^{2+}. The Ca^{2+} entry component following Ca^{2+} replacement in the presence of ATP was clearly reduced in TRPV4$^{-/-}$ cells (Fig. 5 C). In order to distinguish whether TRPV4 participates in ROCE or SOCE mechanisms we use the sarcoplasmic (endoplasmic) reticulum Ca^{2+} pump inhibitor thapsigargin (TG). The ability of TG to empty the intracellular stores and the subsequent activation of SOCE (without the involvement of receptor activation) provides a well known protocol to functionally differentiate SOCE from ROCE (which is defined to require 7 transmembrane receptors, G proteins and PLC activation). The Ca^{2+} entry component following Ca^{2+} replacement after TG (1 μM) stimulation (SOCE) was not different between TRPV4$^{+/+}$ and TRPV4$^{-/-}$ cells (Fig. 5D-F). Also the pattern and amplitude of the peak Ca^{2+} signals generated by ATP and TG in Ca^{2+}-free media (reflecting intracellular Ca^{2+} release) were not significantly different between TRPV4$^{+/+}$ and TRPV4$^{-/-}$ cells (2±0.13 and 1.98±0.09 for ATP; 1.83±0.2 and 1.8±0.2 for TG, respectively; *P*>0.05).

DISCUSSION

This study presents different lines of evidence supporting the role of TRPV4 in the Ca^{2+} signaling of ciliated tracheal cells and its coupling to the regulation of CBF. Our study opens three main points for discussion: 1) the coupling of TRPV4 activity to the regulation of CBF; 2) the minor role of TRPV4 in the autoregulation of CBF in response to increased viscous load and 3) the participation of TRPV4 in ROCE mechanism.

The main task of ciliated cells is the transport of mucus and trapped particles. A primary determinant of mucus transport is the CBF, which can be regulated by different signals (Salathe, 2006), with increase in intracellular Ca^{2+} being particularly relevant (Satir and Sleigh, 1990). Although elevations of intracellular [Ca^{2+}] accelerate CBF, Ca^{2+} signals are most efficient in regulating CBF when produced at the base of the cilia (Tamm, 1994). Therefore, Ca^{2+} entry pathways designed to modulate CBF should be localized close to the base of the cilia within the apical membrane of the cell. Previously, TRPV4 has been principally localized at the base of the cilia of hamster oviduct cells (Fernandes et al., 2008), and its activation by the synthetic agonist 4αPDD increased the CBF in these cells (Andrade et al., 2005). In the present study we show TRPV4-specific immunoreactivity mostly restricted to cilia of $TRPV4^{+/+}$ tracheal epithelial cells, similar to hamster oviduct ciliated cells (Fernandes et al., 2008) and rat ciliated cholangiocytes (Gradilone et al., 2007), while the TRPV4 signal is absent in the cilia of epithelial cells obtained from $TRPV4^{-/-}$ mice.

We found no differences in the basal CBF or the CBF in the absence of extracellular Ca^{2+} between $TRPV4^{+/+}$ and $TRPV4^{-/-}$ cells, in agreement with previous studies suggesting the Ca^{2+}-independent nature of basal CBF and the Ca^{2+} dependency of the stimulated CBF (Ma et al., 2002). We have shown that $TRPV4^{-/-}$ cells, unlike $TRPV4^{+/+}$ cells and cultured human airway epithelial cells (Arniges et al., 2004;Arniges et al., 2006;Grant et al., 2007), are unresponsive to 4αPDD when measuring intracellular [Ca^{2+}]. The CBF response to 4αPDD was also abrogated in $TRPV4^{-/-}$ ciliated cells, providing evidence for the coupling of TRPV4 activity to the regulation of CBF. Interestingly, other 4α-phorbol isomers positively modulate CBF through PKC phosphorylation (Mwimbi et al., 2002). The fact that 4αPDD binds and activates TRPV4 (Vriens et al., 2007) without

the involvement of PKC (Xu et al., 2003), and that the response to 4αPDD is totally lost in different $TRPV4^{-/-}$ cells ((Gevaert et al., 2007) and this study) strongly suggests that 4αPDD effects are mainly TRPV4-mediated, although we can not completely rule out the participation of other pathways.

Both native and heterologously expressed TRPV4 channels respond to warm temperatures, in the range of 30 to 40 ºC, with transient increases in $[Ca^{2+}]$ followed by a slow decay towards the baseline (Guler et al., 2002;Watanabe et al., 2002). $TRPV4^{-/-}$ ciliated tracheal cells show a reduced Ca^{2+} signal and CBF response to changes in temperature from 24ºC to 38ºC, reinforcing the observation that TRPV4 activity can be coupled to CBF regulation. Interestingly, considering that maximal increase in CBF is reached within 33 to 43 ºC in human and bovine airways cells (Sisson et al., 2003;Mwimbi et al., 2003) our data suggests that TRPV4 may play an important role in the control of CBF under physiological temperatures.

The second point for discussion is the role of TRPV4 in the autoregulation of CBF in mouse tracheal cells. Mucus transporting ciliated cells are capable of maintaining their CBF under high viscosity conditions without reducing mucus transport, a process known as CBF autoregulation (Johnson et al., 1991) that depends on Ca^{2+} entry and subsequent activation of cilia (Andrade et al., 2005). TRPV4 has been proposed to participate in the generation of the oscillatory Ca^{2+} signal required to activate this autoregulation in hamster oviduct ciliated cells (Andrade et al., 2005;Fernandes et al., 2008). $TRPV4^{+/+}$ mouse tracheal cells also respond to high viscosity solutions with oscillatory Ca^{2+} signals, although the amplitude of the Ca^{2+} peaks is smaller and the percentage of cells responding with oscillatory signals (26%) is lower than in hamster oviduct cells (76%) (Fernandes et al., 2008). The transient Ca^{2+} signals seen in $TRPV4^{-/-}$ rarely oscillate and resemble the response observed in hamster oviduct ciliated cells in the absence of Ca^{2+} influx (Fernandes et al., 2008). Together, these data confirm the involvement of TRPV4 in the maintenance of Ca^{2+} oscillations under conditions of high viscosity. Mouse tracheal cells (this study) maintain higher CBF in the presence of dextran containing solutions than hamster oviduct cells (Andrade et al., 2005), without significant differences between $TRPV4^{+/+}$ and $TRPV4^{-/-}$ cells. The reason for the relative small contribution of TRPV4 to the autoregulation of CBF in mouse airways, compared to hamster oviduct cells, is

unknown at present, although it may be related to the smaller Ca^{2+} increases generated in the mouse tracheal cells when exposed to high viscous solutions.

The third point of our study refers to the role of TRPV4 in the ATP-triggered response of mouse tracheal ciliated cells. Airway epithelia release ATP in response to a myriad of stimuli including mechanical stimulation induced by tidal breathing (Schwiebert and Zsembery, 2003;Tarran et al., 2005;Okada et al., 2006). The concentration of ATP in airway surface liquid basal can increase from the low nanomolar range (Tarran et al., 2005) up to low micromolar values in response to certain stimuli (Okada et al., 2006). ATP-elicited cellular responses in mouse tracheal epithelia are mainly linked to $P2Y_2$ receptors with minor contribution of other receptors (Homolya et al., 1999). Low micromolar ATP induce a peak release of Ca^{2+} from IP_3-sensitive stores and a more sustained Ca^{2+} influx whereas lower concentrations of ATP typically generate oscillatory Ca^{2+} signals in ciliated cells (Zhang and Sanderson, 2003). Ultimately, the rise in intracellular [Ca^{2+}] triggers activation of CBF (Zhang and Sanderson, 2003). We have shown that targeted disruption of TRPV4 reduced (by 30%) the sustained component of the Ca^{2+} signal generated by ATP (at micromolar concentrations), which should have an important impact on mucociliary transport as small increments in CBF (16%) result in a large increases (56%) in surface liquid velocity and mucus clearance (Seybold et al., 1990). This effect appeared to be related to the participation of TRPV4 in the ROCE but not in the SOCE mechanism, as ciliated tracheal cells from $TRPV4^{-/-}$ mice showed no deficiency in the Ca^{2+} influx elicited by store depletion using TG. The reduced Ca^{2+} influx in $TRPV4^{-/-}$ is also accompanied by a diminished response of the CBF to micromolar concentrations of ATP, confirming again the coupling of TRPV4 to CBF regulation. Interestingly, the lack of TRPV4 does not affect the Ca^{2+} signal (as previously reported for $TRPV4^{-/-}$ urothelial cells (Gevaert et al., 2007)) or CBF acceleration induced by 200nM ATP. At this low ATP concentration the Ca^{2+} signal in tracheal cells is mainly oscillatory, a pattern that, at least for HEK cells, has been associated with the activity of store-operated pathways involving STIM1 and ORAI1 proteins without major contribution of TRP channels (Wedel et al., 2007). Participation of TRPV4 in ciliated tracheal cells ROCE contrasts with the impact of TRPC1 in the Ca^{2+} homeostasis of salivary gland epithelia, where it clearly affects both ROCE and SOCE (Liu et al., 2007). TRPC1, as all TRPC but TRPC7, required STIM1 for its activation by receptor stimulation (Yuan et al., 2007). We suspect that this is not the case for TRPV4 as

no involvement of TRPV4 in SOCE was found. The molecular mechanism linking TRPV4 to ROCE in ciliated tracheal cells is unknown at present, being an interesting focus for future studies. In conclusion, we provided molecular evidence for the physiological function of TRPV4 in receptor-operated calcium entry and regulation of ciliary beat frequency in mouse tracheal epithelial cells.

MATERIAL AND METHODS

Chemicals and solutions. All chemicals were purchased from Sigma-Aldrich except Dextran T-500 (500,000 Daltons; Pharmacia, Uppsala, Sweden), fura2-AM (Molecular Probes), Hank's balanced salt solution (HBSS) (Gibco) and collagen type I from rat tail (Upstate). Isotonic bathing solutions used for imaging experiments contained (in mM): 140 NaCl, 5 KCl, 1.2 CaCl2, 0.5 MgCl2, 5 glucose, 10 HEPES, pH 7.4, and 305 mosmol/l. Ca2+-free extracellular solutions were obtained by replacing CaCl2 with MgCl2 and adding 0.5mM EGTA. Ciliary beat frequency (CBF) measurements were carried out in phenol red-free HBSS supplemented with 25mM HEPES (pH=7,4). ATP and 4αPDD were dissolved in milli-Q distilled water and ethanol, respectively. The viscosity of the isotonic solution was increased by adding 5% or 20% dextran T-500, which does not change the osmolality (305 mosmol/l) of the solution.

Primary cultures of mouse tracheal cells. Adult (10-14 week-old) TRPV4 wild-type mice ($TRPV4^{+/+}$) and null ($TRPV4^{-/-}$) mice generated in a C57Bl6/J background (Liedtke and Friedman, 2003) were used for these studies. Primary cultures of airway tracheal epithelial cells were prepared as previously described (Lansley and Sanderson, 1999). Briefly, the trachea was open and cut into rings, placed onto 1 mg/ml collagen-coated coverslips, and cultured in DMEM containing 1mg/l glucose and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37% in 5% CO_2 for 3-4 days. All experiments were carried out only with beating ciliated cells. Animals were maintained and experiments were performed according to the guidelines issued by both the Institutional Ethics Committees of the Institut Municipal d'Investigació Mèdica (Universitat Pompeu Fabra) and the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Immunodetection. Epithelial cell isolation, immunofluorescence and laser confocal immunolocalization were performed as previously described (Fernandes et al., 2008). Mouse tracheas were fixed with 4% paraformaldehyde in a 3.7% (w/v) sucrose solution prior to cell isolation using 0.005% Protease XIV dissolved in Ca^{2+}-free isotonic solution. Isolated cells were attached to 1.5% gelatin-coated coverslips by spinning at 500 rpm for 3

minutes using a cytospin (Shandon; Thermo Fisher Scientific) and fixation procedure continued for 10 minutes more at room temperature (RT). Single cells were permeabilized with Tween 20 (0.05%) in PBS (15 min at RT) and non-specific interactions were blocked with PBS containing 1.5% BSA, 5% FBS and 0.05% Tween 20. Isolated epithelial cells were incubated overnight at 4ºC with the primary antibodies diluted in the same blocking solution.

The anti-TRPV4 polyclonal antibody (Arniges et al., 2006;Fernandes et al., 2008) was used at 6.4 μg/ml. A commercial anti-α-tubulin (Sigma-Aldrich) was diluted to 1:500. For immunodetection we used a goat anti-rabbit IgG Alexa-488 (Molecular Probes) and a goat anti-mouse IgG Alexa-555 (Molecular Probes) diluted 1:750 in the same solution used with the primary antibodies. Images were taken at RT with an inverted Leica SP2 confocal microscope using a Leica HCX Pl APO 63x 1.32 NA Oil Ph3 CS objective.
Proteins from $TRPV4^{+/+}$ and $TRPV4^{-/-}$ tracheas were also detected by western blot technique using the anti-TRPV4 antibody (1:100) as described previously (Arniges et al., 2004;Andrade et al., 2005;Fernandes et al., 2008).

Measurement of intracellular [Ca^{2+}]. Cytosolic Ca^{2+} signals were determined at RT (~24ºC, unless otherwise indicated) in ciliated cells loaded with 4.5 μM fura-2·AM (45 minutes) as previously described in detail (Fernandez-Fernandez et al., 2002;Arniges et al., 2004). Cytosolic [Ca^{2+}] increases are presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 nm and 380 nm, relative to the ratio measured prior to cell stimulation (ratio 340/380)**.**

Measurement of CBF. CBF of cultured ciliated cells was detected and quantified with high-speed digital imaging system as previously described (Zhang and Sanderson, 2003). In general, phase-contrast images (512 x 512 pixels) were collected at 120-135 frames s-1 (fps) with a high speed CCD (charge-coupled device) camera using a frame grabber and recording software from Video Savant (IO Industries). CBF was determined from the variation in the light intensity of the image that resulted from the repetitive motion of cilia. Video recordings of beating cilia lasting 1s to 2s were analyzed and the frequency of each ciliary beat cycle was determined from the period of each cycle of the grey-intensity waveform.

Statistics. All data were expressed as means±S.E.M. Statistical analysis was performed with Student's paired or unpaired tests, or one-way analysis of variance (ANOVA) using SigmaPlot or OriginPro software. Bonferroni's test was used for post hoc comparison of means. The criterion for a significant difference was a final value of $P<0.05$.

Acknowledgements. Work supported by Spanish Ministries of Education and Science, Health, (Red HERACLES, Fondo de Investigación Sanitaria) and the Generalitat de Catalunya. MJS was supported by grant HL71930 from NIH. We acknowledge G. Horvath for the training with cell cultures; S.A. Serra, A. Garcia-Elias and X. Sanjuan for their help with the immunodetection protocols; B Peñalba and C. Plata for their help with animal care and A. Lindy for her comments and proof-reading.

The authors declare that they have no competing financial interests.

FIGURES.

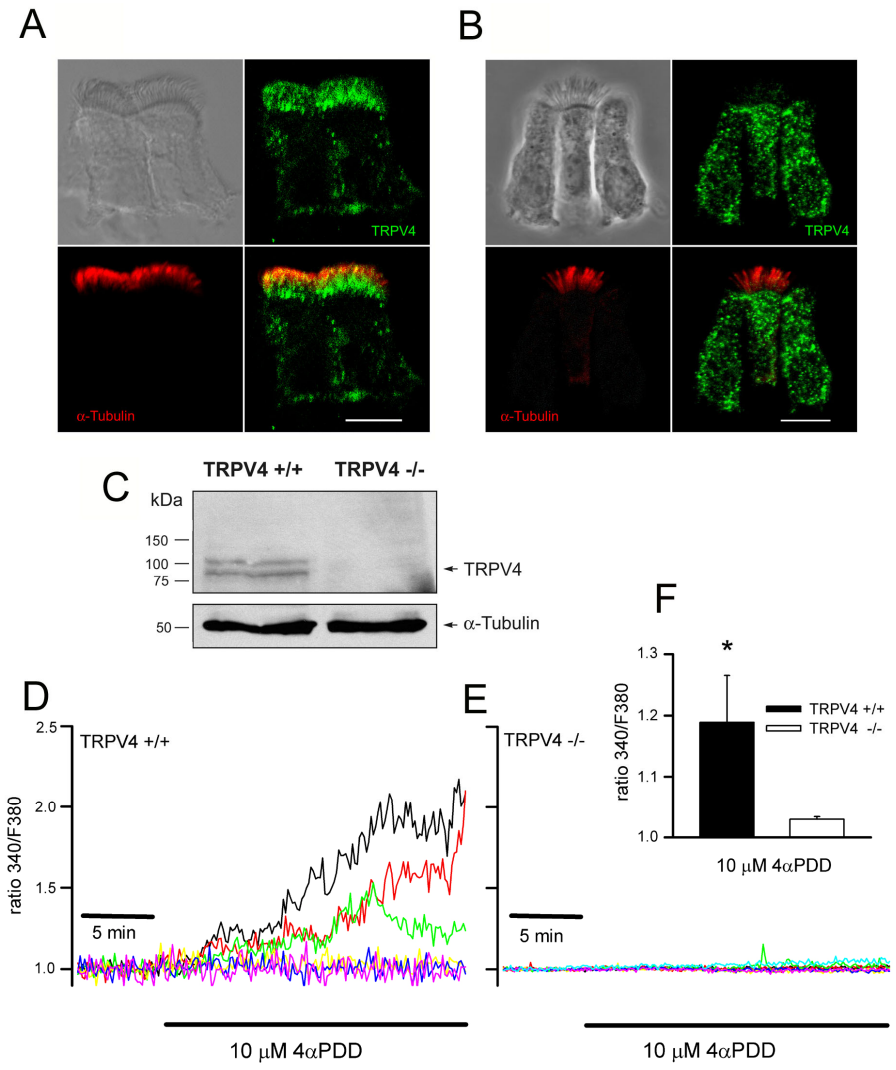


Figure 1. Detection and activity of TRPV4 channel in mouse ciliated tracheal cells. Differential interference contrast (top left), TRPV4 (green, top right), α-tubulin (red, bottom left) and merged (bottom right) images obtained from TRPV4$^{+/+}$ *(A)* and TRPV4$^{-/-}$ *(B)* cells. Co-localization of TRPV4 and tubulin appears as yellow. Scale bar: 10 μm. (C), Western blot showing a typical TRPV4 double band of the predicted molecular size (~100 kD) in TRPV4$^{+/+}$ but not in TRPV4$^{-/-}$ trachea. Tubulin was detected in both TRPV4$^{+/+}$ and in TRPV4$^{-/-}$ trachea. Representative cytosolic Ca^{2+} signals obtained from TRPV4$^{+/+}$ *(D)* and TRPV4$^{-/-}$ *(E)* ciliated tracheal cells exposed

to 10 µM 4αPDD. *(F)*, Average [Ca^{2+}] increases measured after 10 min in 4αPDD. $TRPV4^{+/+}$ (■, n=38) and $TRPV4^{-/-}$ cells (□, n=40). * *P*<0.05, Student's unpaired test.

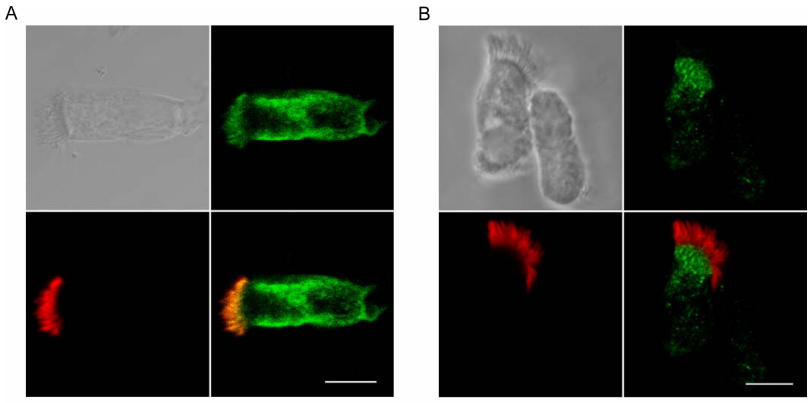


Figure S1. Additional examples of confocal immunofluorescence localization of TRPV4 (green) and tubulin (red) in isolated $TRPV4^{+/+}$ (A) and $TRPV4^{-/-}$ (B) ciliated tracheal cells.

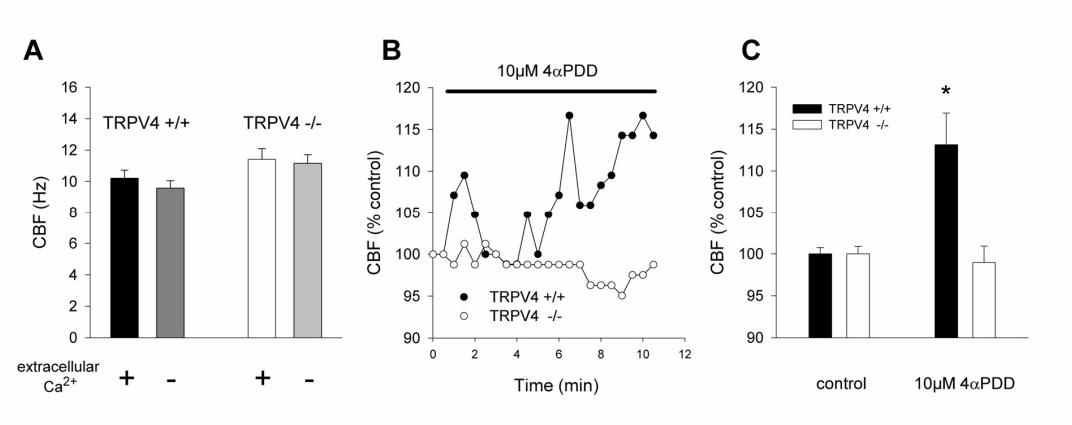


Figure 2. Basal and 4αPDD-stimulated CBF. *(A)*, Mean CBF before and following removal of extracellular Ca^{2+} in $TRPV4^{+/+}$ (■, ■) and $TRPV4^{-/-}$ (□, ■) cells (n=15 for each condition). *P*>0.05, one way ANOVA and Bonferroni *post hoc. (B)*, Representative traces of changes in CBF (% of control) with respect to time of $TRPV4^{+/+}$ (●) and $TRPV4^{-/-}$ (○) cells in response to 10 µM 4αPDD. *(C)*, Mean normalized CBF response (% control) measured after 10 min in 10 µM 4αPDD. $TRPV4^{+/+}$ (■, n=12) and $TRPV4^{-/-}$ cells (□, n=9). * *P*<0.05, one way ANOVA and Bonferroni *post hoc*.

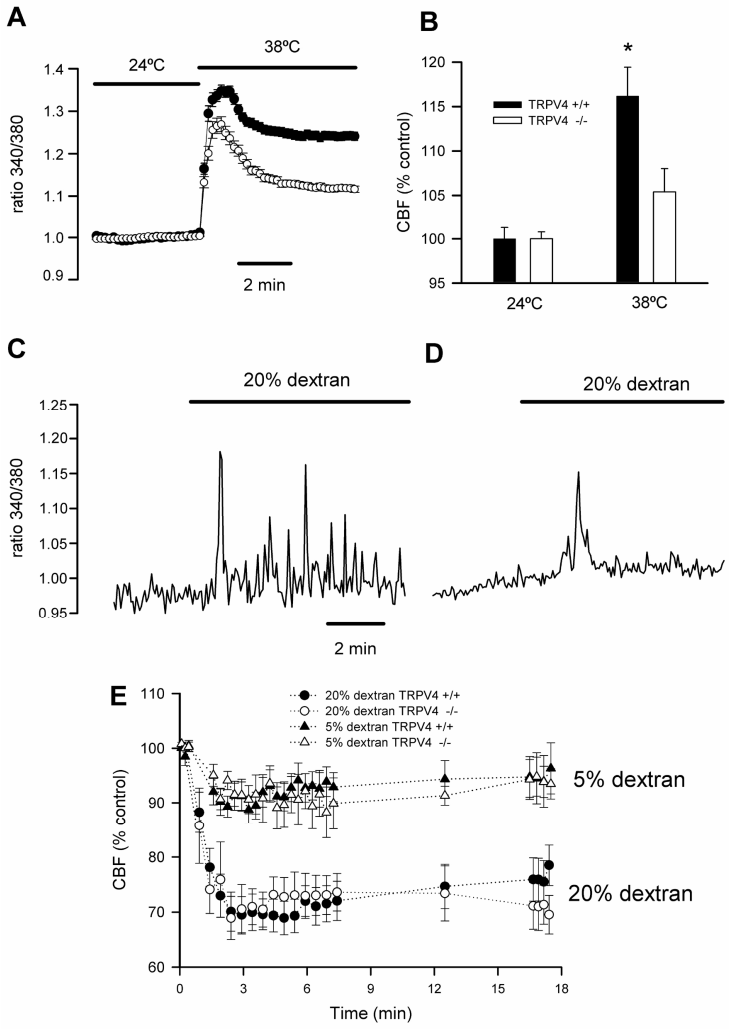


Figure 3. Effect of temperature and high viscous solutions on tracheal cells' Ca^{2+} and CBF responses. *(A)*, Mean [Ca^{2+}] increases in response to a change in the bathing solution temperature from 24 ºC to 38 ºC in TRPV4$^{+/+}$ (●, n=153) and TRPV4$^{-/-}$ cells (○, n=100). *(B)*, Mean normalized CBF response (% control) measured after 10 min at 38 ºC in TRPV4$^{+/+}$ (■, n=27) and TRPV4$^{-/-}$ cells (□, n=17). * P<0.05, for TRPV4$^{+/+}$ (38 ºC) *versus* all other conditions, one way ANOVA and Bonferroni *post hoc*. Different intracellular Ca^{2+} signals (Δ ratio 340/380) obtained from TRPV4$^{+/+}$ *(C)* and TRPV4$^{-/-}$ *(D)* a primary cultures stimulated with 20% dextran solutions. Insets show representative recordings obtained from just one cell. *(E)*, Time course of CBF changes in TRPV4$^{+/+}$ (black; n=9) and TRPV4$^{-/-}$ (white; n=7) tracheal ciliated cells exposed to 5% dextran (▲,△) and 20% dextran (●,○)solutions.

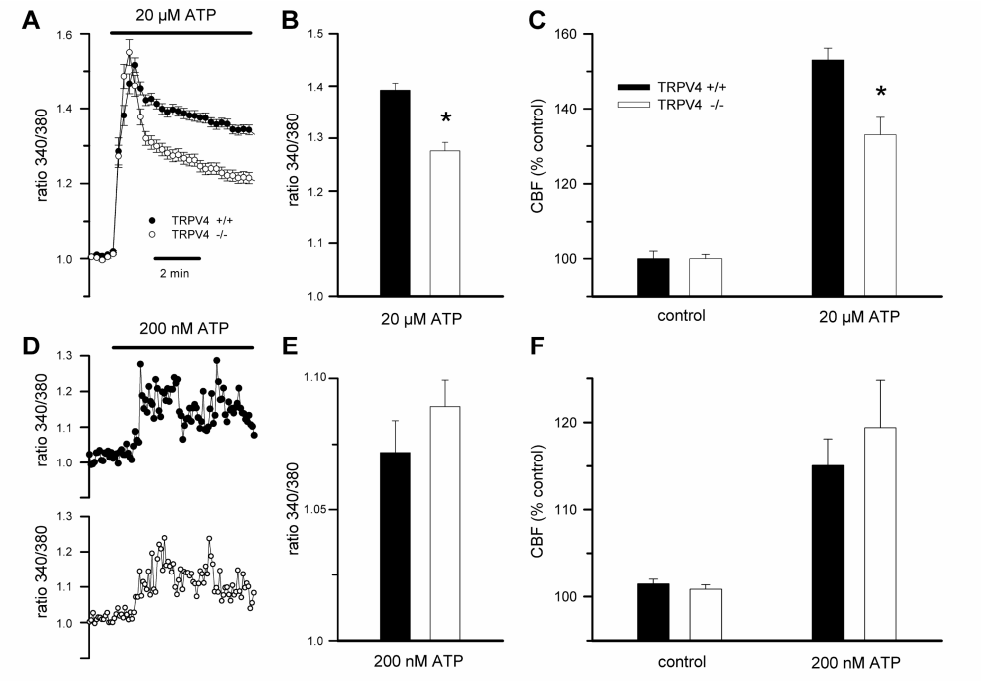


Figure 4. Ca^{2+} and CBF response to ATP in ciliated tracheal cells. *(A)*, Time course of mean Ca^{2+} responses to 20 µM ATP in $TRPV4^{+/+}$ (●) and $TRPV4^{-/-}$ (○) ciliated tracheal cells. *(B)*, Comparison of mean Ca^{2+} responses measured 3 min after the addition of 20 µM ATP. Number of cells for *A* and *B* are: $TRPV4^{+/+}$ (■), n=172 cells; and $TRPV4^{-/-}$ cells (□), n=135. * $P<0.05$, Student's unpaired test. *(C)*, Mean normalized CBF response (% control) measured after 3 min in the presence of 20 µM ATP in $TRPV4^{+/+}$ (■, n=7) and $TRPV4^{-/-}$ cells (□, n=13). * $P<0.05$, for $TRPV4^{+/+}$ *versus* $TRPV4^{-/-}$ response to ATP, one way ANOVA and Bonferroni *post hoc*. Although not marked in the figure the response of both genotypes to ATP is statistically different *versus* the control conditions ($P<0.05$), one way ANOVA and Bonferroni *post hoc. (D)*, Representative time course of Ca^{2+} responses to 200 nM ATP in $TRPV4^{+/+}$ (●) and $TRPV4^{-/-}$ (○) ciliated tracheal cells and *(E)* mean responses after 3 min in 200 nM ATP. $TRPV4^{+/+}$ (■, n=70) and $TRPV4^{-/-}$ cells (□, n=83). $P>0.05$, Student's unpaired test. (F), Mean normalized CBF response (% control) measured after 3 min in the presence of 200 nM ATP in $TRPV4^{+/+}$ (■, n=5) and $TRPV4^{-/-}$ cells (□, n=5). The response of both genotypes to 200 nM ATP is statistically different *versus* the control conditions ($P<0.05$) but not versus each other ($P>0.05$), one way ANOVA and Bonferroni *post hoc.*

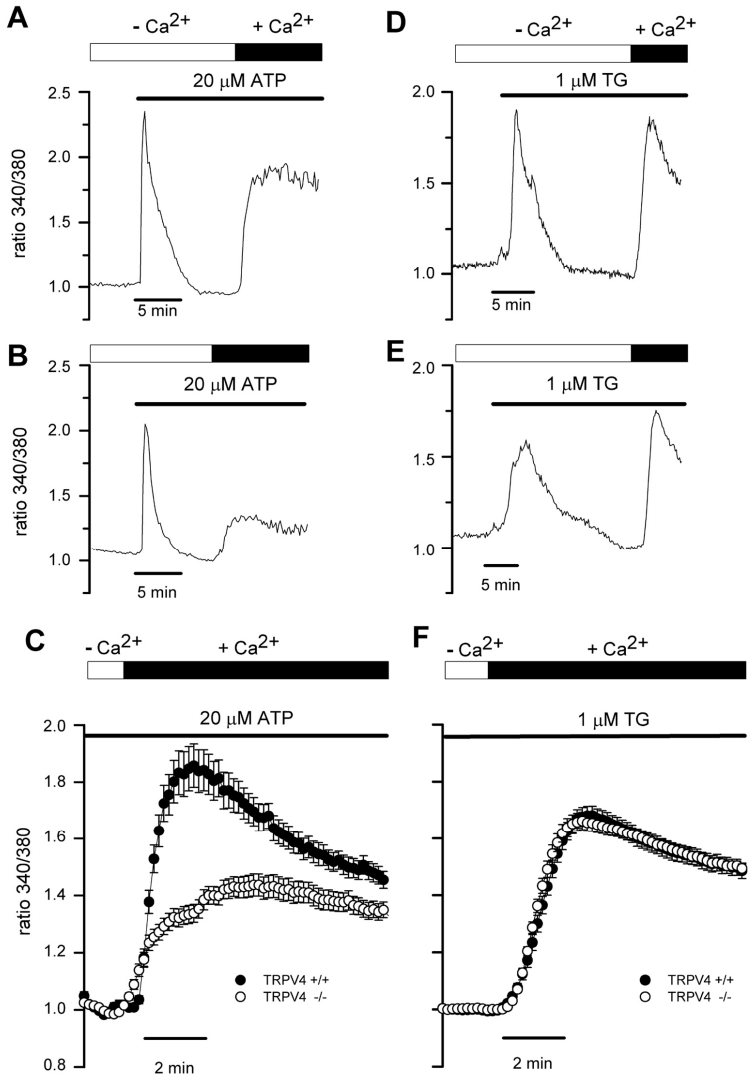


Figure 5. ATP- and thapsigargin-stimulated Ca^{2+} entry in ciliated tracheal cells. Ca^{2+} signals measured in ciliated tracheal cells stimulated with 20 μM ATP (*A-C*) or 1 μM TG (*D-F*) in Ca^{2+}-free solutions (white box, reflecting intracellular Ca^{2+} release), followed by addition of 1.2 mM Ca^{2+} to the bathing solution (black box) to detect Ca^{2+} influx. (*C* and *F*), Mean Ca^{2+} entry from $TRPV4^{+/+}$ (●, n=59) and $TRPV4^{-/-}$ cells (○, n=77).

REFERENCES

1. Alessandri-Haber, N., O.A.Dina, E.K.Joseph, D.Reichling, and J.D.Levine. 2006. A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators. *J. Neurosci.* 26:3864-3874.

2. Andrade, Y.N., J.Fernandes, E.Vazquez, J.M.Fernandez-Fernandez, M.Arniges, T.M.Sanchez, M.Villalon, and M.A.Valverde. 2005. TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *J. Cell Biol.* 168:869-874.

3. Arniges, M., J.M.Fernandez-Fernandez, N.Albrecht, M.Schaefer, and M.A.Valverde. 2006. Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking. *J. Biol. Chem.* 281:1580-1586.

4. Arniges, M., E.Vazquez, J.M.Fernandez-Fernandez, and M.A.Valverde. 2004. Swelling-activated Ca2+ entry via TRPV4 channel is defective in cystic fibrosis airway epithelia. *J. Biol. Chem.* 279:54062-54068.

5. Chuang, H.H., E.D.Prescott, H.Kong, S.Shields, S.E.Jordt, A.I.Basbaum, M.V.Chao, and D.Julius. 2001. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 411:957-962.

6. Fernandes, J., I.M.Lorenzo, Y.N.Andrade, A.Garcia-Elias, S.A.Serra, J.M.Fernandez-Fernandez, and M.A.Valverde. 2008. IP3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid. *J. Cell Biol.* 181:143-155.

7. Fernandez-Fernandez, J.M., M.Nobles, A.Currid, E.Vazquez, and M.A.Valverde. 2002. Maxi K+ channel mediates regulatory volume decrease response in a human bronchial epithelial cell line. *Am. J Physiol Cell Physiol* 283:C1705-C1714.

8. Gao, X., L.Wu, and R.G.O'Neil. 2003. Temperature-modulated diversity of TRPV4 channel gating: activation by physical stresses and phorbol ester derivatives through protein kinase C-dependent and -independent pathways. *J. Biol. Chem.* 278:27129-27137.

9. Gevaert, T., J.Vriens, A.Segal, W.Everaerts, T.Roskams, K.Talavera, G.Owsianik, W.Liedtke, D.Daelemans, I.Dewachter, L.F.Van, T.Voets, R.D.De, and B.Nilius. 2007. Deletion of the transient receptor potential cation channel TRPV4 impairs murine bladder voiding. *J. Clin. Invest* 117:3453-3462.

10. Gradilone, S.A., A.I.Masyuk, P.L.Splinter, J.M.Banales, B.Q.Huang, P.S.Tietz, T.V.Masyuk, and N.F.Larusso. 2007. Cholangiocyte cilia express TRPV4 and detect changes in luminal tonicity inducing bicarbonate secretion. *Proc. Natl. Acad. Sci. U. S. A* 104:19138-19143.

11. Grant, A.D., G.S.Cottrell, S.Amadesi, M.Trevisani, P.Nicoletti, S.Materazzi, C.Altier, N.Cenac, G.W.Zamponi, F.Bautista-Cruz, C.B.Lopez, E.K.Joseph, J.D.Levine, W.Liedtke, S.Vanner, N.Vergnolle, P.Geppetti, and N.W.Bunnett. 2007. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. *J. Physiol* 578:715-733.

12. Guler, A.D., H.Lee, T.Iida, I.Shimizu, M.Tominaga, and M.Caterina. 2002. Heat-evoked activation of the ion channel, TRPV4. *J. Neurosci.* 22:6408-6414.

13. Homolya, L., W.C.Watt, E.R.Lazarowski, B.H.Koller, and R.C.Boucher. 1999. Nucleotide-regulated calcium signaling in lung fibroblasts and epithelial cells from normal and P2Y(2) receptor (-/-) mice. *J. Biol. Chem.* 274:26454-26460.

14. Huang, G.N., W.Zeng, J.Y.Kim, J.P.Yuan, L.Han, S.Muallem, and P.F.Worley. 2006. STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat. Cell Biol.* 8:1003-1010.

15. Johnson, N.T., M.Villalon, F.H.Royce, R.Hard, and P.Verdugo. 1991. Autoregulation of beat frequency in respiratory ciliated cells. Demonstration by viscous loading. *Am. Rev Respir Dis* 144:1091-1094.

16. Lansley, A.B. and M.J.Sanderson. 1999. Regulation of airway ciliary activity by Ca2+: simultaneous measurement of beat frequency and intracellular Ca2+. *Biophys. J.* 77:629-638.

17. Lee, J., S.K.Cha, T.J.Sun, and C.L.Huang. 2005. PIP2 activates TRPV5 and releases its inhibition by intracellular Mg2+. *J. Gen. Physiol* 126:439-451.

18. Lewis, R.S. 2007. The molecular choreography of a store-operated calcium channel. *Nature* 446:284-287.

19. Liao, Y., C.Erxleben, J.Abramowitz, V.Flockerzi, M.X.Zhu, D.L.Armstrong, and L.Birnbaumer. 2008. Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/Icrac channels. *Proc. Natl. Acad. Sci. U. S. A* 105:2895-2900.

20. Liedtke, W. and J.M.Friedman. 2003. Abnormal osmotic regulation in trpv4-/- mice. *Proc. Natl. Acad. Sci. U. S. A* 100:13698-13703.

21. Liu, X., K.T.Cheng, B.C.Bandyopadhyay, B.Pani, A.Dietrich, B.C.Paria, W.D.Swaim, D.Beech, E.Yildrim, B.B.Singh, L.Birnbaumer, and I.S.Ambudkar. 2007. Attenuation of store-operated Ca2+ current impairs salivary gland fluid secretion in TRPC1(-/-) mice. *Proc. Natl. Acad. Sci. U. S. A* 104:17542-17547.

22. Lopez, J.J., G.M.Salido, J.A.Pariente, and J.A.Rosado. 2006. Interaction of STIM1 with endogenously expressed human canonical TRP1 upon depletion of intracellular Ca2+ stores. *J. Biol. Chem.* 281:28254-28264.

23. Lukacs, V., B.Thyagarajan, P.Varnai, A.Balla, T.Balla, and T.Rohacs. 2007. Dual regulation of TRPV1 by phosphoinositides. *J. Neurosci.* 27:7070-7080.

24. Ma, W., S.D.Silberberg, and Z.Priel. 2002. Distinct axonemal processes underlie spontaneous and stimulated airway ciliary activity. *J. Gen. Physiol* 120:875-885.

25. Mwimbi, X.K., R.Muimo, M.W.Green, and A.Mehta. 2003. Making human nasal cilia beat in the cold: a real time assay for cell signalling. *Cell Signal.* 15:395-402.

26. Mwimbi, X.K., R.Muimo, K.J.Treharne, G.Sijumbila, M.Green, and A.Mehta. 2002. 4alpha-Phorbol negates the inhibitory effects of phorbol-12-myristate-13-acetate on human cilia and alters the phosphorylation of PKC. *FEBS Lett.* 530:31-36.

27. Nilius, B., J.Vriens, J.Prenen, G.Droogmans, and T.Voets. 2004. TRPV4 calcium entry channel: a paradigm for gating diversity. *Am. J. Physiol Cell Physiol* 286:C195-C205.

28. Okada, S.F., R.A.Nicholas, S.M.Kreda, E.R.Lazarowski, and R.C.Boucher. 2006. Physiological regulation of ATP release at the apical surface of human airway epithelia. *J. Biol. Chem.* 281:22992-23002.

29. Putney, J.W., Jr. 2007. Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* 42:103-110.

30. Salathe, M. 2006. Regulation of Mammalian Ciliary Beating. *Annu. Rev. Physiol* 69:401-422.

31. Satir, P. and M.A.Sleigh. 1990. The physiology of cilia and mucociliary interactions. *Annu. Rev Physiol* 52:137-155.

32. Schwiebert, E.M. and A.Zsembery. 2003. Extracellular ATP as a signaling molecule for epithelial cells. *Biochim. Biophys. Acta* 1615:7-32.

33. Seybold, Z.V., A.T.Mariassy, D.Stroh, C.S.Kim, H.Gazeroglu, and A.Wanner. 1990. Mucociliary interaction in vitro: effects of physiological and inflammatory stimuli. *J. Appl. Physiol* 68:1421-1426.

34. Sisson, J.H., J.A.Stoner, B.A.Ammons, and T.A.Wyatt. 2003. All-digital image capture and whole-field analysis of ciliary beat frequency. *J. Microsc.* 211:103-111.

35. Tamm, S. 1994. Ca2+ channels and signalling in cilia and flagella. *Trends Cell Biol.* 4:305-310.

36. Tarran, R., B.Button, M.Picher, A.M.Paradiso, C.M.Ribeiro, E.R.Lazarowski, L.Zhang, P.L.Collins, R.J.Pickles, J.J.Fredberg, and R.C.Boucher. 2005. Normal and cystic fibrosis airway surface liquid homeostasis. The effects of phasic shear stress and viral infections. *J. Biol. Chem.* 280:35751-35759.

37. Teilmann, S.C., A.G.Byskov, P.A.Pedersen, D.N.Wheatley, G.J.Pazour, and S.T.Christensen. 2005. Localization of transient receptor potential ion channels in primary and motile cilia of the female murine reproductive organs. *Mol. Reprod. Dev.* 71:444-452.

38. Trebak, M., L.Lemonnier, J.T.Smyth, G.Vazquez, and J.W.Putney, Jr. 2007. Phospholipase C-coupled receptors and activation of TRPC channels. *Handb. Exp. Pharmacol.*593-614.

39. Vazquez, E., M.Nobles, and M.A.Valverde. 2001. Defective regulatory volume decrease in human cystic fibrosis tracheal cells because of altered regulation of intermediate conductance Ca2+- dependent potassium channels. *Proc. Natl. Acad. Sci. U. S. A* 98:5329-5334.

40. Vazquez, E. and M.A.Valverde. 2006. A review of TRP channels splicing. *Semin. Cell Dev. Biol.* 17:607-617.

41. Vriens, J., G.Owsianik, A.Janssens, T.Voets, and B.Nilius. 2007. Determinants of 4 alpha-phorbol sensitivity in transmembrane domains 3 and 4 of the cation channel TRPV4. *J. Biol. Chem.* 282:12796-12803.

42. Watanabe, H., J.Vriens, S.H.Suh, C.D.Benham, G.Droogmans, and B.Nilius. 2002. Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. *J. Biol. Chem.* 277:47044-47051.

43. Wedel, B., R.R.Boyles, J.W.Putney, Jr., and G.S.Bird. 2007. Role of the store-operated calcium entry proteins Stim1 and Orai1 in muscarinic cholinergic receptor-stimulated calcium oscillations in human embryonic kidney cells. *J. Physiol* 579:679-689.

44. Xu, F., E.Satoh, and T.Iijima. 2003. Protein kinase C-mediated Ca2+ entry in HEK 293 cells transiently expressing human TRPV4. *Br. J. Pharmacol.* 140:413-421.

45. Yuan, J.P., W.Zeng, G.N.Huang, P.F.Worley, and S.Muallem. 2007. STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat. Cell Biol.* 9:636-645.

46. Zhang, L. and M.J.Sanderson. 2003. Oscillations in ciliary beat frequency and intracellular calcium concentration in rabbit tracheal epithelial cells induced by ATP. *J. Physiol* 546:733-749.

47. Zhang, Z., H.Okawa, Y.Wang, and E.R.Liman. 2005. Phosphatidylinositol 4,5-bisphosphate rescues TRPM4 channels from desensitization. *J. Biol. Chem.* 280:39185-39192.

3. BOOK CHAPTER

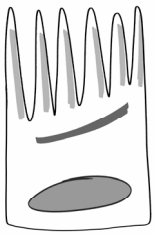
“The TRPV4 channel in ciliated epithelia”

Yaniré N. Andrade, Jacqueline Fernandes, Ivan M. Lorenzo, Maite Arniges, and Miguel A. Valverde

Laboratori de Fisiologia Molecular i Canalopaties, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, PRBB, Barcelona, Spain

Andrade YN, Fernandes J, Lorenzo IM, Arniges M, Valverde MA
The TRPV4 channel in ciliated epithelia

En:
Wofgang B; Liedtke, Stefan Heller.
TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades.
CRC Press, 2006



V. DISCUSSION

1. PREVIOUS HISTORY

Many epithelial functions, including absorption, secretion, response to pathogens, and volume homeostasis are linked to changes in intracellular Ca^{2+} concentration (Zhang and O'Neil, 1999). Cell volume regulation is an important homeostatic mechanism through which cells are able to maintain an optimal volume in an environment where extracellular osmotic pressure differs from intracellular one. Cells exposed to hypotonic or hypertonic extracellular solutions will initially change their volume; swelling or shrinking, respectively. Therefore they have developed cell responses to recover the original size (RVD and RVI) and protect their life and functions (Lang et al., 1998). Although the ability to maintain a constant volume in face of osmotic stress is important for all the cells in the body, the process assumes particular significance in epithelial cells. In airways, the PCL layer which covers the cilia of the luminal face of epithelia, modifies its osmolarity under different situations becoming hyperosmolar during cold or dry ventilation, and hypoosmolar breathing fog (Winters and Yeates, 1997b). Similarly, osmolarity of oviductal fluid also changes with the ovarian cycle (Baltz, 2001). Ciliated epithelia carry out an additional task, the mechanical clearance of mucus and trapped particles from the airways and the transport of ova and embryos through the oviduct (Halbert et al., 1976;Afzelius, 1995;Knowles and Boucher, 2002). A key parameter of the mucociliary transport is the CBF, which is regulated by a variety of chemical and mechanical stimuli (see Introduction). Ciliated epithelia are exposed to physiological changes in mucus viscosity (Rutllant et al., 2002). Despite variations in fluid viscosity, ciliated cells are able to maintain relatively constant their CBF over a range of viscosities. This adapting mechanism called autoregulation, aims to prevent the collapse of mucus transport under high viscous loads (Johnson et al., 1991).

Several intracellular signals have been proposed to mediate the changes of CBF in response to different stimuli: cAMP, cGMP, nitric oxide, and Ca^{2+} (Wyatt et al., 2005;Geary et al., 1995;Jain et al., 1993;Evans and Sanderson, 1999). Among them, the role of Ca^{2+} in the control of CBF autoregulation is particularly interesting as Ca^{2+} has been associated with mechanical stimuli. The response to mechanically induced Ca^{2+} signalling depends on the external Ca^{2+} (Sanderson and Dirksen, 1986). Changes in mucus viscosity might be considered a mechanical stimulus (Spungin and Silberberg, 1984) and the mechanisms linking high viscosity to ciliary activity are poorly studied. Thus, the regulation of Ca^{2+} influx pathway and its implication in the generation of the Ca^{2+} signal is essential to comprehend

ciliated epithelia's response to mechanical stimulation in the context of both physiological and pathological conditions (Houtmeyers et al., 1999;Afzelius, 2004).

Mechanically generated Ca^{2+} signal have been classically attributed to the activation of mechanosensitive Ca^{2+} entry pathways at the plasma membrane or Ca^{2+} release from IP_3-sensitive intracellular stores (McCarty and O'Neil, 1992;Sachs and Morris, 1998). Among the possible candidates to mediate Ca^{2+} entry, TRP channels are well placed, as many of them respond to osmotic and/or mechanical stimuli (Christensen and Corey, 2007). Several members of the TRP family have been found in epithelial tissues, although to date, only TRPV4 and TRPP1-2, have been identified in ciliated cells (see introduction). Previous work in our laboratory provided several pieces of evidences pointing to TRPV4 channel as the molecule through which Ca^{2+} enters into epithelial ciliated cells in response to mechanical/osmotic stimuli, therefore coupling high viscous and hypotonocity stimuli with a cell response: **(1)** TRPV4 is expressed in oviductal ciliated cells and in a human bronchial epithelial cell line (Andrade et al., 2005;Teilmann et al., 2005;Tian et al., 2004;Arniges et al., 2004); **(2)** electrophysiological characterization of high-viscosity and hypotonic-induced cationic currents in epithelial cells (including ciliated cells) coincides with the features of cationic currents induced by the TRPV4-specific agonist 4α-PDD (Arniges et al., 2004;Andrade et al., 2005); **(3)** functional inhibition of the high viscosity-induced cationic current with an antibody against TRPV4 in ciliated oviductal cells (Andrade et al., 2005); and **(4)** TRPV4-cationic currents can be induced by high-viscosity and hypotonic solutions in TRPV4 heterologous expression systems (Andrade et al., 2005;Vriens et al., 2004).

2. PHYSIOLOGICAL ROLE OF TRPV4 IN CILIATED EPITHELIA. COUPLING TRPV4 TO CBF REGULATION

The wide expression of TRPV4 channels and its ability to respond to a broad spectrum of signals, makes TRPV4 an interesting candidate molecule to study in the context of several physiological processes (Nilius et al., 2004). TRPV4 has been shown to be activated by cell swelling (Liedtke et al., 2000;Strotmann et al., 2000;Wissenbach et al., 2000), implicating it in cell volume regulation and shear stress, therefore playing a key sensory role in flow-sensitive tissues such as vascular endothelial cells and renal tubular epithelial cells (Gao et al., 2003;Watanabe et al., 2002b;Wu et al., 2007). Also, hypotonic-induced nociception has been described as a TRPV4-mediated process (Alessandri-Haber

et al., 2003). We have now provided additional evidence for the role of TRPV4 in ciliated epithelia physiology.

2.1. Cellular expression of TRPV4

The main task of ciliated cells is the transport of mucus and the particles trapped within. A primary determinant of mucus transport is the CBF, which can be regulated by different signals, being intracellular Ca^{2+} particularly relevant (Satir and Sleigh, 1990a). It is well known that elevations of intracellular [Ca^{2+}] accelerate CBF (Salathe, 2006). For a useful Ca^{2+} signals, those should be localized at the apical pole of the ciliated cell, close to the base of the cilia (Evans and Sanderson, 1999). The axonemal machinery responsible for the CBF modulation is localized at the base of the cilia (Tamm, 1994). TRPV4 has been previously detected in oviductal ciliated epithelia (Andrade et al., 2005). We now extended the study to its intracellular location, mainly within the apical side of both oviductal and tracheal ciliated cells, where Ca^{2+} is mainly needed for CBF modulation. Subtle differences in TRPV4 staining appear between oviductal and tracheal epithelia. Cilia from the oviduct appear to show a more restricted TRPV4 signal, close to the base, while in tracheal cilia, normally appears all the way through the entire cilia. Whether this apparent difference bears any functional significance it is not known at present. In conclusion, TRPV4 channel is expressed in motile ciliated cells, particullarly in the cilia, exactly where the Ca^{2+} signalling is required for the CBF regulation.

2.2. 4α-PDD agonist

Functional characterization of the TRPV4 channel has greatly benefited from the discovery of the TRPV4-specific agonist 4α-PDD. This compound activates TRPV4 directly, PLA_2-independently, via its interaction with TRPV4 transmembrane domains 3 and 4 (Vriens et al., 2007;Watanabe et al., 2002a). Addition of the agonist 4α-PDD to the extracellular bath activates TRPV4 channels expressed in both oviductal and tracheal ciliated cells. This cause an influx of Ca^{2+} into the cell through TRPV4 channels and therefore an increment of CBF, suggesting that TRPV4 channel activity can be coupled to the regulation of CBF. The fact that 4α-PDD only causes Ca^{2+} signalling and CBF increments in wild type ciliated cells, whereas ciliated cells from the trachea of $TRPV4^{-/-}$

mice never response to the agonist reinforces both the specificity of the agonist 4α-PDD for the TRPV4 channels and the role of the TRPV4 channel in the CBF regulation.

2.3. Temperature stimulus

TRPV4 is a thermo-TRP channel activated by warm temperatures above the thermal threshold of ~25ºC-38ºC (Benham et al., 2003). TRPV4 response to heat is characterized by transient current increases followed by slow decay towards the baseline and a desensitization upon sustained or repeated heat applications above 42ºC, unlike TRPV1 and TRPV3 (Guler et al., 2002a;Watanabe et al., 2002b;Nilius et al., 2004). TRPV4$^{-/-}$ tracheal ciliated cells show a reduced Ca^{2+} signal and CBF response to changes in bath temperature from 24ºC to 38ºC, offering another piece of evidence confirming that TRPV4 channels are coupled to CBF regulation.

Each thermo-TRP has unique characteristics; highlighted by distinct temperature thresholds of activation (see Introduction) and the ability to be modulated by distinct signalling mechanisms further distinguish thermo-TRPs. Of the TRPs detected in the airways, TRPC1, TRPC4, TRPC6, TRPV2, and TRPV4 (Corteling et al., 2004;Kowase et al., 2002;Arniges et al., 2004), only the TRPV2 and TRPV4 are activated by heat. We exclude TRPV2 given that its activation threshold of ~52ºC (Caterina et al., 1999) is much higher than the heat stimulus that we used (~38ºC). TRPV4$^{-/-}$ tracheal ciliated cells still response to milder heat (38º) indicating that other molecules play a role in this response. TRPV1 and TRPV3 could be good candidates to mediate the response to warmth in TRPV4$^{-/-}$ cells. Capsaicin, a TRPV1 agonist, increases CBF (Wong et al., 1990), suggesting the presence of an active TRPV1 channel, although its temperature threshold is higher (~43ºC) than the heat we applied (~38ºC). Considering the heat applied, TRPV3 with a threshold of ~33ºC could be, along with TRPV4, a good candidate to mediate the response to warm temperature in tracheal ciliated cells, but TRPV3 expression in this tissue should be confirmed. Interestingly, CBF dependence on temperature reaches a plateau within 32-33ºC onwards in human and ovine airway ciliated cells (Mwimbi et al., 2003a;Green et al., 1995;Sisson et al., 2003), highlighting the role of TRPV4 channel in the control of CBF under physiological temperatures.

3. TRPV4 ACTIVATION BY MECHANICAL AND OSMOTIC STIMULI

Cellular mechanical stimulation is a generic term describing different stimulatory conditions that cover deformation of cell membranes by means of an osmotic stimulus or direct stretching (Okada et al., 1990;Han et al., 2004;Sanderson et al., 1990;Gheber et al., 1998;Winters et al., 2007;Andrade et al., 2005). Although osmotic cell volume variations are considered mechanical stimuli, we have to notice that other cell alterations different to changes in membrane tension (a mechanical force parallel to the membrane) appear after cell exposure to osmotic stimulation. Thus, other cellular changes appear in cytoskeletal architecture, cellular ion concentrations, and concentration of cytoplasmatic macromolecules, known as macromolecular crowding (Strange, 1994). Throughout my thesis work the mechanical stimuli used were both hypotonic solutions and high viscous loads (without changes in the tonicity of the solution). In this sense, the requirement of epithelial ciliated cells to adapt the CBF response to solutions of high viscosity has been proposed that may be sensed by different ways. **(1)** The flow of the medium with increased viscosity can exert higher shear stress on the cell surface (Winters et al., 2007), **(2)** the viscous resistance of the medium to beating cilia (Fernandes et al., 2008), and/or **(3)** the cell membrane fluctuations (Tuvia et al., 1997). Although several molecules have been proposed to sense the mechanical stimuli: Integrins, cytoskeleton, proteoglycans (Florian et al., 2003), mucins (de et al., 2007), PLA_2 (Lehtonen and Kinnunen, 1995), membrane-bound G-proteins (Gudi et al., 1998), PLC (Ruwhof et al., 2001), stretch activated cation channels (Hansen et al., 1993;Boitano et al., 1994;Felix et al., 1996), among other possibilities.

3.1. TRPV4 as a mechanical transducer

Two TRPV-like channels in *C. elegans* sensory neurons, OSM-9 and OCR-2, are essential for both osmosensory and mechanosensory (nose-touch) behaviours (Liedtke et al., 2003;Liedtke and Kim, 2005). Likewise, two Drosophila TRPV channels, NAN and IAV, are critical for hearing by the mechanosensitive chordotonal organs located in the fly's antennae (Kim et al., 2003). The mechanosensitive nature of the channels appears to be conserved in higher organisms for some TRPV channels. Two vertebrate channels, TRPV2 and TRPV4, are sensitive to hypotonic cell swelling, shear stress (TRPV4), and membrane stretch (TRPV2). In the mammalian osmosensing neurons of the hypothalamus

(circumventricular organs), TRPV4 functions as part of an osmoreceptor complex, in control of vasopressin release (Liedtke, 2005;Liedtke and Friedman, 2003). Finally, in many nonsensory cells expressing TRPV4, such as oviductal ciliated cells, vascular endothelial cells and renal tubular epithelial cells, the channel exhibits well-developed local responses to mechanical processes where high viscous loads, cell swelling and shear stress lead to channel activation (Andrade et al., 2005;Hartmannsgruber et al., 2007b;Cohen, 2005b). Hence, many TRPV channels, or combinations of TRPV channels, display a mechanosensitive nature that underlies multiple mechanosensory processes from worms to mammals (O'Neil and Heller, 2005).

Genetic inactivation of TRPV4 in mice show impairments of volume balance in two independently generated mouse models (Mizuno et al., 2003;Liedtke and Friedman, 2003). In both reports, hyperosmotic challenge of the $TRPV4^{-/-}$ mice induced an increase in serum concentration of antidiuretic hormone. TRPV4 is not only involved in volume balance but also in nociceptive transduction. $TRPV4^{-/-}$ mice show reduced sensitivity to pressure of the tail and acidic nociception (Suzuki et al., 2003). In hot-plate tests, the latency to escape following hyperalgesia was longer in $TRPV4^{-/-}$ mice. Additionally, $TRPV4^{-/-}$ mice show altered thermal selection behaviour. Wild-type mice prefer rooms at 30ºC, whereas TRPV4 knockout mice choose those at 34ºC (Lee H. et al., 2005). In hearing tests, elderly $TRPV4^{-/-}$ mice show hearing impairment with higher thresholds for auditory brainstem responses.

3.2. Cell Signalling Pathways

TRP channels display activation in response to a broad diversity of chemical and physical stimuli. Besides, activation by mechanical stimuli is common to many members of the TRPV subfamily in both lower and higher organisms (O'Neil and Heller, 2005). TRPV4 is activated by diverse chemical and physical stimuli such as heat, phorbol ester compounds, low pH, arachidonic acid (AA) metabolites, high viscous loads and osmotic cell swelling. Previous studies addressing the gating mechanism of TRPV4 by cell swelling exclude potential cellular pathways such as direct membrane stretch (unlike TRPC1), intracellular ionic strength, and membrane-bound G proteins (Strotmann et al., 2000;Nilius et al., 2001).

3.2.1. The PLA_2-AA-EET Pathway

In a number of cell types, mechanical and hypotonic stimuli activate PLA_2 and PLA_2-dependent AA release from membrane phospholipids (Pedersen et al., 2000;Basavappa et al., 1998;Alexander et al., 2004). The enzyme PLA_2 have been proposed as a mechanosensor, responding to the osmotic forces applied on the cellular membrane (Lehtonen and Kinnunen, 1995). Production of AA upon PLA_2 activation and its downstream epoxyeicosatrienoic acid metabolites (5',6'-EET and 8',9'-EET) are responsible for potent TRPV4 activation (Watanabe et al., 2003b). Two mechanisms have been suggested to explain TRPV4 activation by cell swelling: tyrosine kinase activation (Xu et al., 2003b) and PLA_2-AA pathway followed by the production of P450-generated epoxygenase metabolites (EETs) (Vriens et al., 2004). Our laboratory has also demonstrated that TRPV4 channel is activated upon cell stimulation with high viscous loads via PLA_2, coupling for the first time the gating of TRPV4 channels to CBF regulation (Andrade et al., 2005). Thus, up to date, EETs are the only physiological, diffusible molecule known to directly activate TRPV4. Activation of TRPV4 by 4α-PDD and heat is fully independent of PLA_2 and P450 epoxygenase but requires an aromatic residue on position 555 near the N terminus of the third transmembrane domain (Vriens et al., 2004). Interestingly, platelet activating factor (PAF) and muscarinic agonists activate the CBF via PGE_2 production (Hermoso et al., 2001;Gayner and McCaffrey, 1998), therefore suggesting a role between PLA_2 and P450 epoxygenase pathway and CBF regulation.

3.2.2. PLC-IP_3 Pathway

PLC pathway is activated upon mechanical and osmotic stimulation in tracheal ciliated cells (Hansen et al., 1995;Boitano et al., 1994;Sanderson and Dirksen, 1991). Both stimuli release Ca^{2+} from IP_3-sensitive intracellular stores, a response that is also dependent on Ca^{2+} entry (MacLeod, 1994;Boitano et al., 1994). The small G protein Ras is also involved in mechanical and osmotic stimuli (Gudi et al., 2003), but not in TRPV4 activation (Strotmann et al., 2000;Fernandes et al., 2008). Mechanical stimulation of airway epithelial cells causes stretch-activated cation channels and 5'-triphosphate nucleotide-release activation to produce intracellular Ca^{2+} signals and intercellular Ca^{2+} waves (Homolya et al., 2000;Boitano et al., 1994). In airway epithelial ciliated cells, ATP acts mainly via $P2Y_2$ receptors as it is demonstrated using $P2Y_2^{-/-}$ airway epithelial cells (Homolya et al., 1999). Purinergic receptor activation triggers G protein-mediated PLCβ

activation and the subsequent breakdown of PIP_2 molecule into IP_3 and DAG. The rapid rise of IP_3 into the cytoplasm opens IP_3R to produce intracellular Ca^{2+} signals. In parallel, DAG either directly or via PKC (although other pathways may also participate) may open Ca^{2+} channels located on the plasma membrane. The participation of the PLC pathway in the activation of TRPV4 was not initially considered due to the lack of TRPV4 activation with guanosine 5'-O-(3- thiotriphosphate) (GTPγS) (Nilius et al., 2001). This TRPV4 behaviour contrasted with that of other members of the TRPC subfamily that are activated secondary to PLC activation either by a membrane-bound G protein or by tyrosine-kinase coupled-receptors (Abramowitz et al., 2007).

The first study of my Thesis' work shows that high viscous loads and osmotic stimuli triggers the PLC-IP_3 signalling cascade to influence the gating of TRPV4 (Fernandes et al., 2008). Airway epithelial cells express functional P2Y-purinoreceptors that are activated by release of 5'-triphosphate nucleotide (mainly ATP, but also UTP) upon diverse mechanical stimuli (Felix et al., 1996;Okada et al., 2006;Winters et al., 2007). Both mechanical stimuli, high viscous loads and 30% hypotonic solutions, activate PLC pathway in oviductal ciliated cells secondary to ATP release into extracellular medium and activation of purinergic receptors. Consistent with this working model, activation of TRPV4 currents upon high viscous loads stimulation requires not only ATP and purinergic receptors but also activation of membrane-bound G proteins. TRPV4 activation under these conditions is independent of Ca^{2+} release from intracellular stores, as it was demonstrated by patch-clamp studies carried out in the presence of thapsigargin (TG). The presence of IP_3 in the pipette solution bypasses PLC inhibition and supports TRPV4 mechanical activation in the presence of PLC inhibitors, apparently without IP_3 being a direct TRPV4 agonist itself (Fernandes et al., 2008).

In summary, mechanotransduction in oviductal ciliated epithelial cells requires ATP release, purinergic receptors, and the PLCβ/IP_3 cell signalling pathway for TRPV4 channel regulation, but only under conditions maintaining an active PLA_2-AA-EET pathway.

3.2.3. Cross-talk of PLA_2 and PLC Pathways

Patch-clamp and calcium imaging techniques were used to analyse details of cellular pathways involved in the TRPV4 regulation upon high viscous loads and hypotonic stimuli. Oviductal ciliated cells exposed to high viscous loads and hypotonic stimuli use common signalling pathways although their relative contribution depends on the magnitude of the stimulus (see figure 11). With PLA_2 pathway inhibitors there is an abolishment of

both TRPV4 currents and Ca^{2+} signalling under both stimuli (Ca^{2+} imaging data not shown). Inhibiting PLC pathway completely abolishes TRPV4 activation under high viscous load stimulus, whereas only reduces TRPV4 response to 30% hypotonic solutions, also mantaining initial transient Ca^{2+} peak without oscillations. Then the impact of PLC pathway on TRPV4 activation depends on the stimuli used. These results indicate that high viscous stimulus is more dependent on PLC pathway than 30% hypotonic stimulus. Probably both stimuli reflect a different level of PLA_2 activity because: **(1)** 30% hypotonic stimulus is less sensitive to inhibition of PLC pathway, suggesting a higher PLA_2 activity, **(2)** milder 15% hypotonic stimulus turned the TRPV4 activation fully PLC dependent and **(3)** IP_3 metabolite alone at maximal activation of IP_3R (De Smet P. et al., 1999) does not induce TRPV4 activation but rather sensitizes channel response to low [EET] in oviductal ciliated cells and heterogeneous TRPV4 expression system using patch-clamp techniques; and **(4)** the presence of low [ATP] sensitizes TRPV4 channels overexpressed in HeLa cells to mechanical stimuli demonstrated using Ca^{2+} imaging techniques. Similar results have been found in osteoblasts where $P2Y_2$-receptor stimulation by ATP sensitizes mechanically activated calcium channels (Katz et al., 2006). Furthermore, the IP_3 sensitization of TRPV4 requires a functional IP_3 receptor type 3 (IP_3R3) but is independent of the presence of Ca^{2+}, demonstrated by patch-clamp experiments using TG (a blocker of SERCA pumps that depletes passively the intracellular Ca^{2+} stores) and intra- and extracellular EGTA (a Ca^{2+} chelator to ensure the absence of Ca^{2+} entry and intracellular free Ca^{2+}). IP_3R3, but not IP_3R1, has been located to the endoplasmatic reticulum and to the plasma membrane (Dellis et al., 2006) of oviductal ciliated cells in culture (Barrera et al., 2004).

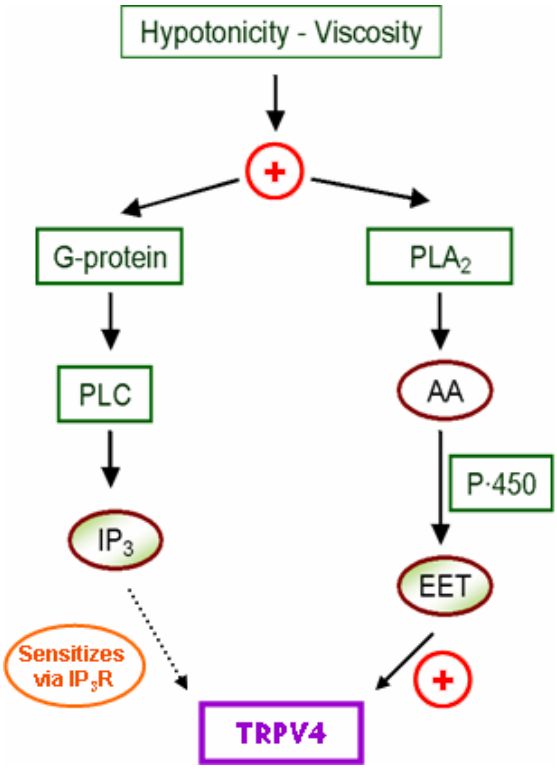


Figure 11. ***Cross-talk of PLA_2 and PLC pathways.*** Hypotonic solutions and high viscosity activate both membrane-bound G protein-PLC-IP_3 and PLA_2-AA-EET pathways. Metabolization of AA by cytochrome P-450 epoxygenase generates EET metabolites for TRPV4 activation. The other activated pathway, membrane-bound G protein-PLC-IP_3, just sensitize TRPV4 channel by the IP_3-IP_3R complex for the gating of TRPV4 by EET. If the stimulus produces low PLA_2 activity, PLC-IP_3 pathway is required for TRPV4 activation.

Altogether, our observation is consistent with the convergence of ATP-PLC-IP_3 and PLA_2-AA-EET signalling for the optimal activation of TRPV4 by high viscous and hypotonic solutions that do not reach the threshold level of PLA_2 activation. IP_3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-EET in a Ca^{2+} independent fashion via functional IP_3R3.

3.3. Calcium oscillations

Calcium oscillations are complex cell signals which are spatially-restricted inside the cell (Shuttleworth, 1999). Intracellular Ca^{2+} oscillations induced by membrane G-protein-coupled receptors provide a versatile encoding mechanism that uses variations in the amplitude, frequency and duration of signals to control cellular processes (Berridge et al., 2000). Ca^{2+} entry is increased during such oscillatory responses and the rate of Ca^{2+} entry during oscillatory response presents a feedback component that also influences oscillations (Shuttleworth, 1999). In some cell types oscillations persist in the absence of Ca^{2+} entry (although their frequency is decreased), whereas in other cell types oscillations show stronger Ca^{2+} entry-dependent component (Shuttleworth et al., 2004;Sneyd et al., 2006). Oviductal ciliated cells exposed to high viscous loads (20% dextran) and hypotonic solutions response with a clear Ca^{2+} oscillatory pattern which depends on Ca^{2+} entry, decreasing until oscillations disappear in the absence of external Ca^{2+}.

The Ca^{2+} oscillatory pattern obesrved upon mechanical stimuli resembles that obtained in response to low concentration of physiological agonist acting on G protein-coupled receptors. This dependency on Ca^{2+} entry for continued oscillations may be interpreted in terms of the biphasic effect of Ca^{2+} on the IP_3R (Sneyd et al., 2006;Goldbeter et al., 1990;Shuttleworth, 1999;Nash et al., 2001). Low and high [Ca^{2+}] inhibit IP_3R while intermediate [Ca^{2+}] facilitate IP_3R activation. The kinetics of the activation and inactivation process also differs, the negative feedback occurs on a slower time scale than activation (Atri et al., 1993). IP_3 increases upon mechanical stimuli (Felix et al., 1996) and low activation of the PLC-IP_3 pathway are associated to oscillatory Ca^{2+} signals. Then, low [IP_3] binds to IP_3R and poorly activates it or sensitizes it for the activation by Ca^{2+}. In other words, influx of Ca^{2+} in close proximity to IP_3R increases the likelihood that low [IP_3] will trigger a repetitive release of Ca^{2+} from the intracellular stores (Shuttleworth and Mignen, 2003). This allows for the possibility that Ca^{2+} oscillations are caused by sequential positive and negative feedback of Ca^{2+} on IP_3R. This Ca^{2+} may be **(1)** released by IP_3R from the stores suggested by the Ca^{2+} transient peak in absence of external Ca^{2+} or **(2)** entered through plasma membrane Ca^{2+} channels as suggest by Ca^{2+} imaging experiments using PLC inhibitors and stimulation with 30% hypotonic solutions. In this sense, the close localization of TRPV4 with IP_3R, suggested by the coimmunoprecipitation experiments, will facilitate the coupling of Ca^{2+} entry to sensitization of IP_3R.

We propose that the PLC-IP_3 pathway is required for PLA_2-dependent TRPV4 activation by dextran solutions and that both active TRPV4 and PLC-IP_3 pathway are

needed to initiate and maintain the oscillatory Ca^{2+} signal. In the case of 30% hypotonic stimuli, TRPV4 activation is more independent of PLC-IP_3 pathway but, again, both active TRPV4 and PLC-IP_3 pathway are needed to initiate and may be to maintain the Ca^{2+} oscillations. Notice the mechanisms of regulation of TRPV4 are well reproduced in heterologus TRPV4 cell expression systems, whereas the Ca^{2+} oscillation of cell response to mechanical stimuli found in ciliated cells was not fully reproduced in Hela cells expressing TRPV4. May be this is due to the different impact of PLC-IP_3 pathway on both ciliated and Hela cells expressing TRPV4. While ciliated cells are independent of ATP addition for the mechanical response, Hela cells expressing TRPV4 need ATP addition to prime the PLC-IP_3 pathway. In contrast to oviductal ciliated cells, Hela cells may require ATP priming for various reasons: **(1)** Hela cells have less ability to release ATP after mechanical stimulus, **(2)** ciliated cells have higher sensitivity of the PLC-IP_3 pathway to released ATP, and **(3)** ciliated cells couple more efficiently ATP-PLC-IP_3 pathway to PLA_2-AA-EET pathway.

Regarding the nature of Ca^{2+} entry during oscillations, it has been largely focussed on the SOCE mechanism. In this sense, recent articles have appeared showing the dependence of Ca^{2+} oscillations on STIM1 and ORAI1 (Wedel et al., 2007;Lewis, 2007). However, several considerations should be taken into account in order to validate this working model (Shuttleworth, 1999): It has been reported a rapid Ca^{2+} entry activation, that may even precede any detectable release of Ca^{2+} from the stores, which contradicts with the SOCE model. Therefore, the participation of the PLC-IP_3 pathway and an additional independent pathway in the Ca^{2+} entry during the oscillatory response has been proposed (Shuttleworth, 1999). In this respect, upon mechanical stimulation, TRPV4 is activated via the PLA_2-AA-EET pathway and modulated by the PLC-IP3 pathway, suggesting the role of TRPV4 in Ca^{2+} entry to initiate and maintain oscillations. The dual regulation of TRPV4 by Ca^{2+}-CaM (potentiation) and [Ca^{2+}] (inhibition at high concentrations) could determine the oscillatory pattern of TRPV4-like currents recorded in oviductal ciliated cells exposed to high viscous solutions (Andrade et al., 2005).

It is well known that TRPV4 channels are involved in the mechanotranduction to convey mechanical stimuli into biochemical signals (Vriens et al., 2004;Andrade et al., 2005). The current hypothesis is that upon mechanically stimulated cells, PLA_2-AA-EET, ATP-PLC-IP_3-IP_3R pathways are involved in the TRPV4 regulation and at the same time, ATP-PLC-IP_3-IP_3R pathway and TRPV4 channels are involved in the initiation and maintaining of Ca^{2+} oscillations response.

3.4. Autoregulation of CBF

Motile ciliated epithelia may be exposed to variations in viscosity and load of the mucus layer (Rutllant et al., 2002;Widdicombe, 2002). Epithelial human airways mucus contain viscosities ranging from 10cP to 5000cP (Puchelle et al., 1987). Tracheal ciliated cells and oviductal ciliated cells are able to maintain the CBF over a range of viscous loads (12-150cP and 30-200cP) (Johnson et al., 1991;Andrade et al., 2005;Lorenzo et al., 2008). Then, motile ciliated epithelia from both trachea and oviduct are capable of preventing the collapse of mucus transport under high viscous loads by activating a mechanism known as CBF autoregulation (Johnson et al., 1991).

CBF autoregulation is generated specifically inside the ciliated cells, without involving nervous stimuli. In culture, epithelial cells are devoid of any innervations; consequently, mechanosensitivity must be unequivocally associated with the ciliated cell. CBF autoregulation is lost when extracellular Ca^{2+} is removed (Andrade et al., 2005). CBF in both water and mucus propelling cilia is known to decrease with increasing viscosity. However the extent of the decrease is dependent on the particular system (Gheber et al., 1998). Our laboratory have previously proposed the participation of TRPV4 in the generation of Ca^{2+} signal required to activate the autoregulatory mechanism in hamster oviductal cells (Andrade et al., 2005). We have now further explored the role of TRPV4 in CBF autoregulation using the TRPV4-KO mouse model.

Ciliated cells from mouse trachea present autoregulation under high viscous loads. The percentage of tracheal ciliated cells responding to high viscous loads (20% dextran) with oscillatory signals (26%) is lower than in oviductal ciliated cells (76%), with also a smaller amplitude (Andrade et al., 2005). Despite the reduced Ca^{2+} signal in tracheal cells, they preserved the autoregulation of CBF. The transient Ca^{2+} signal seen in $TRPV4^{-/-}$ ciliated cells rarely oscillate and resembles the response observed in hamster oviductal ciliated cells in the bathing medium without Ca^{2+}. Although both $TRPV4^{+/+}$ and $TRPV4^{-/-}$ tracheal cells show different Ca^{2+} signalling patterns they decrease CBF to the same extent under high viscous loads, suggesting that CBF autoregulation in the trachea is not so Ca^{2+}- and/or TRPV4-dependent. One reason could be related with the prevalence in tracheal cells of another signalling pathway different to Ca^{2+} for the CBF regulation.

Altogether, our data obtained with the TRPV4-KO model confirms the involvement of TRPV4 in the generation of Ca^{2+} oscillations under conditions of high viscosity, but without a major role in CBF autoregulation.

4. ROCE AND SOCE

Receptor-activated PLC breakdowns PIP_2 to IP_3 and DAG in order to generate Ca^{2+} signalling. Both limbs of the PLC pathway are capable of regulating Ca^{2+} entry. Ca^{2+} signalling is complex, involving two independent and closely coupled components: rapid, transient release of Ca^{2+} stored in the ER, followed by slowly developing extracellular Ca^{2+} entry. Ca^{2+} entry is performed by two distinct mechanisms, known as receptor-operated Ca^{2+} entry (ROCE) and store-operated Ca^{2+} entry (SOCE). Dual regulation of distinct Ca^{2+} entry pathways is not, therefore, simply redundant ways of achieving an increase in cytoplasmatic Ca^{2+}. Rather, different receptors may selectively recruit different Ca^{2+} entry pathways, the activities of the different pathways are carefully coordinated, and Ca^{2+} entering via each channel is likely to be selectively coupled to distinct cellular responses (Taylor C.W., 2002).

SOCE has been classically refereed as the enhanced Ca^{2+} entry that results solely from the emptying of intracellular Ca^{2+} stores. The intracellular agonist-depletion alone is both necessary and sufficient to activate SOCE. The recent molecular discovery of STIM1 has allowed to expand this definition. Nowadays, SOCE can be considered as Ca^{2+} entry through channels that are regulated by STIM1 and require the store depletion-mediated clustering of STIM1 (Yuan et al., 2007). Whereas, ROCE simply is the Ca^{2+} entry secondary to receptor activation but independent of Ca^{2+} stores depletion. A summary of this ideas of the SOCE pathways are depicted in figure 12.

At least two types of SOCE can be distinguished electrophysiologically and now molecularly (Yuan et al., 2007). The first type of SOCE is the classical and well-known, highly Ca^{2+}-selective I_{CRAC} currents that recently were shown to be mediated by the Orai family of proteins (Smyth et al., 2006), and the second type of SOCE is mediated by non-selective, Ca^{2+} permeable TRPC channels. These TRP channels have intrigued the scientific community for several years in the attempt to involve them in the generation of I_{CRAC} currents.

Just both STIM1 and ORAI proteins are able to recapitulate the long-known properties of I_{CRAC} currents. The Orai gene, of which there are three in the mammalian genome, encodes for a plasma membrane protein and appears to have four transmembrane domains. ORAI is a highly Ca^{2+} selective ion channel with the same electrophysiological properties found for the I_{CRAC} currents upon intracellular Ca^{2+} stores depletion (Yeromin et al., 2006). STIM1 belongs to the family of STIM with two known mammalian members: STIM1 and STIM2. Only the knockdown of STIM1 reduces SOCE

and I_{CRAC} in mammalian cells (Liou et al., 2005). STIM1 is a Ca^{2+} sensor within the ER that conveys the Ca^{2+} load of the intracellular stores to the SOC channels at the plasma membrane (Yuan et al., 2007). STIM1 has an amino-terminal EF hand Ca^{2+}-binding domain that resides in the ER lumen. Depletion of ER Ca^{2+} redistributes STIM1 into punctate structures underneath the plasma membrane and subsequent activation of SOCE (Zhang et al., 2005b;Spassova et al., 2006).

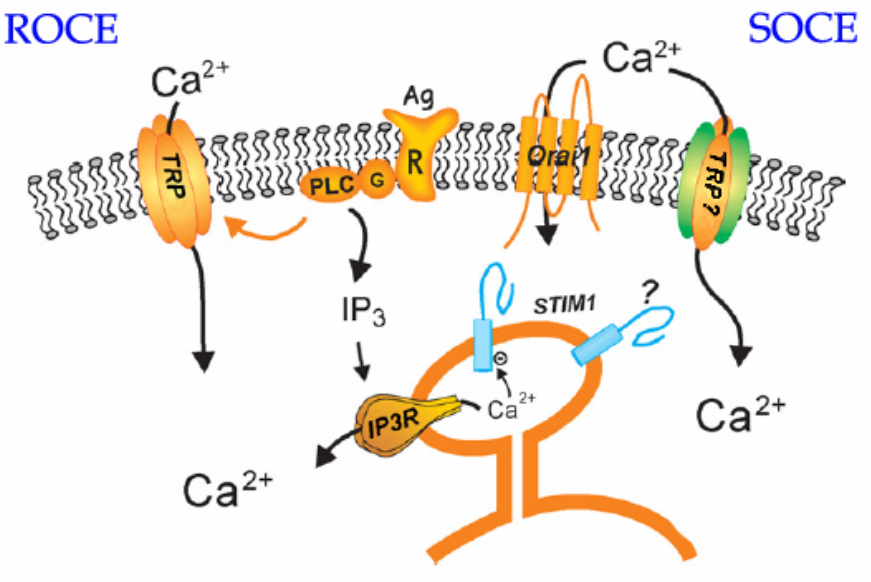


Figure 12. ***Intracellular Ca^{2+} regulation.*** Agonist (Ag) activates plasma membrane receptor (R), a G protein-coupled receptor (G) associates to PLC-β or a tyrosin kinase-coupled receptor associates to PLC-γ. Activated phospholipase C (PLC) generates second messengers, IP_3 and DAG, leading to ROCE and/or SOCE activation. The process of Ca^{2+} entry secondary to receptor activation is the ROCE mechanism. IP_3 diffuses rapidly into the cytosol, open IP_3 receptors (IP_3R) and release Ca^{2+} from stores. Ca^{2+} depletion of the stores gates SOC channels on the plasma membrane by combination of the ER Ca^{2+}-sensor STIM1 and plasma membrane channel ORAI1. The role of TRP channels in SOCE is nowadays controversial and is suggested that may be another kind of SOCE with different biophysical and physiological properties.

The current ideas about STIM1 and ORAI action are consistent with a number of earlier views about I_{CRAC} involvement in SOCE: **(1)** upon depletion of Ca^{2+} stores, Ca^{2+} unbinds from an EF hand motif and STIM1 redistributes into punctate structures underneath the plasma membrane (the same structures found for the generation of I_{CRAC})

and subsequent activation of SOCE; **(2)** the observation that the biophysical properties of I_{CRAC} channels differ substantially from conventional ion channels, predicting that they may also differ substantially in their structure, as been recently demonstrated; **(3)** it has been proposed a close proximity between stores and plasma membrane Ca^{2+} entry channels that would facilitate refilling without further increasing cytosolic [Ca^{2+}]. (Putney, Jr., 2007;Braiman and Priel, 2001).

4.1. TRPs in ROCE and SOCE mechanisms

There are cation channels gated when PLC is activated either by G protein-coupled receptor pathway mediated by PLC-β or by receptor tyrosine kinase signalling pathway mediated by PLC-γ. The PLC-generated second messenger IP_3 releases Ca^{2+} from the stores, depleting them, and triggering plasma membrane calcium entry by SOC channels (SOCE mechanism). The other product of PLC activation, DAG (a membrane-associated fatty acid), might serves as a signal to activate ROC channels directly or via PKC (Spassova et al., 2004).

The role of TRP channels mediating store-operated Ca^{2+} entry is a controversial issue. All TRPC subfamily members: from TRPC1 to TRPC7, have been implicated in ROCE and SOCE mechanisms, although this hypothesis has been disputed at times depending on the experimental model (Abramowitz et al., 2007;Spassova et al., 2004;Dietrich et al., 2005;Trebak et al., 2003;Petersen and Fedirko, 2001;Worley et al., 2007). Also non-TRPC such as TRPM7 has been implicated in SOCE and the regulation the mitotic cell cycle progression of immune cells (Tani et al., 2007).

For the TRPC subfamily members, it seems that their primary mechanism of activation is through PLC activation, although based on the latest definition of SOCE, a Ca^{2+}-entry mechanism regulated by STIM1, they should also be considered SOC channels. STIM1 regulates directly or indirectly all TRPC channels, except TRPC7. However, although STIM1 directly regulates TRPC1, TRPC4, and TRPC5 by direct protein-protein interaction, the regulation of TRPC3 and TRPC6 by STIM1 is mediated by STIM1-dependent heteromultimerization of TRPC3 with TRPC1 and of TRPC6 with TRPC4. STIM1 is required for activation of all TRPC channels by agonist stimulation, but is not essential for channel activation (Yuan et al., 2007).

ROCE can be stimulated as a consequence of agonist activation of receptor-coupled PLC and independently of Ca^{2+} store depletion by one of the next possibilities: **(1)**

generation of DAG, which serves as the signal to activate calcium channels and to translocate and activate the major regulatory kinase, protein kinase C (PKC) that regulates a range of plasma membrane calcium channels (Spassova et al., 2004); **(2)** IP_3 receptor conformational coupling by which IP_3-IP_3R activates TRPC (Boulay et al., 1999;Kiselyov et al., 2007;Rosado et al., 2000); IP_3R is a key player in the activation of calcium entry, as originally predicted by Irvine (Irvine, 1990) and Berridge (Berridge, 1995). IP3 binds and activates nearly every member of TRPC family (Spassova et al., 2004). However, whereas structural evidence for TRP channel-IP3R interactions is compelling, the evidence for a role of IP3R in SOC activation is controversial. More likely, the role of IP3 in the activation of Ca2+ entry is limited to ROCE. **(3)** generation of AA (Shuttleworth et al., 2004); and **(4)** other undefined mechanisms (Liao et al., 2008).

4.2. TRPV4 in ROCE

In the second article of this thesis (Lorenzo et al., 2008) we have addressed the role of TRPV4 in the ATP-triggered Ca^{2+} response of mouse tracheal ciliated cells. Many mechanical stimuli on the ciliated epithelium release ATP to the periciliary liquid (PCL). Mechanical stimuli trigger the release of ATP from ciliated epithelia and the subsequent regulation of CBF (Sanderson and Dirksen, 1986;Winters et al., 2007). The Ca^{2+} response pattern depends on the extracellular [ATP] and the sensitivity of each ciliated cell (Zhang and Sanderson, 2003). Released ATP acts via G-protein coupled receptors on $P2Y_2$ receptors to activate PLC-β and induce both the release of Ca^{2+} from stores by IP_3 metabolites and the sustained Ca^{2+} entry at high [ATP]. Ciliated tracheal cells from $TRPV4^{-/-}$ mice show a significant reduction of the sustained component by around 30%, therefore, involving TRPV4 in the generation of the sustained component of the ATP-triggered Ca^{2+} response.

The sustained component involves Ca^{2+} entry via ROCE and/or SOCE. We used Ca^{2+} imaging protocols to demonstrate the possible role of TRPV4 in Ca^{2+} entry mechanisms. Our results suggest the selective participation of TRPV4 in ROCE mechanism, discarding a possible role on SOCE. The initial Ca^{2+} peak released from the stores is not statistically different between the two genotypes, suggesting that TRPV4 is not involved in this first Ca^{2+} release after large [IP_3] increments generated upon submaximal ATP stimulation. Similarly, the rate of Ca^{2+} release induced by TG is not

affected by TRPV4. The lack of TRPV4 involved in SOCE suggests that TRPV4 activation is independent of the intracellular Ca^{2+} sensor STIM1.

4.3. Pathways involved in TRPV4 activation by ATP stimuli

TRPV4 channels are opened by diverse physical and chemical stimuli such as cell swelling (Vriens et al., 2004;Arniges et al., 2004), shear stress (Gao et al., 2003;O'Neil and Heller, 2005), high viscosity (Andrade et al., 2005), moderate heat (Gao et al., 2003;Vriens et al., 2004), low pH (Suzuki et al., 2003) and synthetic non-PKC-activating 4α-PDD phorbol ester (Watanabe et al., 2002a;Vriens et al., 2004;Vriens et al., 2007). The signalling pathways involved in the channel responses are also diverse and at times controversial: tyrosine kinases (Xu et al., 2003b), PKC phosphorylation (Gao et al., 2003;Watanabe et al., 2002a), endogenous agonists like 5'-6'-EET, a metabolite from the PLA_2-AA-cytocrome P450 epoxygenase pathway (Watanabe et al., 2003b), biphasic modulation by Ca^{2+} and Ca^{2+}-CaM (Watanabe et al., 2003a;Strotmann et al., 2003) and IP_3-dependent modulation (Fernandes et al., 2008). Besides, several of this signals also participate in both CBF regulation (Salathe, 2006) and ROCE (Putney, Jr., 2007). Cross-talk between different signalling pathways is common. For example, in osteoblasts, ATP sensitization of mechanically-activated Ca^{2+} channels depends on PLC, Src kinases and PKC-mediated cytoskeleton reorganization (Katz et al., 2006;Khatib et al., 2004). Similarly, hypotonic stimuli potentiate acetylcholine-stimulated CBF through ATP release in rat tracheal ciliated cells (Kawakami M. et al., 2004). Therefore, the mechanism involved in TRPV4 activation leading to ROCE is still an open question.

Phosphatidylinositol 4,5-biphosphate (PIP_2), the substrate of PLC, activates the cold-sensitive TRPM8 channel (Rohacs, 2007) but inhibits TRPV1, a close relative of TRPV4 (Chuang et al., 2001). The modular binding site of TRPV1 is not conserved in TRPV4, but exist a low-homology site with six basic amino acids between residues 400 and 446 whose possible functional impact is still unknown. Then, PIP_2 may be good candidate to modulate TRPV4 channel (probably without being direct activator) by decreasing or increasing its relative concentration in the plasma membrane.

The PLC generated membrane-bound metabolite, DAG, has been proposed as a messenger involved in ROCE. DAG has a rapid and direct role in TRPC3, TRPC6, and TRPC7 activation but at the same time reports exist implicating DAG in the activation of PKC, which negatively regulates all TRPC channels (Venkatachalam et al., 2003). This

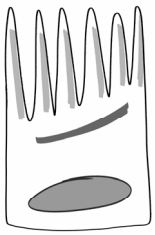
inhibitory regulation most likely does not apply to TRPV1 and TRPV4, as they are activated by PKC-dependent phosphorylation mechanisms (Nilius et al., 2004). Besides, PKC also decreases the CBF. Phosphorylation of PKC targets has been implicated in slowing ciliary beating in the airways of different species (Wong et al., 1998;Salathe et al., 1993), thus, unlikely to be involved in TRPV4-dependent ROCE mechanism.

TRPV4 displays a dual regulation by Ca^{2+}, there is both a Ca^{2+}-CaM-dependent potentiation and inactivation by Ca^{2+} ions (Strotmann et al., 2003) (Watanabe et al., 2003a). Half-maximal CaM binding to TRPV4 is at a [Ca^{2+}] of 200nM (Strotmann et al., 2003), while IC_{50} for TRPV4 channel inactivation by Ca^{2+} is around 400-600nM (Watanabe et al., 2003a). Considering that ATP-triggered sustained Ca^{2+} response in mammalian ciliated cells displays maximal increments of intracellular [Ca^{2+}] around 500-700nM (Zhang and Sanderson, 2003;Korngreen and Priel, 1996) TRPV4 channels should be half-active, and susceptible to be regulated by CaM. On the other hand, regulation of CBF by CaM is a controversial issue, with voices claiming a direct regulation by Ca^{2+} instead of Ca^{2+}-CaM (Salathe, 2006). Then, if CaM plays any role in ROCE mechanisms via TRPV4 may be limited to the modulation of the channel rather than been an activator.

Tyrosine phosphorylation of TRPV4 has been implicated in the channel activation in response to cell swelling (Xu et al., 2003b). Association of TRPV4 with Src family kinases, probably Lyn, requires an intact tyrosine phosphorylation site, Y253, located in a Src family kinase SH2 domain. However, opposing views exists regarding whether tyrosine phosphorylation of TRPV4 is necessary for its hypotonic-induced activation. While David Cohen and coworkers showed loss of function changing tyrosine 253 to alanine, Bernd Nilius' lab found normal hypotonic stimulation independently of which amino acid is placed at position 253 (Vriens et al., 2004). Tyrosine kinases have been also implicated in the enhanced osmotransduction and TRPV4-mediated hyperalgesia (Alessandri-Haber et al., 2004). Interestingly, tyrosine kinases are involved in ROCE mechanism. Evidence has accumulated in the literature showing that tyrosine phosphorylation is a common consequence associated with stimulation of cells that signal through the Gp-PLC pathway (Babnigg et al., 1997;Gutkind and Robbins, 1992;Igishi and Gutkind, 1998). Thus, tyrosine kinase-dependent phosphorylation of TRPV4 should evaluate as a possible mechanism linking channel activation to ROCE.

TRPV4 gating in response to mechanical and/or osmotic stimuli is primarily dependent on PLA_2-AA-EET pathway activation. A previous study has reported that in UTP stimulation of endothelial cells, $cPLA_2$-AA pathway is critical for normal endothelial Ca^{2+} response via TRPV4 (Marrelli S.P. et al., 2007). The authors propose that purinergic

stimulation results in PLC-IP_3 pathway activation, an increase of intracellular Ca^{2+}, activation of the Ca^{2+}-dependent $cPLA_2$ and subsequent production of AA. Finally, AA via P450 epoxygenase will generate EETs, final activators of TRPV4 channels. There are two major pathways responsible for the generation of AA in cells. One possibility is that following stimulation of tracheal ciliated cells with ATP, there is a $cPLA_2$ activation and production of AA. Based on this model, we propose that low ATP concentrations (200nM) do not produce enough AA and subsequent EET for TRPV4 activation, whereas higher doses of ATP result in stronger PLA_2 activation. Moreover, co-existence of PLA_2-AA-EET and IP_3-IP_3R pathways will potentiate TRPV4 gating (Fernandes et al., 2008). This signalling cross-talk may even involve other pathways. It is known that $cPLA_2$ can be also activated by MAPK-dependent phosphorylation (Lin L.L. et al., 1993). P2Y purinergic receptors activation by ATP leads to ERK and MAPK pathway activation (Katz et al., 2006;Neary J.T. et al., 2003;Igishi and Gutkind, 1998). The other possibility is the liberation of AA from DAG by the sequential actions of two lipases: DAG and monoacylglycerol (MAG) lipases (Burgoyne R. and Morgan A., 1990). The role of AA generation in the TRPV4-mediated ROCE mechanisms should be the focus of future research.



VI. CONCLUSIONS

1. TRPV4 channels are expressed in native ciliated cells of hamster oviduct and mouse trachea. The protein shows a polarized distribution, mainly at base of the cilia, where the Ca^{2+} signal is required for CBF regulation.

2. TRPV4 channels activity can be coupled to CBF increments in response to TRPV4-activating stimuli such as the specific TRPV4 agonist 4α-PDD, warmth and ATP, thereby confirming the participation of TRPV4 in CBF regulation.

3. TRPV4 channels present low impact in CBF autoregulatory mechanism under high viscous loads in mouse tracheal ciliated cells compared to hamster oviductal ciliated cells.

4. The gating of TRPV4 under mechanical and osmotic stimuli can be modulated by the ATP-PLC-IP_3 pathway. IP_3, through its receptor, sensitizes TRPV4 to the mechano- and osmotransducing 5',6'-EET messenger but not to thermal stimulation.

5. Osmo- and mechanically stimulated cells that do not reach a threshold level of PLA_2 activity, require IP_3-mediated sensitization mechanism for TRPV4 activation.

6. Ciliated cells stimulated with mechanical and hypotonic stimuli are dependent on the functional coupling between IP_3-IP_3R and TRPV4 channels to display complete Ca^{2+} responses with oscillatory pattern.

7. TRPV4 channels are also involved in the cell's response to ATP, one of the main physiological CBF activators in mouse tracheal ciliated cells.

8. TRPV4 participates in ATP-triggered ROCE mechanism, but not in SOCE.

VII. REFERENCES

1. Abramowitz, J., E.Yildirim, and L.Birnbaumer. 2007. The TRPC Family Ion channels. *In* TRP Ion Channel Function in Sensory Transduction and Cellular Signalling Cascades. W.Liedtke and S.Heller, editors. 1-30.

2. Afzelius, B.A. 1995. Role of cilia in human health. *Cell Motil. Cytoskeleton* 32:95-97.

3. Afzelius, B.A. 2004. Cilia-related diseases. *J. Pathol.* 204:470-477.

4. Afzelius, B.A. 1976. A human syndrome caused by immotile cilia. *Science* 193:317-319.

5. Alessandri-Haber, N., O.A.Dina, J.J.Yeh, C.A.Parada, D.B.Reichling, and J.D.Levine. 2004. Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. *J. Neurosci.* 24:4444-4452.

6. Alessandri-Haber, N., J.J.Yeh, A.E.Boyd, C.A.Parada, X.Chen, D.B.Reichling, and J.D.Levine. 2003. Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* 39:497-511.

7. Alexander, L.D., S.Alagarsamy, and J.G.Douglas. 2004. Cyclic stretch-induced cPLA2 mediates ERK 1/2 signaling in rabbit proximal tubule cells. *Kidney Int.* 65:551-563.

8. Amiri, H., G.Schultz, and M.Schaefer. 2003. FRET-based analysis of TRPC subunit stoichiometry. *Cell Calcium* 33:463-470.

9. Andrade, Y.N., J.Fernandes, E.Vazquez, J.M.Fernandez-Fernandez, M.Arniges, T.M.Sanchez, M.Villalon, and M.A.Valverde. 2005. TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *J. Cell Biol.* 168:869-874.

10. Arniges, M., J.M.Fernandez-Fernandez, N.Albrecht, M.Schaefer, and M.A.Valverde. 2006. Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking. *J. Biol. Chem.* 281:1580-1586.

11. Arniges, M., E.Vazquez, J.M.Fernandez-Fernandez, and M.A.Valverde. 2004. Swelling-activated Ca2+ entry via TRPV4 channel is defective in cystic fibrosis airway epithelia. *J. Biol. Chem.* 279:54062-54068.

12. Atri, A., J.Amundson, D.Clapham, and J.Sneyd. 1993. A single-pool model for intracellular calcium oscillations and waves in the Xenopus laevis oocyte. *Biophys. J.* 65:1727-1739.

13. Babnigg, G., S.R.Bowersox, and M.L.Villereal. 1997. The role of pp60c-src in the regulation of calcium entry via store-operated calcium channels. *J Biol. Chem.* 272:29434-29437.

14. Baltz, J.M. 2001. Osmoregulation and cell volume regulation in the preimplantation embryo. *Curr. Top. Dev. Biol.* 52:55-106.

15. Bandell, M., L.J.Macpherson, and A.Patapoutian. 2007. From chills to chilis: mechanisms for thermosensation and chemesthesis via thermoTRPs. *Curr. Opin. Neurobiol.* 17:490-497.

16. Barrera, N.P., B.Morales, and M.Villalon. 2004. Plasma and intracellular membrane inositol 1,4,5-trisphosphate receptors mediate the Ca(2+) increase associated with the ATP-induced increase in ciliary beat frequency. *Am. J. Physiol Cell Physiol* 287:C1114-C1124.

17. Basavappa, S., S.F.Pedersen, N.K.Jorgensen, J.C.Ellory, and E.K.Hoffmann. 1998. Swelling-induced arachidonic acid release via the 85-kDa cPLA2 in human neuroblastoma cells. *J. Neurophysiol.* 79:1441-1449.

18. Bateman, J.R., D.Pavia, N.F.Sheahan, J.E.Agnew, and S.W.Clarke. 1983. Impaired tracheobronchial clearance in patients with mild stable asthma. *Thorax* 38:463-467.

19. Benham, C.D., M.J.Gunthorpe, and J.B.Davis. 2003. TRPV channels as temperature sensors. *Cell Calcium* 33:479-487.

20. Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. *Nature* 361:315-325.

21. Berridge, M.J. 1995. Capacitative calcium entry. *Biochem. J.* 312 (Pt 1):1-11.

22. Berridge,M.J. Module2: Cell signalling pathways. Cell Signalling Biology - www.cellsignallingbiology.org . 2006.

23. Berridge, M.J. 1997. The AM and FM of calcium signalling. *Nature* 386:759-760.

24. Berridge, M.J., M.D.Bootman, and H.L.Roderick. 2003. Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* 4:517-529.

25. Berridge, M.J. and R.F.Irvine. 1989. Inositol phosphates and cell signalling. *Nature* 341:197-205.

26. Berridge, M.J., P.Lipp, and M.D.Bootman. 2000. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1:11-21.

27. Birder, L.A., A.J.Kanai, W.C.de Groat, S.Kiss, M.L.Nealen, N.E.Burke, K.E.Dineley, S.Watkins, I.J.Reynolds, and M.J.Caterina. 2001. Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells. *Proc. Natl. Acad. Sci. U. S. A* 98:13396-13401.

28. Birder, L.A., Y.Nakamura, S.Kiss, M.L.Nealen, S.Barrick, A.J.Kanai, E.Wang, G.Ruiz, W.C.de Groat, G.Apodaca, S.Watkins, and M.J.Caterina. 2002. Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1. *Nat. Neurosci.* 5:856-860.

29. Boitano, S., M.J.Sanderson, and E.R.Dirksen. 1994. A role for Ca(2+)-conducting ion channels in mechanically-induced signal transduction of airway epithelial cells. *J. Cell Sci.* 107 (Pt 11):3037-3044.

30. Boucher, R.C. 2003. Regulation of airway surface liquid volume by human airway epithelia. *Pflugers Arch.* 445:495-498.

31. Boucher, R.C. 2002. An overview of the pathogenesis of cystic fibrosis lung disease. *Adv. Drug Deliv. Rev.* 54:1359-1371.

32. Boulay, G., D.M.Brown, N.Qin, M.Jiang, A.Dietrich, M.X.Zhu, Z.Chen, M.Birnbaumer, K.Mikoshiba, and L.Birnbaumer. 1999. Modulation of Ca(2+) entry by polypeptides of the inositol 1,4, 5-trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP3R in store depletion-activated Ca(2+) entry. *Proc. Natl. Acad. Sci. U. S. A* 96:14955-14960.

33. Braiman, A. and Z.Priel. 2001. Intracellular stores maintain stable cytosolic Ca(2+) gradients in epithelial cells by active Ca(2+) redistribution. *Cell Calcium* 30:361-371.

34. Brokaw, C.J. 1982. Introduction: generation of the bending cycle in cilia and flagella. *Prog. Clin. Biol. Res.* 80:137-141.

35. Brokaw, C.J. 1994. Control of flagellar bending: a new agenda based on dynein diversity. *Cell Motil. Cytoskeleton* 28:199-204.

36. Burgoyne R. and Morgan A. The control of free arachidonic acid levels. Trends in Biochemical Science 15, 365-366. 1990.
Ref Type: Abstract

37. Caterina, M.J., T.A.Rosen, M.Tominaga, A.J.Brake, and D.Julius. 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398:436-441.

38. Chilvers, M.A. and C.O'Callaghan. 2000. Local mucociliary defence mechanisms. *Paediatr. Respir. Rev.* 1:27-34.

39. Christensen, A.P. and D.P.Corey. 2007. TRP channels in mechanosensation: direct or indirect activation? *Nat. Rev. Neurosci.* 8:510-521.

40. Chuang, H.H., E.D.Prescott, H.Kong, S.Shields, S.E.Jordt, A.I.Basbaum, M.V.Chao, and D.Julius. 2001. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 411:957-962.

41. Chung, M.K., H.Lee, A.Mizuno, M.Suzuki, and M.J.Caterina. 2004. TRPV3 and TRPV4 mediate warmth-evoked currents in primary mouse keratinocytes. *J. Biol. Chem.* 279:21569-21575.

42. Ciura, S. and C.W.Bourque. 2006. Transient receptor potential vanilloid 1 is required for intrinsic osmoreception in organum vasculosum lamina terminalis neurons and for normal thirst responses to systemic hyperosmolality. *J. Neurosci.* 26:9069-9075.

43. Clapham, D.E. 2003. TRP channels as cellular sensors. *Nature* 426:517-524.

44. Clarke, S.W. 1989. Rationale of airway clearance. *Eur. Respir. J. Suppl* 7:599s-603s.

45. Cohen, D.M. 2005b. TRPV4 and the mammalian kidney. *Pflugers Arch.* 451:168-175.

46. Cohen, D.M. 2005a. SRC family kinases in cell volume regulation. *Am. J. Physiol Cell Physiol* 288:C483-C493.

47. Colbert, H.A., T.L.Smith, and C.I.Bargmann. 1997. OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in Caenorhabditis elegans. *J. Neurosci.* 17:8259-8269.

48. Corey, D.P., J.Garcia-Anoveros, J.R.Holt, K.Y.Kwan, S.Y.Lin, M.A.Vollrath, A.Amalfitano, E.L.Cheung, B.H.Derfler, A.Duggan, G.S.Geleoc, P.A.Gray, M.P.Hoffman, H.L.Rehm, D.Tamasauskas, and D.S.Zhang. 2004. TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. *Nature* 432:723-730.

49. Corteling, R.L., S.Li, J.Giddings, J.Westwick, C.Poll, and I.P.Hall. 2004. Expression of transient receptor potential C6 and related transient receptor potential family members in human airway smooth muscle and lung tissue. *Am. J. Respir. Cell Mol. Biol.* 30:145-154.

50. Cortright, D.N. and A.Szallasi. 2004. Biochemical pharmacology of the vanilloid receptor TRPV1. An update. *Eur. J. Biochem.* 271:1814-1819.

51. De Smet P., J.B.Parys, S.Vanlingen, G.Bultynck, G.Callewaert, A.Galione, De Smedt H., and L.Missiaen. 1999. The relative order of IP3 sensitivity of types 1 and 3 IP3 receptors is pH dependent. *Pflugers Arch.* 438:154-158.

52. de, N.E., F.X.Real, and F.Posas. 2007. Mucins, osmosensors in eukaryotic cells? *Trends Cell Biol.* 17:571-574.

53. Delany, N.S., M.Hurle, P.Facer, T.Alnadaf, C.Plumpton, I.Kinghorn, C.G.See, M.Costigan, P.Anand, C.J.Woolf, D.Crowther, P.Sanseau, and S.N.Tate. 2001. Identification and characterization of a novel human vanilloid receptor-like protein, VRL-2. *Physiol Genomics* 4:165-174.

54. Dellis, O., S.G.Dedos, S.C.Tovey, U.R.Taufiq, S.J.Dubel, and C.W.Taylor. 2006. Ca2+ entry through plasma membrane IP3 receptors. *Science* 313:229-233.

55. Delmotte, P. and M.J.Sanderson. 2006. Ciliary beat frequency is maintained at a maximal rate in the small airways of mouse lung slices. *Am. J. Respir. Cell Mol. Biol.* 35:110-117.

56. Dhaka, A., V.Viswanath, and A.Patapoutian. 2006. Trp ion channels and temperature sensation. *Annu. Rev. Neurosci.* 29:135-161.

57. Di, P.F., I.A.Belyantseva, H.J.Kim, T.F.Vogt, B.Kachar, and K.Noben-Trauth. 2002. Mutations in Mcoln3 associated with deafness and pigmentation defects in varitint-waddler (Va) mice. *Proc. Natl. Acad. Sci. U. S. A* 99:14994-14999.

58. Dietrich, A., M.Schnitzler, H.Kalwa, U.Storch, and T.Gudermann. 2005. Functional characterization and physiological relevance of the TRPC3/6/7 subfamily of cation channels. *Naunyn Schmiedebergs Arch. Pharmacol.* 371:257-265.

59. Earley, S., T.J.Heppner, M.T.Nelson, and J.E.Brayden. 2005. TRPV4 forms a novel Ca2+ signaling complex with ryanodine receptors and BKCa channels. *Circ. Res.* 97:1270-1279.

60. Engelke, M., O.Friedrich, P.Budde, C.Schafer, U.Niemann, C.Zitt, E.Jungling, O.Rocks, A.Luckhoff, and J.Frey. 2002. Structural domains required for channel function of the mouse transient receptor potential protein homologue TRP1beta. *FEBS Lett.* 523:193-199.

61. Erler, I., D.Hirnet, U.Wissenbach, V.Flockerzi, and B.A.Niemeyer. 2004. Ca^{2+}-selective transient receptor potential V channel architecture and function require a specific ankyrin repeat. *J. Biol. Chem.* 279:34456-34463.

62. Evans, J.H. and M.J.Sanderson. 1999. Intracellular calcium oscillations regulate ciliary beat frequency of airway epithelial cells. *Cell Calcium* 26:103-110.

63. Felix, J.A., M.L.Woodruff, and E.R.Dirksen. 1996. Stretch increases inositol 1,4,5-trisphosphate concentration in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 14:296-301.

64. Feranchak, A.P., R.B.Doctor, M.Troetsch, K.Brookman, S.M.Johnson, and J.G.Fitz. 2004. Calcium-dependent regulation of secretion in biliary epithelial cells: the role of apamin-sensitive SK channels. *Gastroenterology* 127:903-913.

65. Fernandes, J., I.M.Lorenzo, Y.N.Andrade, A.Garcia-Elias, S.A.Serra, J.M.Fernandez-Fernandez, and M.A.Valverde. 2008. IP3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid. *J. Cell Biol.* 181:143-155.

66. Fernandez-Fernandez, J.M., M.Nobles, A.Currid, E.Vazquez, and M.A.Valverde. 2002. Maxi K+ channel mediates regulatory volume decrease response in a human bronchial epithelial cell line. *Am. J Physiol Cell Physiol* 283:C1705-C1714.

67. Fink, J.B. 2007. Forced expiratory technique, directed cough, and autogenic drainage. *Respir. Care* 52:1210-1221.

68. Florian, J.A., J.R.Kosky, K.Ainslie, Z.Pang, R.O.Dull, and J.M.Tarbell. 2003. Heparan sulfate proteoglycan is a mechanosensor on endothelial cells. *Circ. Res.* 93:e136-e142.

69. Forteza, R., T.Lieb, T.Aoki, R.C.Savani, G.E.Conner, and M.Salathe. 2001. Hyaluronan serves a novel role in airway mucosal host defense. *FASEB J.* 15:2179-2186.

70. Gao, X., L.Wu, and R.G.O'Neil. 2003. Temperature-modulated diversity of TRPV4 channel gating: activation by physical stresses and phorbol ester derivatives through protein kinase C-dependent and -independent pathways. *J. Biol. Chem.* 278:27129-27137.

71. Garcia-Sanz, N., A.Fernandez-Carvajal, C.Morenilla-Palao, R.Planells-Cases, E.Fajardo-Sanchez, G.Fernandez-Ballester, and A.Ferrer-Montiel. 2004. Identification of a tetramerization domain in the C terminus of the vanilloid receptor. *J. Neurosci.* 24:5307-5314.

72. Gayner, S.M. and T.V.McCaffrey. 1998. Muscarinic ciliostimulation requires endogenous prostaglandin production. *Am. J. Rhinol.* 12:203-207.

73. Geary, C.A., C.W.Davis, A.M.Paradiso, and R.C.Boucher. 1995. Role of CNP in human airways: cGMP-mediated stimulation of ciliary beat frequency. *Am. J Physiol* 268:L1021-L1028.

74. Gertsberg, I., V.Hellman, M.Fainshtein, S.Weil, S.D.Silberberg, M.Danilenko, and Z.Priel. 2004. Intracellular Ca2+ regulates the phosphorylation and the dephosphorylation of ciliary proteins via the NO pathway. *J. Gen. Physiol* 124:527-540.

75. Gheber, L., A.Korngreen, and Z.Priel. 1998. Effect of viscosity on metachrony in mucus propelling cilia. *Cell Motil. Cytoskeleton* 39:9-20.

76. Goldbeter, A., G.Dupont, and M.J.Berridge. 1990. Minimal model for signal-induced Ca2+ oscillations and for their frequency encoding through protein phosphorylation. *Proc. Natl. Acad. Sci. U. S. A* 87:1461-1465.

77. Green, A., L.A.Smallman, A.C.Logan, and A.B.Drake-Lee. 1995. The effect of temperature on nasal ciliary beat frequency. *Clin. Otolaryngol. Allied Sci.* 20:178-180.

78. Grimm, C., R.Kraft, S.Sauerbruch, G.Schultz, and C.Harteneck. 2003. Molecular and functional characterization of the melastatin-related cation channel TRPM3. *J. Biol. Chem.* 278:21493-21501.

79. Gudi, S., I.Huvar, C.R.White, N.L.McKnight, N.Dusserre, G.R.Boss, and J.A.Frangos. 2003. Rapid activation of Ras by fluid flow is mediated by Galpha(q) and Gbetagamma subunits of heterotrimeric G proteins in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 23:994-1000.

80. Gudi, S., J.P.Nolan, and J.A.Frangos. 1998. Modulation of GTPase activity of G proteins by fluid shear stress and phospholipid composition. *Proc. Natl. Acad. Sci. U. S. A* 95:2515-2519.

81. Guler, A.D., H.Lee, T.Iida, I.Shimizu, M.Tominaga, and M.Caterina. 2002a. Heat-evoked activation of the ion channel, TRPV4. *J. Neurosci.* 22:6408-6414.

82. Guler, A.D., H.Lee, T.Iida, I.Shimizu, M.Tominaga, and M.Caterina. 2002b. Heat-evoked activation of the ion channel, TRPV4. *J. Neurosci.* 22:6408-6414.

83. Gutkind, J.S. and K.C.Robbins. 1992. Activation of transforming G protein-coupled receptors induces rapid tyrosine phosphorylation of cellular proteins, including p125FAK and the p130 v-src substrate. *Biochem. Biophys. Res. Commun.* 188:155-161.

84. Halbert, S.A., P.Y.Tam, and R.J.Blandau. 1976. Egg transport in the rabbit oviduct: the roles of cilia and muscle. *Science* 191:1052-1053.

85. Hallows, R.H. and P.A.Knauf. 2008. Principles of cell volume regulation. *In* Cellular and Molecular Physiology of Cell Volume Regulation. K.Strange, editor. Boca Raton: CRC Press, 3-29.

86. Han, B., X.H.Bai, M.Lodyga, J.Xu, B.B.Yang, S.Keshavjee, M.Post, and M.Liu. 2004. Conversion of mechanical force into biochemical signaling. *J. Biol. Chem.* 279:54793-54801.

87. Hansen, M., S.Boitano, E.R.Dirksen, and M.J.Sanderson. 1993. Intercellular calcium signaling induced by extracellular adenosine 5'-triphosphate and mechanical stimulation in airway epithelial cells. *J. Cell Sci.* 106 (Pt 4):995-1004.

88. Hansen, M., S.Boitano, E.R.Dirksen, and M.J.Sanderson. 1995. A role for phospholipase C activity but not ryanodine receptors in the initiation and propagation of intercellular calcium waves. *J. Cell Sci.* 108 (Pt 7):2583-2590.

89. Harteneck, C. and G.Schultz. 2007. TRPV4 and TRPM3 as Volume-Regulated Cation Channels. *In* TRP Ion Channel Function in Sensory Transduction and Cellular Signalling Cascades. W.Liedtke and S.Heller, editors. 141-150.

90. Hartmannsgruber, V., W.T.Heyken, M.Kacik, A.Kaistha, I.Grgic, C.Harteneck, W.Liedtke, J.Hoyer, and R.Kohler. 2007a. Arterial response to shear stress critically depends on endothelial TRPV4 expression. *PLoS. ONE.* 2:e827.

91. Hartmannsgruber, V., W.T.Heyken, M.Kacik, A.Kaistha, I.Grgic, C.Harteneck, W.Liedtke, J.Hoyer, and R.Kohler. 2007b. Arterial response to shear stress critically depends on endothelial TRPV4 expression. *PLoS. ONE.* 2:e827.

92. Harvey Lodish, Arnold Berk, Lawrence S.Zipursky, Paul Matsudaira, David Baltimore, and James Darnell. 2008. Molecular Cell Biology. Freeman W.H., editor.

93. Hellwig, N., N.Albrecht, C.Harteneck, G.Schultz, and M.Schaefer. 2005. Homo- and heteromeric assembly of TRPV channel subunits. *J. Cell Sci.* 118:917-928.

94. Hermoso, M., N.Barrera, B.Morales, S.Perez, and M.Villalon. 2001. Platelet activating factor increases ciliary activity in the hamster oviduct through epithelial production of prostaglandin E2. *Pflugers Arch.* 442:336-345.

95. Hilgemann, D.W., S.Feng, and C.Nasuhoglu. 2001. The complex and intriguing lives of PIP2 with ion channels and transporters. *Sci. STKE.* 2001:RE19.

96. Hille, B. 1992. Ionic channels of excitable membranes. Sinauer Associates Inc., Sunderland,Massachuusetts.

97. Hoenderop, J.G., B.Nilius, and R.J.Bindels. 2003a. Epithelial calcium channels: from identification to function and regulation. *Pflugers Arch.* 446:304-308.

98. Hoenderop, J.G., T.Voets, S.Hoefs, F.Weidema, J.Prenen, B.Nilius, and R.J.Bindels. 2003b. Homo- and heterotetrameric architecture of the epithelial Ca2+ channels TRPV5 and TRPV6. *EMBO J.* 22:776-785.

99. Hoffmann, E.K. 2000. Intracellular signalling involved in volume regulatory decrease. *Cell Physiol Biochem* 10:273-288.

100. Hoffmann, E.K. and P.B.Dunham. 1995. Membrane mechanisms and intracellular signalling in cell volume regulation. *Int. Rev. Cytol.* 161:173-262.

101. Homolya, L., T.H.Steinberg, and R.C.Boucher. 2000. Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia. *J. Cell Biol.* 150:1349-1360.

102. Homolya, L., W.C.Watt, E.R.Lazarowski, B.H.Koller, and R.C.Boucher. 1999. Nucleotide-regulated calcium signaling in lung fibroblasts and epithelial cells from normal and P2Y(2) receptor (-/-) mice. *J. Biol. Chem.* 274:26454-26460.

103. Houtmeyers, E., R.Gosselink, G.Gayan-Ramirez, and M.Decramer. 1999. Regulation of mucociliary clearance in health and disease. *Eur. Respir. J.* 13:1177-1188.

104. Hoyer, J., A.Distler, W.Haase, and H.Gogelein. 1994. Ca2+ influx through stretch-activated cation channels activates maxi K+ channels in porcine endocardial endothelium. *Proc. Natl. Acad. Sci. USA* 91:2367-2371.

105. Huang,C.L. The Transient Receptor Potential Superfamily of Ion Channels. J Am Soc Nephrol 15, 1690-1699. 2004.

106. Igishi, T. and J.S.Gutkind. 1998. Tyrosine kinases of the Src family participate in signaling to MAP kinase from both Gq and Gi-coupled receptors. *Biochem. Biophys. Res. Commun.* 244:5-10.

107. Irvine, R.F. 1990. 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates--a possible mechanism. *FEBS Lett.* 263:5-9.

108. Jain, B., I.Rubinstein, R.A.Robbins, K.L.Leise, and J.H.Sisson. 1993. Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem. Biophys. Res. Commun.* 191:83-88.

109. Jakab, M., J.Furst, M.Gschwentner, G.Botta, M.L.Garavaglia, C.Bazzini, S.Rodighiero, G.Meyer, S.Eichmueller, E.Woll, S.Chwatal, M.Ritter, and M.Paulmichl. 2002. Mechanisms sensing and modulating signals arising from cell swelling. *Cell Physiol Biochem* 12:235-258.

110. Janssen, L.J. and C.Y.Kwan. 2007. ROCs and SOCs: What's in a name??? *Cell Calcium* 41:245-247.

111. Jia, Y., X.Wang, L.Varty, C.A.Rizzo, R.Yang, C.C.Correll, P.T.Phelps, R.W.Egan, and J.A.Hey. 2004. Functional TRPV4 channels are expressed in human airway smooth muscle cells. *Am. J. Physiol Lung Cell Mol. Physiol* 287:L272-L278.

112. Johnson, N.T., M.Villalon, F.H.Royce, R.Hard, and P.Verdugo. 1991. Autoregulation of beat frequency in respiratory ciliated cells. Demonstration by viscous loading. *Am. Rev Respir Dis* 144:1091-1094.

113. Katz, S., R.Boland, and G.Santillan. 2006. Modulation of ERK 1/2 and p38 MAPK signaling pathways by ATP in osteoblasts: involvement of mechanical stress-activated calcium influx, PKC and Src activation. *Int. J. Biochem. Cell Biol.* 38:2082-2091.

114. Kawakami M., Nagira T., Hayashi T., Shimamoto C., Kubota T., Mori H., Yoshida H., and Nakahari T. Hypo-osmotic potentiation of acetycholine-stimulated ciliary beat frequency through ATP release in rat tracheal ciliary cells. Exp Physiol. 89[6], 739-751. 2004.

115. Kerem, E., T.Bistritzer, A.Hanukoglu, T.Hofmann, Z.Zhou, W.Bennett, E.MacLaughlin, P.Barker, M.Nash, L.Quittell, R.Boucher, and M.R.Knowles. 1999. Pulmonary epithelial sodium-channel dysfunction and excess airway liquid in pseudohypoaldosteronism. *N. Engl. J. Med.* 341:156-162.

116. Khatib, L., D.E.Golan, and M.Cho. 2004. Physiologic electrical stimulation provokes intracellular calcium increase mediated by phospholipase C activation in human osteoblasts. *FASEB J.* 18:1903-1905.

117. Kim,J., Chung Y.D., Park D.Y., Choi S., Shin D.W., Soh H., Lee H.W., Son W., Yim J., Park C.S., Kernan M.J., and Kim C. A TRPV family ion channel required for hearing in Drosophila. Nature 424, 81-84. 2003.

118. Kiselyov, K., D.M.Shin, J.Y.Kim, J.P.Yuan, and S.Muallem. 2007. TRPC channels: interacting proteins. *Handb. Exp. Pharmacol.*559-574.

119. Knowles, M.R. and R.C.Boucher. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest* 109:571-577.

120. Korngreen, A. and Z.Priel. 1996. Purinergic stimulation of rabbit ciliated airway epithelia: control by multiple calcium sources. *J. Physiol* 497 (Pt 1):53-66.

121. Kowase, T., Y.Nakazato, O.Yoko, A.Morikawa, and I.Kojima. 2002. Immunohistochemical localization of growth factor-regulated channel (GRC) in human tissues. *Endocr. J.* 49:349-355.

122. Kultgen, P.L., S.K.Byrd, L.E.Ostrowski, and S.L.Milgram. 2002. Characterization of an A-kinase anchoring protein in human ciliary axonemes. *Mol. Biol. Cell* 13:4156-4166.

123. LaMotte, R.H. and J.N.Campbell. 1978. Comparison of responses of warm and nociceptive C-fiber afferents in monkey with human judgments of thermal pain. *J. Neurophysiol.* 41:509-528.

124. Lang, F. 2007. Mechanisms and significance of cell volume regulation. *J. Am. Coll. Nutr.* 26:613S-623S.

125. Lang, F., G.L.Busch, M.Ritter, H.Volkl, S.Waldegger, E.Gulbins, and D.Haussinger. 1998. Functional significance of cell volume regulatory mechanisms. *Physiol Rev.* 78:247-306.

126. Lansley, A.B. and M.J.Sanderson. 1999. Regulation of airway ciliary activity by Ca^{2+}: simultaneous measurement of beat frequency and intracellular Ca^{2+}. *Biophys. J.* 77:629-638.

127. Lazarowski, E.R., R.C.Boucher, and T.K.Harden. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol. Pharmacol.* 64:785-795.

128. Lee H., Iida T., Mizuno A., Suzuki M., and Caterina M.J. Altered thermal selection behavior in mice lacking TRPV4. Journal of Neuroscience 25, 1304-1310. 2005.

129. Lehtonen, J.Y. and P.K.Kinnunen. 1995. Phospholipase A2 as a mechanosensor. *Biophys. J.* 68:1888-1894.

130. Lewis, R.S. 2007. The molecular choreography of a store-operated calcium channel. *Nature* 446:284-287.

131. Liao, Y., C.Erxleben, J.Abramowitz, V.Flockerzi, M.X.Zhu, D.L.Armstrong, and L.Birnbaumer. 2008. Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/Icrac channels. *Proc. Natl. Acad. Sci. U. S. A* 105:2895-2900.

132. Liedtke, W. 2005. TRPV4 as osmosensor: a transgenic approach. *Pflugers Arch.* 451:176-180.

133. Liedtke, W., Y.Choe, M.A.Marti-Renom, A.M.Bell, C.S.Denis, A.Sali, A.J.Hudspeth, J.M.Friedman, and S.Heller. 2000. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* 103:525-535.

134. Liedtke, W. and J.M.Friedman. 2003. Abnormal osmotic regulation in trpv4-/- mice. *Proc. Natl. Acad. Sci. U. S. A* 100:13698-13703.

135. Liedtke, W. and C.Kim. 2005. Functionality of the TRPV subfamily of TRP ion channels: add mechano-TRP and osmo-TRP to the lexicon! *Cell Mol. Life Sci.* 62:2985-3001.

136. Liedtke, W., D.M.Tobin, C.I.Bargmann, and J.M.Friedman. 2003. Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A* 100 Suppl 2:14531-14536.

137. Lin L.L., Wartmann M., Lin a.Y., Knopf J.L., Seth A., and Davis R.J. cPLA2 is phosphorylated and activated by MAK kinase. Cell 72, 269-278. 1993.

138. Lin, S.Y. and D.P.Corey. 2005. TRP channels in mechanosensation. *Curr. Opin. Neurobiol.* 15:350-357.

139. Lindberg, S. 1994. Morphological and functional studies of the mucociliary system during infections in the upper airways. *Acta Otolaryngol. Suppl* 515:22-24.

140. Liou, J., M.L.Kim, W.D.Heo, J.T.Jones, J.W.Myers, J.E.Ferrell, Jr., and T.Meyer. 2005. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr. Biol.* 15:1235-1241.

141. Lorenzo,I.M., Liedtke W., M.J.Sanderson, and M.A.Valverde. TRPV4 channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airways epithelial cells. *PNAS 2008 in press*.

142. Lumpkin, E.A. and M.J.Caterina. 2007. Mechanisms of sensory transduction in the skin. *Nature* 445:858-865.

143. Lyons, R.A., E.Saridogan, and O.Djahanbakhch. 2006. The reproductive significance of human Fallopian tube cilia. *Hum. Reprod. Update.* 12:363-372.

144. Ma, W., S.D.Silberberg, and Z.Priel. 2002. Distinct axonemal processes underlie spontaneous and stimulated airway ciliary activity. *J. Gen. Physiol* 120:875-885.

145. Machemer, H. 1972. Ciliary activity and the origin of metachrony in Paramecium: effects of increased viscosity. *J. Exp. Biol.* 57:239-259.

146. MacLeod, R.J. 1994. How an epithelial cell swells is determinant of the signaling pathways that activate RVD. *In* Cellular and mollecular physiology of cell volume regulation. K.Strange, editor. CRC Press, Boca Raton. 191-213.

147. Maroto, R., A.Raso, T.G.Wood, A.Kurosky, B.Martinac, and O.P.Hamill. 2005. TRPC1 forms the stretch-activated cation channel in vertebrate cells. *Nat. Cell Biol.* 7:179-185.

148. Marrelli S.P., O'Neil R.G., Brown R.C., and Bryan R.M.Jr. PLA2 and TRPV4 channels regulate endothelial calcium in cerebral arteries. Am J Physiol Heart Circ Physiol 292, H1390-H1397. 2007.

149. Matsui, H., S.H.Randell, S.W.Peretti, C.W.Davis, and R.C.Boucher. 1998. Coordinated clearance of periciliary liquid and mucus from airway surfaces. *J. Clin. Invest* 102:1125-1131.

150. McCarty,N.A. and R.G.O'Neil. Calcium signalling in cell volume regulation. Physiol.Rev. 72, 1037-1061. 1992.

151. McKemy, D.D. 2005. How cold is it? TRPM8 and TRPA1 in the molecular logic of cold sensation. *Mol. Pain* 1:16.

152. McKemy, D.D., W.M.Neuhausser, and D.Julius. 2002. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* 416:52-58.

153. Meyer, F.A. and A.Silberberg. 1980. The rheology and molecular organization of epithelial mucus. *Biorheology* 17:163-168.

154. Mizuno, A., N.Matsumoto, M.Imai, and M.Suzuki. 2003. Impaired osmotic sensation in mice lacking TRPV4. *Am. J. Physiol Cell Physiol* 285:C96-101.

155. Montell, C. 2005. The TRP superfamily of cation channels. *Sci. STKE.* 2005:re3.

156. Montell, C., L.Birnbaumer, and V.Flockerzi. 2002. The TRP channels, a remarkably functional family. *Cell* 108:595-598.

157. Morris, C.E. 1990. Mechanosensitive ion channels. *J. Membrane Biol.* 113:93-107.

158. Morse, D.M., J.L.Smullen, and C.W.Davis. 2001. Differential effects of UTP, ATP, and adenosine on ciliary activity of human nasal epithelial cells. *Am. J. Physiol Cell Physiol* 280:C1485-C1497.

159. Muglia, U. and P.M.Motta. 2001. A new morpho-functional classification of the Fallopian tube based on its three-dimensional myoarchitecture. *Histol. Histopathol.* 16:227-237.

160. Mwimbi, X.K., R.Muimo, M.W.Green, and A.Mehta. 2003b. Making human nasal cilia beat in the cold: a real time assay for cell signalling. *Cell Signal.* 15:395-402.

161. Mwimbi, X.K., R.Muimo, M.W.Green, and A.Mehta. 2003a. Making human nasal cilia beat in the cold: a real time assay for cell signalling. *Cell Signal.* 15:395-402.

162. Nagata, K., A.Duggan, G.Kumar, and J.Garcia-Anoveros. 2005. Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. *J. Neurosci.* 25:4052-4061.

163. Nash, M.S., K.W.Young, R.A.Challiss, and S.R.Nahorski. 2001. Intracellular signalling. Receptor-specific messenger oscillations. *Nature* 413:381-382.

164. Nauli, S.M., F.J.Alenghat, Y.Luo, E.Williams, P.Vassilev, X.Li, A.E.Elia, W.Lu, E.M.Brown, S.J.Quinn, D.E.Ingber, and J.Zhou. 2003. Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat. Genet.* 33:129-137.

165. Nauli, S.M. and J.Zhou. 2004. Polycystins and mechanosensation in renal and nodal cilia. *BIOESSAYS* 26:844-856.

166. Neary J.T., Kang Y., Willoughby K.A., and Ellis E.F. Activation of extracellular signal-regulated kinase by stretch-induced injury in astrocytes involves extracellular ATP and P2 purinergic receptors. Journal of Neuroscience 23, 2348-2356. 2003.
Ref Type: Abstract

167. Nijenhuis, T., J.G.Hoenderop, B.Nilius, and R.J.Bindels. 2003. (Patho)physiological implications of the novel epithelial Ca^{2+} channels TRPV5 and TRPV6. *Pflugers Arch.* 446:401-409.

168. Nilius, B., J.Prenen, U.Wissenbach, M.Bodding, and G.Droogmans. 2001. Differential activation of the volume-sensitive cation channel TRP12 (OTRPC4) and volume-regulated anion currents in HEK-293 cells. *Pflugers Arch.* 443:227-233.

169. Nilius, B., J.Vriens, J.Prenen, G.Droogmans, and T.Voets. 2004. TRPV4 calcium entry channel: a paradigm for gating diversity. *Am. J. Physiol Cell Physiol* 286:C195-C205.

170. North, R.A. 2002. Molecular physiology of P2X receptors. *Physiol Rev.* 82:1013-1067.

171. O'Neil, R.G. and S.Heller. 2005. The mechanosensitive nature of TRPV channels. *Pflugers Arch.* 451:193.

172. Okada, S.F., R.A.Nicholas, S.M.Kreda, E.R.Lazarowski, and R.C.Boucher. 2006. Physiological regulation of ATP release at the apical surface of human airway epithelia. *J. Biol. Chem.* 281:22992-23002.

173. Okada, Y., A.Hazama, and W.-L.Yuan. 1990. Stretch-induced activation of Ca^{2+}-permeable ion channels is involved in the volume regulation of hypotonically swollen epithelial cells. *Neur. Res.* 12:S5-S13.

174. Palmblad, J., B.Mossberg, and B.A.Afzelius. 1984. Ultrastructural, cellular, and clinical features of the immotile-cilia syndrome. *Annu. Rev. Med.* 35:481-492.

175. Paradiso, A.M., E.H.Cheng, and R.C.Boucher. 1991. Effects of bradykinin on intracellular calcium regulation in human ciliated airway epithelium. *Am. J. Physiol* 261:L63-L69.

176. Parekh, A.B. and R.Penner. 1997. Store depletion and calcium influx. *Physiol Rev.* 77:901-930.

177. Parker, J.C. 1993. In defense of cell volume? *Am. J. Physiol* 265:C1191-C1200.

178. Pasantes-Morales, H. and Morales-Mulia S. 2000. Influence of calcium on regulatory volume decrease: role of potassium channels. *Nephron* 86:414-427.

179. Pavia, D. 1987. Acute respiratory infections and mucociliary clearance. *Eur. J. Respir. Dis.* 71:219-226.

180. Pedersen, S., I.H.Lambert, S.M.Thoroed, and E.K.Hoffmann. 2000. Hypotonic cell swelling induces translocation of the alpha isoform of cytosolic phospholipase A2 but not the gamma isoform in Ehrlich ascites tumor cells. *Eur. J. Biochem.* 267:5531-5539.

181. Pedersen, S.F., E.K.Hoffmann, and J.W.Mills. 2001. The cytoskeleton and cell volume regulation. *Comp Biochem. Physiol A Mol. Integr. Physiol* 130:385-399.

182. Peier, A.M., A.Moqrich, A.C.Hergarden, A.J.Reeve, D.A.Andersson, G.M.Story, T.J.Earley, I.Dragoni, P.McIntyre, S.Bevan, and A.Patapoutian. 2002. A TRP channel that senses cold stimuli and menthol. *Cell* 108:705-715.

183. Perozo, E., D.M.Cortes, P.Sompornpisut, A.Kloda, and B.Martinac. 2002. Open channel structure of MscL and the gating mechanism of mechanosensitive channels. *Nature* 418:942-948.

184. Petersen, O.H. and N.V.Fedirko. 2001. Calcium signalling: store-operated channel found at last. *Curr. Biol.* 11:R520-R523.

185. Plant, T.D. and R.Strotmann. 2007. TPRV4: A Multifunctional Nonselective Cation Channel with Complex Regulation. *In* TRP Ion Channel Function in Sensory Transduction and Cellular Signalling Cascades. W.Liedtke and S.Heller, editors. 125-140.

186. Praetorius, H.A. and K.R.Spring. 2005. A physiological view of the primary cilium. *Annu. Rev. Physiol* 67:515-529.

187. Puchelle, E., J.M.Zahm, and D.Quemada. 1987. Rheological properties controlling mucociliary frequency and respiratory mucus transport. *Biorheology* 24:557-563.

188. Putney, J.W., Jr. 2007. Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* 42:103-110.

189. Putney, J.W., Jr. 1987. Formation and actions of calcium-mobilizing messenger, inositol 1,4,5-trisphosphate. *Am. J. Physiol* 252:G149-G157.

190. Putney, J.W., Jr. 1998. Calcium signaling: up, down, up, down...what's the point? *Science* 279:191-192.

191. Quinton,P.M. Physiological basis of cystic fibrosis: a historical perspective. Physiol.Rev. 79[1], S3-S22. 1999.

192. Ramsey, I.S., M.Delling, and D.E.Clapham. 2006. An introduction to TRP channels. *Annu. Rev. Physiol* 68:619-647.

193. Ratjen, F. and G.Doring. 2003. Cystic fibrosis. *Lancet* 361:681-689.

194. Rohacs, T. 2007. Regulation of TRP channels by PIP(2). *Pflugers Arch.* 453:753-762.

195. Rosado, J.A. 2006. Discovering the mechanism of capacitative calcium entry. *Am. J. Physiol Cell Physiol* 291:C1104-C1106.

196. Rosado, J.A., S.Jenner, and S.O.Sage. 2000. A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. *J. Biol. Chem.* 275:7527-7533.

197. Rutllant, J., M.Lopez-Bejar, P.Santolaria, J.Yaniz, and F.Lopez-Gatius. 2002. Rheological and ultrastructural properties of bovine vaginal fluid obtained at oestrus. *J. Anat.* 201:53-60.

198. Ruwhof, C., J.T.van Wamel, L.A.Noordzij, S.Aydin, J.C.Harper, and L.A.van der. 2001. Mechanical stress stimulates phospholipase C activity and intracellular calcium ion levels in neonatal rat cardiomyocytes. *Cell Calcium* 29:73-83.

199. Sachs, F. and C.E.Morris. 1998. Mechanosensitive ion channels in nonspecialized cells. *Rev Physiol Biochem. Pharmacol.* 132:1-77.

200. Salathé M., O'Riordan T.G., and Wanner A. 1997. Mucociliary clearance. *In* The lung: Scientific fundations. Crystal R.G., West J.B., Barnes P.J, and Weibel E.R, editors. Philadelphia. 2295-2308.

201. Salathe, M. 2006. Regulation of Mammalian Ciliary Beating. *Annu. Rev. Physiol* 69:401-422.

202. Salathe, M. and R.J.Bookman. 1999. Mode of Ca2+ action on ciliary beat frequency in single ovine airway epithelial cells. *J. Physiol* 520 Pt 3:851-865.

203. Salathe, M., E.J.Lipson, P.I.Ivonnet, and R.J.Bookman. 1997. Muscarinic signaling in ciliated tracheal epithelial cells: dual effects on Ca2+ and ciliary beating. *Am. J. Physiol* 272:L301-L310.

204. Salathe, M., M.M.Pratt, and A.Wanner. 1993. Protein kinase C-dependent phosphorylation of a ciliary membrane protein and inhibition of ciliary beating. *J. Cell Sci.* 106 (Pt 4):1211-1220.

205. Sanderson,M.J. Sanderson's web page. http://users.umassmed.edu/michael.sanderson/mjslab/index.htm . 2008.

206. Sanderson, M.J., A.C.Charles, and E.R.Dirksen. 1990. Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. *Cell Regul.* 1:585-596.

207. Sanderson, M.J. and E.R.Dirksen. 1986. Mechanosensitivity of cultured ciliated cells from the mammalian respiratory tract: implications for the regulation of mucociliary transport. *Proc. Natl. Acad. Sci. U. S. A* 83:7302-7306.

208. Sanderson, M.J. and E.R.Dirksen. 1991. Hypotonic conditions elevate intracellular calcium in traqueal epithelial cells. *Biophys. J.* 59:650a.

209. Sanderson, M.J. and M.A.Sleigh. 1981. Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. *J. Cell Sci.* 47:331-347.

210. Satir, P. and M.A.Sleigh. 1990a. The physiology of cilia and mucociliary interactions. *Annu. Rev. Physiol* 52:137-155.

211. Satir, P. and M.A.Sleigh. 1990b. The physiology of cilia and mucociliary interactions. *Annu. Rev Physiol* 52:137-155.

212. Schaefer, M. 2005. Homo- and heteromeric assembly of TRP channel subunits. *Pflugers Arch.*

213. Schlingmann, K.P., S.Weber, M.Peters, N.L.Niemann, H.Vitzthum, K.Klingel, M.Kratz, E.Haddad, E.Ristoff, D.Dinour, M.Syrrou, S.Nielsen, M.Sassen, S.Waldegger, H.W.Seyberth, and M.Konrad. 2002. Hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family. *Nat. Genet.* 31:166-170.

214. Schwiebert, E.M., D.J.Benos, M.Egan, M.J.Stutts, and W.B.Guggino. 1999. CFTR is a conductance regulator as well as a chloride channel. *Physiol. Rev.* 79:S145-S166.

215. Seybold, Z.V., A.T.Mariassy, D.Stroh, C.S.Kim, H.Gazeroglu, and A.Wanner. 1990. Mucociliary interaction in vitro: effects of physiological and inflammatory stimuli. *J. Appl. Physiol* 68:1421-1426.

216. Sheppard,D.N. and M.J.Welsh. Structure and function of the CFR chloride channel. Physiol.Rev. 79[1], S23-S45. 1999.

217. Shin, J.B., D.Adams, M.Paukert, M.Siba, S.Sidi, M.Levin, P.G.Gillespie, and S.Grunder. 2005b. Xenopus TRPN1 (NOMPC) localizes to microtubule-based cilia in epithelial cells, including inner-ear hair cells. *Proc. Natl. Acad. Sci. U. S. A* 102:12572-12577.

218. Shin, J.B., D.Adams, M.Paukert, M.Siba, S.Sidi, M.Levin, P.G.Gillespie, and S.Grunder. 2005a. Xenopus TRPN1 (NOMPC) localizes to microtubule-based cilia in epithelial cells, including inner-ear hair cells. *Proc. Natl. Acad. Sci. U. S. A* 102:12572-12577.

219. Shuttleworth, T.J. 1999. What drives calcium entry during [Ca2+]i oscillations?--challenging the capacitative model. *Cell Calcium* 25:237-246.

220. Shuttleworth, T.J. and O.Mignen. 2003. Calcium entry and the control of calcium oscillations. *Biochem. Soc. Trans.* 31:916-919.

221. Shuttleworth, T.J., J.L.Thompson, and O.Mignen. 2004. ARC channels: a novel pathway for receptor-activated calcium entry. *Physiology. (Bethesda.)* 19:355-361.

222. Sisson, J.H., J.A.Stoner, B.A.Ammons, and T.A.Wyatt. 2003. All-digital image capture and whole-field analysis of ciliary beat frequency. *J. Microsc.* 211:103-111.

223. Sleigh, M.A. 1983. Ciliary function in transport of mucus. *Eur. J. Respir. Dis. Suppl* 128 (Pt 1):287-292.

224. Sleigh, M.A., J.R.Blake, and N.Liron. 1988. The propulsion of mucus by cilia. *Am. Rev. Respir. Dis.* 137:726-741.

225. Smith, P.L., K.N.Maloney, R.G.Pothen, J.Clardy, and D.E.Clapham. 2006. Bisandrographolide from Andrographis paniculata activates TRPV4 channels. *J. Biol. Chem.* 281:29897-29904.

226. Smyth, J.T., W.I.Dehaven, B.F.Jones, J.C.Mercer, M.Trebak, G.Vazquez, and J.W.Putney, Jr. 2006. Emerging perspectives in store-operated Ca^{2+} entry: roles of Orai, Stim and TRP. *Biochim. Biophys. Acta* 1763:1147-1160.

227. Sneyd, J., K.Tsaneva-Atanasova, V.Reznikov, Y.Bai, M.J.Sanderson, and D.I.Yule. 2006. A method for determining the dependence of calcium oscillations on inositol trisphosphate oscillations. *Proc. Natl. Acad. Sci. U. S. A* 103:1675-1680.

228. Spassova, M.A., J.Soboloff, L.P.He, T.Hewavitharana, W.Xu, K.Venkatachalam, D.B.van Rossum, R.L.Patterson, and D.L.Gill. 2004. Calcium entry mediated by SOCs and TRP channels: variations and enigma. *Biochim. Biophys. Acta* 1742:9-20.

229. Spassova, M.A., J.Soboloff, L.P.He, W.Xu, M.A.Dziadek, and D.L.Gill. 2006. STIM1 has a plasma membrane role in the activation of store-operated Ca(2+) channels. *Proc. Natl. Acad. Sci. U. S. A* 103:4040-4045.

230. Spungin, B. and A.Silberberg. 1984. Stimulation of mucus secretion, ciliary activity, and transport in frog palate epithelium. *Am. J. Physiol* 247:C299-C308.

231. Stephens, R.E. and G.Prior. 1992. Dynein from serotonin-activated cilia and flagella: extraction characteristics and distinct sites for cAMP-dependent protein phosphorylation. *J. Cell Sci.* 103 (Pt 4):999-1012.

232. Strange, K. 1994. Are all cell volume changes the same? *News Physiol. Sci.* 9:223-228.

233. Strange, K. 2004. Cellular volume homeostasis. *Adv. Physiol Educ.* 28:155-159.

234. Strotmann, R., C.Harteneck, K.Nunnenmacher, G.Schultz, and T.D.Plant. 2000. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat. Cell Biol.* 2:695-702.

235. Strotmann, R., G.Schultz, and T.D.Plant. 2003. Ca2+-dependent potentiation of the nonselective cation channel TRPV4 is mediated by a C-terminal calmodulin binding site. *J. Biol. Chem.* 278:26541-26549.

236. Sutto, Z., G.E.Conner, and M.Salathe. 2004. Regulation of human airway ciliary beat frequency by intracellular pH. *J. Physiol* 560:519-532.

237. Suzuki, M., A.Mizuno, K.Kodaira, and M.Imai. 2003. Impaired pressure sensation in mice lacking TRPV4. *J. Biol. Chem.* 278:22664-22668.

238. Svartengren, M., C.H.Ericsson, K.Philipson, B.Mossberg, and P.Camner. 1989. Tracheobronchial clearance in asthma-discordant monozygotic twins. *Respiration* 56:70-79.

239. Svartengren, M., B.Mossberg, K.Philipson, and P.Camner. 1986. Mucociliary clearance in relation to clinical features in patients with bronchiectasis. *Eur. J. Respir. Dis.* 68:267-278.

240. Tamm, S. 1994. Ca^{2+} channels and signalling in cilia and flagella. *Trends Cell Biol.* 4:305-310.

241. Tamm, S.L. and M.Terasaki. 1994. Visualization of calcium transients controlling orientation of ciliary beat. *J. Cell Biol.* 125:1127-1135.

242. Tani, D., M.K.Monteilh-Zoller, A.Fleig, and R.Penner. 2007. Cell cycle-dependent regulation of store-operated I(CRAC) and Mg2+-nucleotide-regulated MagNuM (TRPM7) currents. *Cell Calcium* 41:249-260.

243. Tarran, R., B.R.Grubb, J.T.Gatzy, C.W.Davis, and R.C.Boucher. 2001. The relative roles of passive surface forces and active ion transport in the modulation of airway surface liquid volume and composition. *J. Gen. Physiol* 118:223-236.

244. Taylor C.W. Controlling Calcium Entry. Cell 111, 767-769. 2002.

245. Teilmann, S.C., A.G.Byskov, P.A.Pedersen, D.N.Wheatley, G.J.Pazour, and S.T.Christensen. 2005. Localization of transient receptor potential ion channels in primary and motile cilia of the female murine reproductive organs. *Mol. Reprod. Dev.* 71:444-452.

246. Tian, W., Y.Fu, D.H.Wang, and D.M.Cohen. 2005. Regulation of TRPV1 by a novel renally expressed rat TRPV1 splice variant. *Am. J. Physiol Renal Physiol*.

247. Tian, W., M.Salanova, H.Xu, J.N.Lindsley, T.T.Oyama, S.Anderson, S.Bachmann, and D.M.Cohen. 2004. Renal expression of osmotically responsive cation channel TRPV4 is restricted to water-impermeant nephron segments. *Am. J. Physiol Renal Physiol* 287:F17-F24.

248. Tominaga, M. and M.J.Caterina. 2004. Thermosensation and pain. *J. Neurobiol.* 61:3-12.

249. Trebak, M., G.Vazquez, G.S.Bird, and J.W.Putney, Jr. 2003. The TRPC3/6/7 subfamily of cation channels. *Cell Calcium* 33:451-461.

250. Tsavaler, L., M.H.Shapero, S.Morkowski, and R.Laus. 2001. Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. *Cancer Res.* 61:3760-3769.

251. Tuvia, S., A.Almagor, A.Bitler, S.Levin, R.Korenstein, and S.Yedgar. 1997. Cell membrane fluctuations are regulated by medium macroviscosity: evidence for a metabolic driving force. *Proc. Natl. Acad. Sci. U. S. A* 94:5045-5049.

252. Uzlaner, N. and Z.Priel. 1999. Interplay between the NO pathway and elevated [Ca^{2+}]i enhances ciliary activity in rabbit trachea. *J. Physiol* 516 (Pt 1):179-190.

253. Vazquez, E., M.Nobles, and M.A.Valverde. 2001. Defective regulatory volume decrease in human cystic fibrosis tracheal cells because of altered regulation of intermediate conductance Ca^{2+}- dependent potassium channels. *Proc. Natl. Acad. Sci. U. S. A* 98:5329-5334.

254. Vazquez-Juarez, E., G.Ramos-Mandujano, R.Hernandez-Benitez, and H.Pasantes-Morales. 2008. On the role of G-protein coupled receptors in cell volume regulation. *Cell Physiol Biochem* 21:1-14.

255. Venkatachalam, K., F.Zheng, and D.L.Gill. 2003. Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. *J. Biol. Chem.* 278:29031-29040.

256. Verdugo, P. 1984. Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: a new model. *Ciba Found. Symp.* 109:212-225.

257. Verdugo, P. 1991. Mucin exocytosis. *Am. Rev. Respir. Dis.* 144:S33-S37.

258. Verdugo, P. 1980. Ca^{2+}-dependent hormonal stimulation of ciliary activity. *Nature* 283:764-765.

259. Verdugo, P., B.V.Raess, and M.Villalon. 1983. The role of calmodulin in the regulation of ciliary movement in mammalian epithelial cilia. *J. Submicrosc. Cytol.* 15:95-96.

260. Voets, T., J.Prenen, J.Vriens, H.Watanabe, A.Janssens, U.Wissenbach, M.Bodding, G.Droogmans, and B.Nilius. 2002. Molecular determinants of permeation through the cation channel TRPV4. *J Biol. Chem.*

261. Voets, T., K.Talavera, G.Owsianik, and B.Nilius. 2005. Sensing with TRP channels. *Nat. Chem. Biol.* 1:85-92.

262. Vriens, J., G.Owsianik, B.Fisslthaler, M.Suzuki, A.Janssens, T.Voets, C.Morisseau, B.D.Hammock, I.Fleming, R.Busse, and B.Nilius. 2005. Modulation of the Ca2+ Permeable Cation Channel TRPV4 by Cytochrome P450 Epoxygenases in Vascular Endothelium. *Circ. Res.* 97:908-915.

263. Vriens, J., G.Owsianik, A.Janssens, T.Voets, and B.Nilius. 2007. Determinants of 4 alpha-phorbol sensitivity in transmembrane domains 3 and 4 of the cation channel TRPV4. *J. Biol. Chem.* 282:12796-12803.

264. Vriens, J., H.Watanabe, A.Janssens, G.Droogmans, T.Voets, and B.Nilius. 2004. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc. Natl. Acad. Sci. U. S. A* 101:396-401.

265. Wanner, A. 1990. The role of mucus in chronic obstructive pulmonary disease. *Chest* 97:11S-15S.

266. Warwick, W.J. 1983. Mechanisms of mucous transport. *Eur. J. Respir. Dis. Suppl* 127:162-167.

267. Watanabe, H., J.B.Davis, D.Smart, J.C.Jerman, G.D.Smith, P.Hayes, J.Vriens, W.Cairns, U.Wissenbach, J.Prenen, V.Flockerzi, G.Droogmans, C.D.Benham, and B.Nilius. 2002a. Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. *J Biol. Chem.* 277:13569-13577.

268. Watanabe, H., J.Vriens, A.Janssens, R.Wondergem, G.Droogmans, and B.Nilius. 2003a. Modulation of TRPV4 gating by intra- and extracellular Ca^{2+}. *Cell Calcium* 33:489-495.

269. Watanabe, H., J.Vriens, J.Prenen, G.Droogmans, T.Voets, and B.Nilius. 2003b. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* 424:434-438.

270. Watanabe, H., J.Vriens, S.H.Suh, C.D.Benham, G.Droogmans, and B.Nilius. 2002b. Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. *J. Biol. Chem.* 277:47044-47051.

271. Watson, P.A. 1991. Function follows form: generation of intracellular signals by cell deformation. *FASEB J.* 5:2013-2019.

272. Wedel, B., R.R.Boyles, J.W.Putney, Jr., and G.S.Bird. 2007. Role of the store-operated calcium entry proteins Stim1 and Orai1 in muscarinic cholinergic receptor-stimulated calcium oscillations in human embryonic kidney cells. *J. Physiol* 579:679-689.

273. Wehner, F., H.Olsen, H.Tinel, E.Kinne-Saffran, and R.K.Kinne. 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev. Physiol Biochem. Pharmacol.* 148:1-80.

274. Weiss, T., L.Gheber, V.Shoshan-Barmatz, and Z.Priel. 1992. Possible mechanism of ciliary stimulation by extracellular ATP: involvement of calcium-dependent potassium channels and exogenous Ca2+. *J. Membr. Biol.* 127:185-193.

275. Widdicombe, J.H. 2002. Regulation of the depth and composition of airway surface liquid. *J Anat.* 201:313-318.

276. Willumsen, N.J. and R.C.Boucher. 1992. Intracellular pH and its relationship to regulation of ion transport in normal and cystic fibrosis human nasal epithelia. *J. Physiol* 455:247-269.

277. Winters, S.L. and D.B.Yeates. 1997a. The interaction between ion transporters and the mucociliary transport system in the dog and baboon. *J. Appl. Physiol* 83:1348-1359.

278. Winters, S.L., C.W.Davis, and R.C.Boucher. 2007. Mechanosensitivity of mouse tracheal ciliary beat frequency: roles for Ca2+, purinergic signaling, tonicity, and viscosity. *Am. J. Physiol Lung Cell Mol. Physiol* 292:L614-L624.

279. Winters, S.L. and D.B.Yeates. 1997b. Roles of hydration, sodium, and chloride in regulation of canine mucociliary transport system. *J Appl. Physiol* 83:1360-1369.

280. Wissenbach, U., M.Bodding, M.Freichel, and V.Flockerzi. 2000. Trp12, a novel Trp related protein from kidney. *FEBS Lett.* 485:127-134.

281. Wong, L.B., I.F.Miller, and D.B.Yeates. 1990. Stimulation of tracheal ciliary beat frequency by capsaicin. *J. Appl. Physiol* 68:2574-2580.

282. Wong, L.B., C.L.Park, and D.B.Yeates. 1998. Neuropeptide Y inhibits ciliary beat frequency in human ciliated cells via nPKC, independently of PKA. *Am. J. Physiol* 275:C440-C448.

283. Worley, P.F., W.Zeng, G.N.Huang, J.P.Yuan, J.Y.Kim, M.G.Lee, and S.Muallem. 2007. TRPC channels as STIM1-regulated store-operated channels. *Cell Calcium* 42:205-211.

284. Wu, L., X.Gao, R.C.Brown, S.Heller, and R.G.O'Neil. 2007. Dual role of the TRPV4 channel as a sensor of flow and osmolality in renal epithelial cells. *Am. J. Physiol Renal Physiol* 293:F1699-F1713.

285. Wyatt, T.A., M.A.Forget, J.M.Adams, and J.H.Sisson. 2005. Both cAMP and cGMP are required for maximal ciliary beat stimulation in a cell-free model of bovine ciliary axonemes. *Am. J. Physiol Lung Cell Mol. Physiol* 288:L546-L551.

286. Xu, F., E.Satoh, and T.Iijima. 2003a. Protein kinase C-mediated Ca^{2+} entry in HEK 293 cells transiently expressing human TRPV4. *Br. J. Pharmacol.* 140:413-421.

287. Xu, H., Y.Fu, W.Tian, and D.M.Cohen. 2006. Glycosylation of the osmoresponsive transient receptor potential channel TRPV4 on Asn-651 influences membrane trafficking. *Am. J. Physiol Renal Physiol* 290:F1103-F1109.

288. Xu, H., H.Zhao, W.Tian, K.Yoshida, J.B.Roullet, and D.M.Cohen. 2003b. Regulation of a transient receptor potential (TRP) channel by tyrosine phosphorylation. SRC family kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its response to hypotonic stress. *J. Biol. Chem.* 278:11520-11527.

289. Xu, X.Z., H.S.Li, W.B.Guggino, and C.Montell. 1997. Coassembly of TRP and TRPL produces a distinct store-operated conductance. *Cell* 89:1155-1164.

290. Yang, H., S.Mergler, X.Sun, Z.Wang, L.Lu, J.A.Bonanno, U.Pleyer, and P.S.Reinach. 2005. TRPC4 knockdown suppresses epidermal growth factor-induced store-operated channel activation and growth in human corneal epithelial cells. *J. Biol. Chem.* 280:32230-32237.

291. Yeromin, A.V., S.L.Zhang, W.Jiang, Y.Yu, O.Safrina, and M.D.Cahalan. 2006. Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai. *Nature* 443:226-229.

292. Yuan, J.P., W.Zeng, G.N.Huang, P.F.Worley, and S.Muallem. 2007. STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat. Cell Biol.* 9:636-645.

293. Yule, D.I. and D.V.Gallacher. 1988. Oscillations of cytosolic calcium in single pancreatic acinar cells stimulated by acetylcholine. *FEBS Lett.* 239:358-362.

294. Zhang, L., D.Han, and M.J.Sanderson. 2005a. Effect of isoproterenol on the regulation of rabbit airway ciliary beat frequency measured with high-speed digital and fluorescence microscopy. *Ann. Otol. Rhinol. Laryngol.* 114:399-403.

295. Zhang, L. and M.J.Sanderson. 2003. Oscillations in ciliary beat frequency and intracellular calcium concentration in rabbit tracheal epithelial cells induced by ATP. *J. Physiol* 546:733-749.

296. Zhang, M.I. and R.G.O'Neil. 1999. The diversity of calcium channels and their regulation in epithelial cells. *Adv. Pharmacol.* 46:43-83.

297. Zhang, S.L., Y.Yu, J.Roos, J.A.Kozak, T.J.Deerinck, M.H.Ellisman, K.A.Stauderman, and M.D.Cahalan. 2005b. STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. *Nature* 437:902-905.

298. Zhou, X.L., A.F.Batiza, S.H.Loukin, C.P.Palmer, C.Kung, and Y.Saimi. 2003. The transient receptor potential channel on the yeast vacuole is mechanosensitive. *Proc. Natl. Acad. Sci. U. S. A* 100:7105-7110.

Back cover: confocal microscopy immunofluorescence of three ciliated epithelial cells isolated from hamster oviduct showing the actin cytosqueleton (red), the nuclei (blue) and TRPV4 (green).