PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Ciencias Fisiológicas

TESIS DOCTORAL

CONTROL OF BASAL CILIARY ACTIVITY AND RESPONSE TO MECHANICAL AND CHEMICAL STIMULI DEPEND ON EXTRACELLULAR ATP THROUGH AN AUTOCRINE/PARACRINE MECHANISM

Por

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Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias Biológicas con mención en Ciencias Fisiológicas

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Dedicada a mis hijos, como ejemplo de esfuerzo y perseverancia.

Para la felicidad y orgullo de mis padres.

Agradecida de mi esposo y de Dios.

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List of Abbreviations

8-SPT: 8-(p-Sulfophenyl)theophylline

ADO: adenosine

ADP: adenosine monophosphate

ALI: air-liquid interface

AMP: adenosine-monophosphate

APY: apyrase

ATP: adenosine-triphosphate

cAMP: cyclic adenosine monophosphate

CBF: ciliary beat frequency

CBX: carbenoxolone

Cx: connexin

DAPI: 4',6-diamidino-2-phenylindole

eATP: extracellular ATP

GA: glycyrrhetinic acid

HCs: hemichannels

$[Ca^{2+}]_i$: intracellular calcium concentration

IP3: inositol triphosphate

MFD: microphotodensitometry

MS: mechanical stimulation

oATP: oxidized ATP

P2X7-R: ionotropic purinergic receptor subtype 7

P2Y2-R: metabotropic purinergic receptor subtype 2

Panx: pannexin

PKA: protein kinase A

PKC: protein kinase C

PKG: protein kinase G

PROB: probenecid

SAVA: Sisson Ammons Video Analysis

SUR: suramin

Abstract

In mucociliary epithelia from respiratory tract and the oviduct, ciliary beat frequency (CBF) is the main factor that determines the effectiveness of mucociliary clearance (MCC). CBF can be measured in both, basal or enhanced conditions following mechanical or chemical stimulation. ATP is known to increase CBF in ciliated cells, effect that is mediated by purinergic receptor activation. The increase of CBF induced by ATP depends on Ca^{2+} release from intracellular compartments and Ca^{2+} entry from the extracellular medium. In respiratory epithelial cells, ATP is released constitutively or following mechanical stimulation (MS) through a mechanism possibly mediated by pannexin 1 (Panx1) or connexin 43 (Cx43) hemichannels (HCs). The aim of this study was to determine if ATP released from mucociliary epithelium contributes in the control mechanism of basal ciliary activity and mediates the increase of CBF induced by mechanical or chemical signals.

We used primary cultures of mouse trachea, human adenoid and rat oviduct epithelium to obtain confluent monolayers of ciliated cells. A reduction of extracellular ATP concentration (e[ATP]), using apyrase (APY), correlated with a reduction of basal CBF (~50%) and $[Ca^{2+}]_i$. Concomitant treatment with CBX, a Cx and Panx formed channel inhibitor, oATP, a P2X7-R antagonist and APY further reduced the basal CBF (~85%). MS produced an increase in CBF, occurrence of an intercellular calcium wave (ICW) and increased e[ATP], effects that were prevented by the pretreatment with APY. Increase of CBF and $[Ca^{2+}]_i$ induced by an exogenous stimulus of ATP were blocked by CBX, which also reduced e[ATP]. Our results suggest that ATP release from epithelial ciliated cells is necessary to maintain the basal ciliary activity and to mediate the CBF increase induced by mechanical and chemical stimulation. The underlying molecular mechanism might involve HCs for ATP release and P2X7-R activation by ATP in epithelial ciliated cells.

Resumen

En el epitelio mucociliado de las vías respiratorias y del oviducto, la frecuencia de batido ciliar (CBF) es el principal factor que determina la efectividad del transporte mucociliar. CBF se puede medir en un estado basal o incrementada por un estimulo químico o mecánico. ATP es un conocido estimulante de la CBF, efecto que es mediado por la activación de receptores purinérgicos. El incremento en la CBF inducido por ATP depende de la liberación de Ca^{2+} de almacenes intracelulares y de la entrada de Ca^{2+} desde el medio extracelular. En células del epitelio respiratorio, ATP es liberado al medio extracelular de manera constitutiva o en respuesta a una estimulación mecánica (MS), por un mecanismo que involucraría hemicanales (HCs) de panexina 1 (Panx1) o de conexina 43 (Cx43). El objetivo de este estudio fue determinar si ATP liberado por el epitelio mucociliado contribuye en el mecanismo de control de la actividad ciliar basal y si el incremento de la CBF inducido por una estimulación química y mecánica es mediado por la liberación de ATP. Se utilizaron cultivos primarios de células ciliadas de epitelio de tráquea de ratón, de adenoides de humano y de oviducto de rata. Una disminución en las concentraciones extracelulares de ATP con apirasa (APY), se correlacionó con una disminución de la CBF basal (~50%) y de $[Ca^{2+}]_i$. El tratamiento conjunto con CBX, un inhibidor de los HCs o canales formados por Cx y Panx, oATP, un antagonista de los receptores P2X7 y apirasa redujeron aún más la CBF basal (~85%). La estimulación mecánica produjo un incremento en la CBF, propagación de una onda de calcio y un incremento en las e[ATP]. Todos estos cambios fueron prevenidos con APY. El incremento en la CBF y en las $[Ca^{2+}]_i$ por un estímulo químico de ATP fueron bloqueados por CBX, el cual, también disminuyó la e[ATP]. Nuestros resultados sugieren que la liberación de ATP por las células de epitelio ciliado es necesaria para mantener la actividad ciliar basal y para mediar el incremento en la CBF inducido por una estimulación química y mecánica. El mecanismo molecular subyacente involucraría HCs para la liberación de ATP y la activación del receptor P2X7-R.

Introduction

1.1 Ciliary activity in mucociliary epithelia

Motile cilia are hair-like organelles that project from the plasma membrane of cells. They are involved in cell motility or fluid transport on the cell surface from unicellular to vertebrate organisms. In mammalian, ciliated cells cover the luminal surface of the respiratory tract and oviductal epithelia. The coordinated movement of the cilia propels the mucus produced by secretory cells, cleaning harmful particles or mobilizing the oocyte and embryos. This mechanism, called mucociliary transport or mucociliary clearance (MCC), plays a key role in preventing lung infections or infertility (Schmid & Salathe, 2011). MCC velocity is determined by the ciliary beat frequency (CBF) -the synchronic movement of cilia (metachrony) and the rheological properties of mucus. Correlation studies between CBF and mucus transport rate suggests that CBF is the main factor that determines the MCC (Duchateau et al., 1985). Basal CBF varies from 6 to 15 Hz depending on the species and the anatomic location of the epithelium under study (Nakahari et al., 2007); however few studies clarify its controlling mechanism.

Ciliary movement is produced by microtubule sliding inside the cilium. The energy for this process comes from the hydrolysis of intracellular ATP by dynein ATPase (Satir, 1980). In isolated cilia where the cell membrane had been extracted with detergent, spontaneous movement is partially recovered in presence of ATP and Mg^{2+} (Naitoh & Kaneko, 1972; Navarrette et al., 2012; Hard et al., 1988), indicating that intracellular ATP is necessary but does not account for all the controlling mechanisms of basal CBF.

Studies in unicellular organisms as *Paramecium* showed that membrane-regulated changes in the $[Ca^{2+}]_i$ control ciliary activity. The removal of extracellular Ca^{2+} with EGTA stops ciliary activity

and the subsequent injection of Ca^{2+} reactivates the movement of the cilia and the locomotion of *Paramecium*. In addition, an increase in $[Ca^{2+}]_i$ produces a shift in the direction of ciliary beating along with an increase in frequency. In the absence of stimulation, Ca^{2+}, that slowly leaks into the cell is pumped out at a similar rate to maintain a steady state (Eckert & Murakami, 1971; Eckert, 1972), thus, the influx of Ca^{2+} is necessary to maintain the ciliary activity. In respiratory and oviductal ciliated cells, elevation in $[Ca^{2+}]_i$ increases CBF via an initial action on the cilia and subsequently via cross talk with cAMP or cGMP pathways (Salathe, 2007; Schmid & Salathe, 2011).

1.2 Control mechanism of CBF

Many studies have characterized chemical signals that enhance the ciliary activity in ciliated cells from respiratory tract and oviduct. These signals, associated to neurotransmitters and inflammatory mediators, including acetylcholine (Salathe et al., 1997; Salathe, 2007; Kawakami et al., 2004), β-adrenergic agonists (Lindberg et al., 1995), UTP (Morse et al., 2001), ATP, adenosine (ADO) (González et al., 2013; Morales et al., 2000; Morse et al., 2001), tumor necrosis factor α (TNF-α) (Perez, 2011), prostaglandins (PGF2α) (Villalón et al., 1989) and bacterial components (LPS) (Carreño, 2013) can increase CBF.

ATP is a powerful activator of CBF in ciliated epithelial cells derived from frog esophagus (Levin et al., 1997), oviduct and respiratory tract from human, mouse, hamster, rat and rabbit (Villalón et al., 1989; Morales et al., 2000; Kerr, 2004; Jiao et al., 2012; Kawakami et al., 2004; Korngreen & Priel, 1996; Morse et al., 2001). The CBF increase induced by ATP is mediated by purinergic P2 receptor activation. There are two families of P2-receptors. The first family consists of P2X-

receptors, which are ligand-gated ion channels that allow the influx of extracellular cations, including Na^+ and Ca^{2+}, and leads to cellular depolarization (Li et al., 2010). The second family constitutes P2Y-receptors, which belong to the group of G-protein-coupled receptors. Ciliated cells from the respiratory tract express P2X4, P2X7, P2Y2, P2Y6 and P2Y11 (Kim et al., 2004; Hayashi et al., 2005; Ma et al., 2006; Kawakami et al., 2004). It has been demonstrated that the increase in CBF induced by ATP in primary cultures of oviductal ciliated cells is mediated by P2Y2-R activation (Morales et al., 2000). The increase in CBF induced by ATP has two components, which are, a fast initial increase component followed by a sustained component that decays gradually. The first component of the response is accounted for Ca^{2+} mobilization from intracellular Ca^{2+} stores induced by IP3 receptor activation. Subsequently, the amplification or maintenance of the CBF response induced by ATP is dependent of Ca^{2+} influx (Barrera et al., 2004; Kerr, 2004). In respiratory ciliated cells, the sustained component of the CBF in response to ATP is regulated by Ca^{2+} influx through P2X7-R (Surprenant and North, 2009; Ma et al., 1999 and 2006; Li et al., 2010). Additionally, the sustained component depends on ADO, a product of ATP hydrolysis, which increases cAMP levels through the A2b receptor activation (Morse et al., 2001). Finally, CBF increase induced by ATP is controlled by a signaling network that involves $[Ca^{2+}]_i$, cAMP, cGMP, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), nitric oxide (NO), PKG, and PKA (Barrera et al., 2004; Kerr, 2004; Braiman & Priel, 2008).

Mechanical and hyposmotic stimulation causes release of ATP in respiratory epithelial cells (Bodin & Burnstock, 2001; Yegutkin et al., 2000; Hansen et al., 1993; Kawakami et al., 2004; Button et al., 2007). Compressive stress increase apical e[ATP] in human tracheobronchial epithelial cells maintained under air-liquid interface (ALI) culture conditions. The measured increase in apical [ATP] was abolished by preincubating the cultures with apyrase, an enzyme that rapidly degrades ATP to AMP (Button et al., 2007). ATP can also be released constitutively

by resting epithelia (Lazarowski et al., 2004). e[ATP] (1-5 nM) were detected in human nasal and bronchial epithelial ALI cultures (Lazarowski et al., 2004). However, its role in the control mechanisms of basal CBF is unknown.

1.3 Synchronous mechanisms of ciliary activity in mucociliary epithelia

In mucociliary epithelia, cilia movements are synchronized in patterns known as metachronal waves, which are essential to maintain an efficient MCC. The mechanism by which cilia coordinate their overall motion has been opened to speculation. Hydrodynamic interactions have long been proposed as a mechanism, but there also remains the possibility of biochemical signaling (Brumley et al., 2012; Mitran, 2007).

Ciliated epithelial cells are permanently exposed to mechanical forces associated to changes in mucus viscosity, cell membrane deformation due to shear stress or pressure changes by normal breathing, coughing, sneezing or nose blowing (Button et al., 2007). A mechanical stimulation (MS) on the surface of a single cell induces an increase in CBF (Sanderson & Dirksen, 1986), which is transmitted to adjacent cells. This increase in CBF is associated with a rise in $[Ca^{2+}]_i$, a mechanism known as intercellular calcium wave (ICW) (Sanderson et al., 1990; Lansley & Sanderson, 1999). ICW has two potential mechanisms of transmission: first, the mechanically stimulated cell generates IP3, which permeates to adjacent cells via gap junctions inducing Ca^{2+} release from internal stores (Sanderson et al., 1990; Hansen et al., 1993); and second, a paracrine propagation through ATP release from the stimulated cell into the extracellular space, activating purinergic receptors in adjacent cells (Winters et al., 2007; Homolya et al., 2000). In respiratory and oviductal epithelium, the mechanism of ICW remains controversial.

Gap junctions are aggregates of intercellular channels formed by hemichannels (HCs) of connexins (Cx). Cx HCs of adjacent cells form a channel , allowing small molecules (<1,000 D) to pass through the cell membrane barrier (Eckert, 2006). The oviduct expresses Cx43 and Cx26 (Hermoso et al., 1997). Among Cxs 26, 32 and 43, only Cx43 was found in nasal mucosa and cultured epithelial cells (Yeh, 2003). Cx43 is the responsible for dye transfer through gap junctions in nasal epithelial cells (Yeh et al., 2007). Pannexin family (Panx1-3) is conformed for transmembrane channels or HCs with a similar structure to connexin. Panx1 is predominantly expressed in respiratory epithelium; however, rather than forming gap junctions, it operates as an HCs allowing the exchange of molecules between the cytoplasm and the extracellular space (Bruzzone et al., 2003; Ransford et al., 2009; Dahl and Keane, 2012; Ohbuchi et al., 2013).

Release of ATP is mediated by vesicular or channel mechanisms. Many channels, including CxHCs, Panx1, voltage dependent anion channel (VDAC), volume regulated anion channel (VRAC), cystic fibrosis transmembrane regulator (CFTR), maxi-anion channel and P2X7-R have been proposed to release ATP (Verdugo et al., 1991; Schock et al., 2008; Schwiebert & Zsembery, 2003; Locovei et al., 2006; Locovei et al., 2006b ; Huang et al., 2007). However, pharmacological studies, using the dual Cx/Panx HC blocker carbenoxolone (10-100 μM), the dual Panx1/ABC transporter blocker probenecid (1 mM) (Montalbetti et al., 2011; Barr et al., 2013) and specific blocking peptides (Orellana et al., 2013), have determined that Panx1 and Cx43 are the most relevant channels involved in ATP release. Panx1 HCs provide the pathway for ATP release in astrocytes (Orellana et al., 2013), erythrocytes (Montalbetti et al., 2011) and skeletal muscle fiber (Riquelme et al., 2013). Cx43 HCs release ATP in melanoma cells (Ohshima et al., 2012), astrocyte cultures (Stehberg et al., 2012) and keranocytes (Barr et al., 2013). In mouse trachea epithelium, ATP can be released by mucin granules from secreting cells and through Panx1 HCs (Okada et al., 2011; Sesma et al., 2013). Both mechanisms contribute independently to the release of ATP in

respiratory epithelial cells (Sesma et al., 2013). Panx1 HCs mediate the ATP release induced by hypotonic stress after TRPV4 stimulation (Seminario-Vidal et al., 2011). Hayoz et al. (2012) demonstrated that stimulation with ATP induces the release of more ATP by mouse neonatal olfactory epithelial cells. In addition, Panx1 and Cx43 HCs have been frequently associated to P2X7-R, where extracellular ATP (eATP) activates P2X7-R allowing the entry of Ca^{2+}. $[Ca^{2+}]_i$ regulates the opening of HCs (Panx1/Cx43) and consequently, the release of molecules, such as ATP (Kim & Kang, 2011; North, 2002; Pelegrin et al., 2006; Ohshima et al., 2012). However it is unknown whether eATP itself induces its own release in respiratory cells and which is the mechanism leading to ATP release and determines the basal CBF.

In summary, basal ciliary activity is modified by mechanical and chemical signals. Recent evidence indicates that ATP is constitutively released by the ciliated epithelium and that the addition of ATP increases ciliary activity. However, the role of eATP in the control of basal ciliary activity and increases in CBF induced by mechanical and chemical stimulation (ATP) is unknown. Therefore, this thesis proposed the following hypothesis:

Hypothesis

Activation of purinergic receptors (P2Y2/P2X7) by ATP released through hemichannels (Panx1/Cx43) contributes to the control mechanism of basal ciliary activity and mediates the increase on CBF induced by mechanical and chemical signals.

General Objective

Determine whether extracellular ATP, released by hemichannels (Panx/Cx) and associated to activation of purinergic receptors (P2Y2/P2X7), contributes to control the basal ciliary activity and mediates the increase of ciliary activity observed in response to mechanical and chemical stimuli.

Specific objectives

1. Determine whether modification of extracellular [ATP] affects basal ciliary activity in primary cultures of ciliated cells.

2. Determine the participation of HCs (Panx/Cx) and purinergic receptors in the control of basal ciliary activity in primary cultures of ciliated cells.

3. Determine whether the increase in CBF induced by a mechanical stimulus depends on ATP release in primary cultures of ciliated cells.

4. Determine whether the increase in CBF induced by a chemical stimulation with ATP depends on ATP release in primary cultures of ciliated cells.

Materials and Methods

4.1 Reagents

Adenosine 5′-triphosphate (ATP) disodium salt hydrate (A6144), Adenosine (ADO) hemisulfate salt (A7636), Carbenoxolone (CBX) disodium salt (C4790), Apyrase from potato (APY) (A7647), Levamisole hydrochloride (L9756), Adenosine 5´-triphosphate periodate oxidized sodium salt (oATP) (A6779),TNP-ATP hydrate (T4193), Gadolinium (III) chloride hexahydrate (G7532), 8-(p-sulfophenyl theophylline hydrate (A013), Adenosine 5′-diphosphate sodium salt (ADP) (A2754), KN-62 (I2142), Lanthanum (III) chloride heptahydrate (262072), 18β-Glycyrrhetinic acid (G10105), B, γ- methylene-ATP disodium salt (AMP-PCP) (M7510), Ebselen (E3520), Ethylene glycol-bis (2-aminoethylether) tetraacetic acid (EGTA) (E4378), Hank´s balanced salts (HBSS) (H1387) were obtained from Sigm-Aldrich (St. Louis, MO, USA). Probenecid (P36400) and DMEM/F12 were obtained from Invitrogen (CA, USA). Bapta-AM (B-6769) and Fura 2-AM, cell permeant (F-1221) were obtained from Molecular Probes (Eugene, OR, USA). Enliten ATP Assay System Bioluminescence detection kit for ATP measurement (FF2000) was obtained from Promega. Reagents from NHS medium: NaCl, KCl, Glucosa, NaHPO4, NaH2PO4, KH2PO4, vitamins, were obtained from Merck (NJ, USA). MgCl2, CaCl2, gelatin (G9391), pronase (P5147), sodium piruvate, aminoacids, phenol red were obtained from Sigma Aldrich (St Louis, MO, USA). Streptomycin, penicillin G and amphotericin B (15240) were obtained from Gibco. Bovine fetal serum (FBS) was obtained from Biological Industries (Israel).

Antibodies were Anti-Connexin 43 (P17302 Millipore); Anti-P2X7 (ab77413 Abcam); Anti-Panx1 (AV42783 Sigma); Anti-Panx2 (Diatheva); Anti-Panx3 (433270 Life technology); and Anti-P2Y2-R (#APR-010, Alomone Labs, Israel).

4.2 Epithelium tissue samples

4.2.1 Mouse trachea tissue samples

Tracheas from adult BALB/c male mice, weighting between 25-30 gr., 6 to 8 weeks, were obtained from The Research Animal Facility of Pontificia Universidad Católica de Chile. Animals were kept under controlled environment (temperature 23°C–25°C and 14-hour light, 10-hour dark cycles, food and water *ad libitum*). Animal euthanasia was performed by cervical dislocation. Tracheas were removed and washed with DMEM/F12 supplemented with antibiotics (10 μg/mL streptomycin, 100 U/mL penicillin G, 0.125 μg/mL amphotericin B) to remove traces of blood.

4.2.2 Rat oviduct epithelium samples

Oviducts from Sprague- Dawley female rats, weighting between 200 and 300 gr. and maintained under controlled environment (temperature 21°C–24°C and 12-hour light, 12-hour dark cycles, food and water *ad libitum*) were obtained. Animal euthanasia was performed by cervical dislocation, previous anesthesia. In the first day of estral cycle, oviducts were removed and placed in Hank´s balanced salt solution (HBSS) (pH 7.4, supplemented with antibiotics (10 μg/mL streptomycin, 100 U/mL penicillin G, 0.125 µg/mL amphotericin B) at room temperature.

All protocols were approved by the Bioethics and Biosafety Committee of the School of Biological Sciences, Pontificia Universidad Católica de Chile.

4.2.3 Adenoids tissue samples

Adenoid tissue was obtained from pediatric patients -aged between 3 and 12 years- undergoing adenoidectomy for obstructive pathology (adenoid or adenotonsillar hypertrophy) with parental informed consent. Exclusion criteria: Indication of adenoidectomy for otitis media with effusion, recurrent acute otitis media or chronic sinusitis, allergy and user of orally inhaled corticosteroids for asthma. The study design and informed consent were reviewed and approved by the Ethics Committee of Pontificia Universidad Católica de Chile.

Adenoid tissue was placed in a HBSS medium, pH 7.4, supplemented with antibiotics (10 μg/mL streptomycin, 100 U/mL penicillin G, 0.125 μg/mL amphotericin B).

4.2.4 Human sinonasal tissue

Patients over the age of 18 were recruited from the Division of Rhinology of the Department of Otorrhinolaryngology–Head and Neck Surgery at the University of Pennsylvania, as well as the otorrhinolaryngology clinic at the Philadelphia Veterans Administration Medical Center. Approval for the study was obtained from the institutional review boards at both institutions. Informed consent was obtained during the preoperative clinic visit or in the preoperative waiting room. Selection criterion for recruitment was scheduled sinonasal surgery. Exclusion criteria included a history of systemic granulomatous disease, active smoking, or the diagnosis of primary ciliary dyskinesia. Control patients had no history of chronic rhinosinsusitis and no evidence of sinonasal mucosal inflammation or infection on nasal endoscopy. All patients recruited for the study had stopped all intranasal medication, as well as systemic steroids and antibiotics, 4 wk prior to surgery. Prior to surgery, oxymetazoline-soaked pledgets were inserted

into each nasal cavity and subsequently removed. Specimens were acquired at the initiation of surgery prior to injection with lidocaine or epinephrine using a Blakesley forceps. Samples were 5 x 5 mm in size and were immediately placed in saline and placed on ice prior to transport to the laboratory.

4.3 Ciliated primary cultures from mouse tracheas, human adenoids and rat oviducts

Primary cultures of ciliated epithelium cells were obtained from mouse trachea, human adenoid and rat oviduct using a modified culture technique from Verdugo et al. (1980). Mouse trachea (with a previous longitudinally cut to expose the epithelium) or human adenoid biopsy were placed in a DMEM/F12 medium with antibiotics and pronase 0.05 % w/v and left half hour in CO_2 chamber at 37°C or overnight at 4°C respectively. Then, the tissue was transferred into a Petri dish with DMEM-F12 and 5% bovine fetal serum. The epithelium was mechanically removed with forceps and scissors, cut in 2- 4 mm^2 pieces and soaked in NHS (137 mM NaCl; 5.09 mM KCl; 1.14 mM Na_2HPO_4 x $2H_2O$; 0.18 mM KH_2PO_4; 0.923 mM $MgCl_2$ x $6H_2O$; 0.91 mM $CaCl_2$ x $2H_2O$; 4.07 mM $NaHCO_3$; 21.5 mM glucose, pH 7.4), supplemented with 1% of vitamins, 1% essential amino acids, 1% non-essential amino acid, 1% pyruvate and antibiotics: 0.2 mg/mL neomycin and 0.12 mg/mL penicillin. The pieces of mouse trachea, human adenoid and rat oviduct epithelia were placed on a coverslip pre-treated with 0.1 % gelatin in Rose chambers. Explants were covered with a sterile dialysis membrane (Spectra/Por*2, MWCO 12-14,000; #25218-468, VWR Scientific) pre-hydrated with distilled water. Rose chambers were filled with 2 mL of NHS medium; containing 10% heat inactivated horse serum (pH 7.2-7.4) and kept in an incubator at 37°C. The culture medium was renewed every 48h.

In these cultures a monolayer of ciliated cells grew surrounding the explants extracted from the respiratory or reproductive epithelium. In this study, it was not determined if the monolayer was formed by cell migration from the explants or by proliferating cells followed by differentiation. A culture was ready for use when a monolayer of ciliated cells showed spontaneous ciliary activity. Generally, primary cultures from mouse trachea were used in the fifth day of culture, and human adenoid and rat oviduct were used in the seventh day of culture.

4.4 Air-liquid interface (ALI) cultures

ALI cultures from mouse nasal septal, etmoides and trachea epithelium and from human seno nasal tissue were obtained using a culture technique described previously (Antunes et al., 2007; Woodworth et al., 2007). Mouse tissue epithelial cells from surgically excised nasal septum, etmoides or trachea were harvested following pronase (1.4 mg/mL) digestion and grown on Costar 6.5-mm-diameter transwell permeable filter supports (Corning Inc. Life Sciences, Lowell, MA, USA) submerged in culture medium DMEM and Nutrient Mixture Ham`s F-12 medium containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2% NuSerum™ (BD Biosciences). The medium was removed from the surface of the monolayers on day 7 after reaching confluence, and the cells were fed via the basal chamber.

Differentiation and ciliogenesis occurred within 10 to 14 days. Cultures were used between 21 and 30 days of growing.

4.5 Ciliary Beat Frequency (CBF) measurements

CBF was recorded using two different techniques: microphotodensitometric (MFD) measurement and the Sisson-Ammons Video Analysis (SAVA) system:

MFD technique: CBF was monitored and recorded by using MFD technique (Villalón et al., 1989). The spectral structure of light-scattering fluctuations produced by moving cilia of a single cell were detected with a photodiode, and the signal was processed on-line by a digital spectrum computer card (model R360, Rapid System) installed on a personal computer. The CBF was derived from the power spectrum obtained by fast Fourier transform of the data. The instant mean spectrum was recorded for further analysis. CBF was recorded in individual ciliated cells and was measured every minute.

The Sisson-Ammons Video Analysis (SAVA) system: explants of ciliated primary cultures were recorded at 35°C, with a high-speed video camera (Basler scA640) (100 frames/sec) mounted on a Nikon TE200 (40X) microscope. The video, 2.26 sec of length, was captured and files were reloaded and analyzed with the SAVA software to provide an immediate analysis of the CBF.
The software performs a power spectrum analysis (using a fast Fourier transform) and displays the number of readings vs. frequencies to determine the dominant frequency present in the whole field or in a region of interest (ROI) (Sisson et al., 2003); Whole Field Analysis was used, except when debris interfered with the measurement. To determine the calibration of SAVA system a low frequency oscillator model 202c was used. SAVA system is unable to determine frequency lower than 2 Hz, for this reason, the lack of movement of the cilia was determined visually. Recordings with less to 1000 points of movement and higher to 6000 points of movements (corresponding to ~<3 and >20 cells) were excluded.

In both techniques of CBF measurements, primary cultures were washed three times with warm HBSS. Cultures were kept for 15 min at 35°C to stabilize the basal CBF. CBF was recorded for 5 min to determine the baseline CBF (0%). After that, different treatments were added to the culture medium and CBF was measured for the next 10 or 20 min. Protocols with ALI cultures were done at 27°C. Considering that the CBF fell significantly as temperature decreased (Smith et al. 2008), each independent experiment was performed with a constant temperature in both set of cultures.

4.6 Quantification of extracellular ATP, ADP, AMP and ADO

Luminometric technique: Cultures were washed with HBSS medium three times. Then, cultures were kept for 20 min at 35°C. A sample of culture medium of 100 µl from primary cultures or 10 µl from the apical compartment of ALI-cultures was obtained. Sample was used directly or diluted in HBSS medium. Immediately, the sample was boiled 1 min to prevent the degradation of ATP by ecto-nucleotidases (Lazarowski et al., 2004). Samples were maintained in -70°C previously to the measurement. A calibration standard curve (between 0.05 nM to20 nM) was done in each experiment. Luciferin/luciferase (100 µL) was added on each well using a microinjector and luminescence was recorded for 10 sec (Luminoskan Ascent 2.5; Thermo Labsystems).

Nucleotide separation by HPLC and fluorescence detection: sample of 200 µl, obtained from the cultures, were derivatized for sensitive quantitation of adenyl purines as fluorescent 1,***N***6-etheno species (Todorov et al., 1996; Buvinic et al., 2009). To each sample was added 100 µl of phosphate-citrate buffer (77 mM Na2HPO4, 61 mM citric acid, pH 4) and maintained on ice until derivatization. After the addition of 10 µl of chloroacetaldehyde, samples were heated for 40 min

at 80 °C. The reaction was stopped by cooling the samples in ice for 5 min. Samples were kept for 24 h at 4 °C. Merck/Hitachi HPLC apparatus equipped with a fluorescence detector (VWR-Hitachi) was used for the identification and quantification of ethenylated species at excitation and emission wavelengths of 230 and 420 nm, respectively. Each sample (20 µl) was injected into a reverse-phase column (Chromolith® Performance RP-18e, 100-3 mm) equilibrated with the mobile phase (200 mm $NaH_2PO_4 \cdot H_2O$, 200 mm $Na_2HPO_4 \cdot 2H_2O$, 5 mm tetrabutylammonium, adjusted to pH 6 using H_3PO_4) at 1.5 ml/min. Data from test samples were compared against known concentrations of derivatized ADO, AMP, ADP, and ATP.

As primary cultures are not confluents, the area occupied by the cells in the glass was calculated and ATP levels were expressed as pmoles/cm^2. While, ALI cultures are confluent, mass of ATP levels was expressed directly.

4.7 Measurements of Ca^{2+}_i levels

Ca^{2+}_i level was determined using a fluorometric technique described previously (Barrera et al., 2004). Primary cultures of ciliated cells were loaded with 1.5 µM Fura-2AM for 1 h at 37°C. Cultures were washed three times and stabilized for 30 min. Experiments were performed at room temperature. Fluorescence images were acquired at excitation wavelength of 340 and 380 nm and detected at 510 nm with an Olympus fluorescence microscope coupled to an image acquisition system (Metafluor, Universal Imaging Corporation, v6.1). To evaluate the intercellular calcium wave, region of interest (ROI) of 10x10 pixels were performed from stimulated cell to the border of the image in 8 different directions. Increase over 20% from the basal ratio (340/380) in ROI was considered as a $[Ca^{2+}]_i$ increase to determine the distance reached by the calcium wave.

4.8 Mechanical Stimulation

Three approximations were used to induce a MS on the epithelial cells, pipetting MS, air MS and touch a single cell.

Pipetting: using a micropipette P1000 three gently movement were performed in the culture medium of the explants.

Touch a single cell: a single cell was stimulated with a glass micropipette (1 μm tip) directed with a micromanipulator.

Air pressure: Delivery of timed bursts of compressed air (nitrogen 78%, oxygen 21%, and argon 1%; Airgas Corp., Radnor, PA, USA) was regulated by a Pico-Spritzer II 2-valve microinjector ejector (General Valve Corp., Fairfield, NJ, USA), and delivered to the apical surface of the ALI cultures via a 22-gauge plastic catheter (BD, Franklin Lakes, NJ, USA). Unless stated otherwise, 40 and 600 μl medium with or without drugs was kept in the apical and basolateral side of transwell inserts (3470; Corning), respectively. To keep the air pressure delivered to the cell surface the same in all experiments, the catheter was connected, via a rigid plastic tube to a micromanipulator, allowing an accurate, precise, and reproducible placement (6 mm above the apical fluid and 1 mm from the wall of the transwell), thus leaving the center of the transwell unobstructed for observation and video recording. All experiments used cells in the center of the transwell filter.

Calibration of the pressure delivered to the culture was performed by sealing the apical surface of the transwell insert with a fitted stopper with two 22-gauge holes. Through one hole, the pressure was applied, while the other hole was connected to a manometer (Traceable Manometer/Pressure/Vacuum Gauge, 3460; Control Co., Friendswood, TX, USA).

In collaboration with Dr. Cohen´s laboratory, it was published that ALI cultures from mouse septum stimulated with air MS showed a pressure dependant CBF increase. All cultures stimulated with an air pressure of 55 mmHg delivered during 50 msec responded with a CBF increase of ≥15% (Zhao et al., 2012), so, this pressure/time was used as air MS.

4.9 Scanning electron microscopy (SEM)

Primary cultures of epithelial cells were fixed with 2% PFA in PBS, pH 7.2, for 45 min at 4 ºC. Then, tissue was washed, dehydrated in a progression of increasing ethanol concentrations, up to 100% ethanol and critical point dried (CPDS Model-2002). Samples were sputter-coated with gold palladium using the Varian/Vacuum equipment Evaporator division PS 10E and observed with a JEOL JSM-25 S II Scanning Microscope.

4.10 Immunofluorescence

The presence of Panx1, Panx2, Panx3, and P2X7-R was detected by immunofluorescence in tissue section of mouse trachea and in primary culture from mouse trachea and human adenoids.

Tracheas were removed from mouse and fixed in paraformaldehyde 4% (PAF) in PBS for 24 hours. PAF was dissolved in water with drops of NaOH 10N to help dissolution and finally, pH was adjusted to 7. After fixation, tissues were dehydrated with immersion in ethanol 70%, 80%, 90% and 100% I; 12 h in ethanol 100% II, 1 h each, 1 h in Xylene I and finally 12 h in Xylene II. After dehydration, organs were included in Paraplast (Tyco/Health Care, USA) and cut in 8 μm sections and mounted on Xylane-(3-aminopropyl) triethoxysilane (Sigma- Aldrich) treated slides.

The sections were desparaffinized by immersion in two consecutive xylene baths, 15 min each and rehydrated with five washes in 100% I, 100% II, 90%, 80%, 70% ethanol for 5 min each and finally rinsed in PBS.

In the immunofluorescence analysis of cell culture of trachea epithelium, cell grown in coverslides were fixed with PAF 4 % for 1 h at 4°C. Afterward, the antibody-binding site were exposed through the incubation the slides with cell cultures with Triton X-100 1 % for 10 min and by boiling the slides with tissue sections for 10 min in sodium citrate 0.01 M pH 6.0.

Non-specific binding was blocked by the incubation with PBS/BSA 3 % at 25°C during 1 h. The cells were hybridized overnight at 4°C with the primary antibody Panx1 (anti-rabbit), Panx2 (anti-chicken), Panx3 (anti-rabbit) and P2X7-R (anti-rabbit) dissolved in PBS/BSA 3%. Then, slides were washed with PBS and tissue were incubated with the following secondary antibody according to the primary antibody: Alexa Fluo® 488 anti-rabbit (A11008), Alexa Fluo® 568 anti-rabbit (A100042), Alexa Fluo® 488 anti-chicken (A11039) or Alexa Fluo® 488 anti-goat (A21202), (life technologies, 1:2000), dissolved in PBS/BSA 3% for 2 h at room temperature. Slides were washed with PBS and cell nucleus stained with DAPI (4', 6-Diamidino-2-Phenylindole, D-3571). After washing, slides were mounted using a drop of Fluoromount G and cells were visualized using a fluorescence Bx51 Olympus microscope. In some experiments, the primary antibody was omitted as a negative control. Brain sections were used as positive controls (data not shown).

4.11 Western Blot

Ciliated cells from bottle cultures were harvested in cold PBS and the pellet resuspended in lysis buffer (150 mM NaCl; 25 mM Tris-HCl, pH7.6; 1% NP-40; 1% Sodium deoxicholate; 0,1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Pierce). The lysate was sonicated and centrifuged at 13500 g for 20 min at 4°C to separate membrane (pellet) and cytosolic (supernatant) fractions. Protein concentration was determined by Lowry assay (Bio-Rad D_c Protein Assay). one hundred µg of crude membrane extract was loaded in each lane, separated by 8 % poliacrilamyde gel electrophoresis in the presence of sodium dodecylsulphate (SDS), transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline 0.1 % Tween-20 (TBST) 5 % non-fat milk for 1 h at room temperature and incubated overnight with primary antibodies polyclonal rabbit P2Y2-R antibody, 1:150, Anti-Cx43 and anti-Panx1. Secondary antibody was goat anti rabbit coupled to hydrogen peroxidase (1:500). The reaction was developed with chemiluminescence using ECL western blot analysis system (NEN, Western lightning, Perkin–Elmer, Boston, MA).

4.12 Data analysis

Data are expressed as the mean ± standard error (SEM). Statistical analysis was performed with GraphPad Prism5 (GraphPad Software, San Diego, CA, USA). *t* test or One-way ANOVA with the Tukey's post test were performed. The changes of the data were analyzed after arcsine transformation. The criterion for a significant difference was a final value of $p < 0.05$.

4.13 Experimental procedure

Most experiments were performed in primary cultures of mouse trachea. However, other cellular models of respiratory and reproductive epithelia were used to establish the contribution of eATP in the control mechanism of ciliary activity. Primary cultures from human adenoid; air-liquid interface cultures of respiratory epithelium from mouse and human; and primary cultures from rat oviducts were used. Characterization of basal CBF and basal e[ATP] were performed in all models.

To assess whether e[ATP] affected basal ciliary activity, primary cultures of mouse trachea were exposed to apyrase (5-100 U/mL), a commercial ecto-nucleotidase that hydrolyze ATP. Ciliary beat frequency (CBF) and $[Ca^{2+}]_i$ was recorded for 20 min after apyrase was added to the culture. Also, to evaluate the participation of adenosine in the control of basal ciliary activity, CBF was measured in the presence of 8-SPT (100 μM), an ADO receptor antagonist. In addition, to evaluate the participation of HCs (Panx1/Cx) and purinergic receptors in the control mechanism of basal ciliary activity, CBF, e[ATP] and $[Ca^{2+}]_i$ were measured in the presence of HC and purinergic receptor blockers (carbenoxolone (CBX 10-200 μM), probenecid (PROB 1 mM), La^{3+} (100 μM), Gd^{2+} (100 μM), suramin (SUR 200 μM) and oxidized ATP (oATP 100 μM)). CBF and $[Ca^{2+}]_i$ were measured during 20 min after HC blockers were added. e[ATP] was measured in medium samples taken 5, 10 and 20 min after the addition of the corresponding blocker.

To determine if the increase in CBF induced by a mechanical stimulation (MS) depend on ATP release, CBF and e[ATP] were measured after the application of the MS (pipetting) was on primary cultures of respiratory ciliated cells. CBF was measured during 10 min and e[ATP] was measured in medium samples taken before and after (1, 3, 5 and 10 min) MS. In addition, $[Ca^{2+}]_i$ was measured following MS (touch a single cell) during 3 min and the effect of apyrase was

determined on $[Ca^{2+}]_i$ in the stimulated cell and on the propagation of calcium waves in the culture.

To evaluate whether the increase in CBF induced by a chemical stimulation with ATP depend on ATP release through HCs, the effect of ATP (10 μM) on CBF was evaluated in the presence of HC blockers (CBX and PROB). ATP was added 10 or 20 min after HC blocker addition. e[ATP] was measured in medium samples taken 4 and 20 min following ATP addition in the presence of CBX added after ATP.

Results

5.1 eATP controls the basal ciliary activity in ciliated cells from mouse trachea, human adenoid and rat oviduct

5.1.1 Characterization of basal CBF in primary cultures from mouse trachea, human adenoid and rat oviduct

Scanning electron microphotography of the primary cultures from mouse trachea after five day of culture (Figure 1A) showed a monolayer of ciliated cells with cilia grouped on the apical surface. The basal CBF was measured in 858 whole fields using SAVA system. A whole field with 3713 points of movement and power spectrum with mean CBF of 13.1 Hz is showed in Figure 1B inset. Basal CBF follows a Gaussian distribution with a basal CBF of 13.3 ± 2.9 Hz (Mean ± SD) (Figure 1B).

Scanning electron microphotography of the primary cultures from human adenoid after eight days of culture showed a monolayer of ciliated cells with cilia grouped on the apical surface (Figure 2A and published in Gonzalez et al., 2013). In these cultures, basal CBF was measured in a total of 55 cells using the MFD technique. It was possible to observe ciliated cells with different basal CBF, showing a Gaussian distribution with a basal CBF of 10.3 ± 1.3 Hz (Mean ± SD) (Figure 2B). The instant mean spectrum obtained of a ciliated cell with a CBF of 13.5 Hz is showed in Figure 2B inset.

In primary cultures from rat oviduct, the basal CBF was measured with the SAVA system in a total of 265 whole fields corresponding to 83 cultures. Basal CBF follows a Gaussian distribution with a mean of 11.5 Hz ± 2.8 (Mean ± SD). 60.6% of analyzed fields were between 8.6 and 14.9 Hz

(data not shown). CBF obtained in primary cultures from rat oviducts are not different from CBF obtained in previous studies in our laboratory using MFD techniques (10.7 ± 2.0 Hz, n=46; Kerr, 2004).

In an internship at Dr. Noam Cohen`s laboratory (University of Pennsylvania), we determined the basal CBF in air-liquid-interfase (ALI) cultures from mouse septum, mouse etmoides and mouse trachea. These cultures showed a basal CBF of 4.7 ± 0.6, 4.4 ± 0.4 and 13.2 ± 0.8 Hz (Mean ± SEM, n=3, 5 and 6) respectively with 27°C. ALI cultures from human senonasal epithelium showed a basal CBF of 5.1 ± 0.4 Hz (Mean ± SEM, n=6) (Data not shown). These results were consistent with the basal CBF obtained previously in Cohen´s laboratory (Woodworth et al., 2007).

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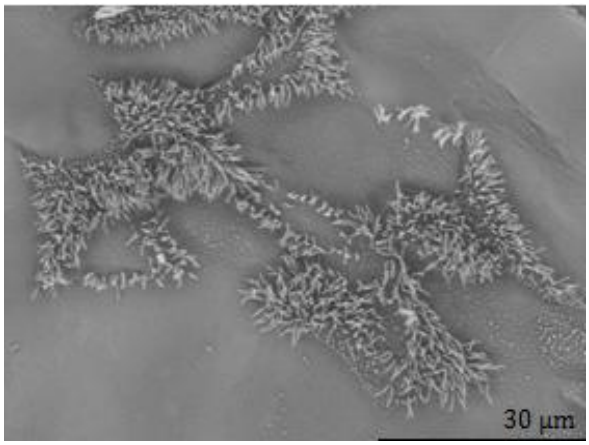

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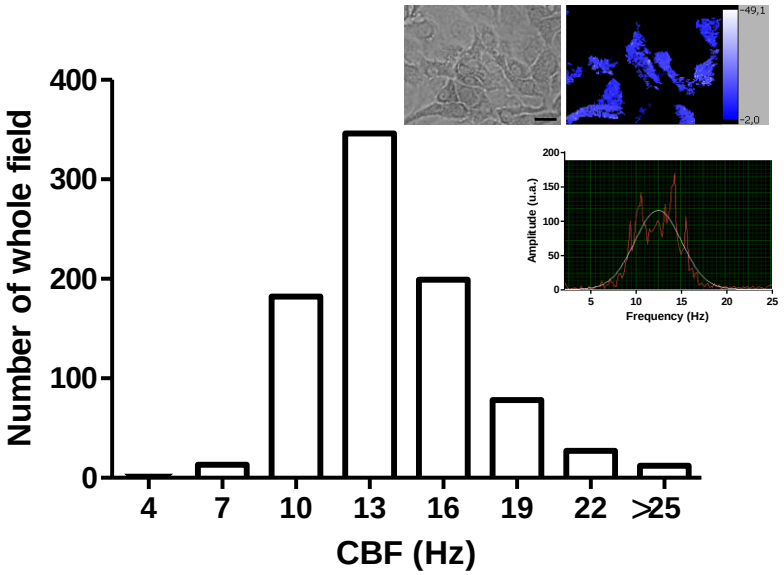

Figure 1. Characterization of basal CBF in primary cultures from mouse trachea.

A. Scanning electron microphotography of ciliated cells of primary culture from mouse trachea epithelium in day 5 of culture. A monolayer of ciliate cells with cilia grouped on the apical surface is showed.

B. Basal CBF was measured using SAVA system in primary cultures from mouse trachea. Frequency histogram of basal CBF follows a Gaussian distribution with a mean of 13.3 ± 2.9 Hz (mean ± SD, n=858). Inset: (upper left) representative microphotograpy obtains from video recorder. This video was recorder in 40X with a high-speed video camera; (upper right) In blue scale is showed the frequency limited by amplitude of the 3713 points of movements corresponding to a ciliary movement detected in the video recorder; (down) Frequency by amplitude of the whole field power spectrum of CBF obtained from the video recorder with mean of 13.1 Hz. (Bar = 30µm).

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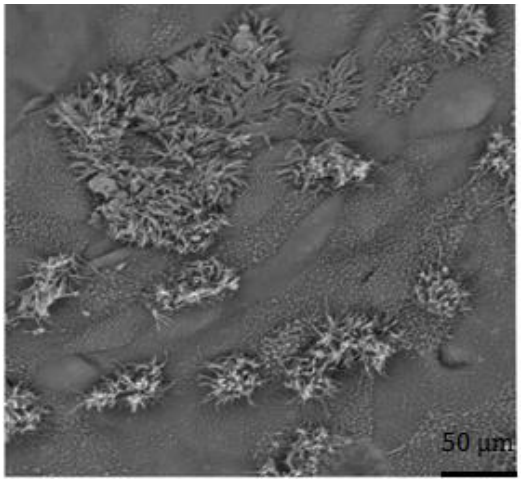

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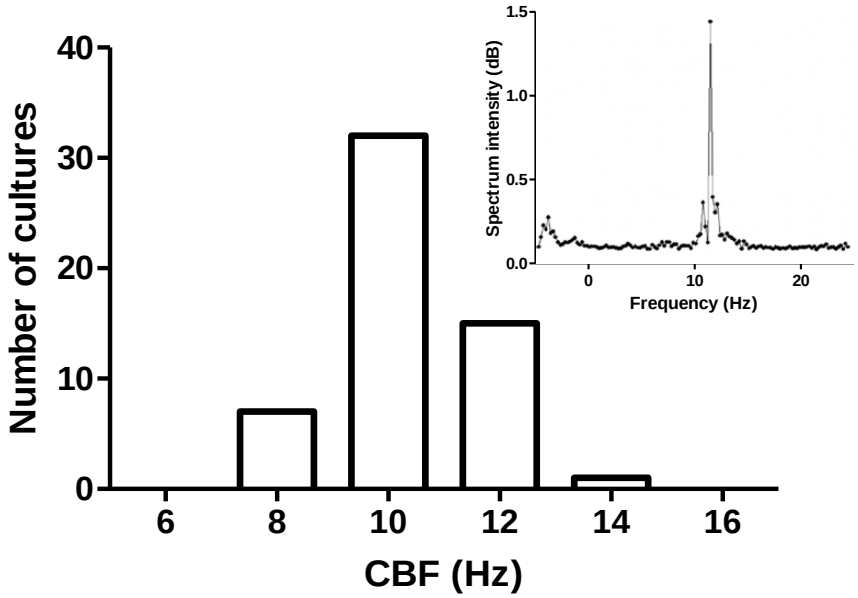


Figure 2. Characterization of basal CBF in primary cultures from biopsies of human adenoid.

A. Scanning electron microphotography of ciliated cells in a primary culture from human adenoid epithelium in day 8 of culture. A monolayer of ciliated cells with cilia grouped at the center of the cell on the apical surface is showed (published in González et al., 2013).
B. Distribution of basal CBF in cultures measured with the microphotodensitometric (MFD) technique. Basal CBF follows a Gaussian distribution with a mean of 10.3 ± 1.3 Hz (Mean ± SD, n=55). Inset show a representative power spectrum of ciliary activity obtained from a single ciliated cell with a CBF peak of 13.5 Hz.

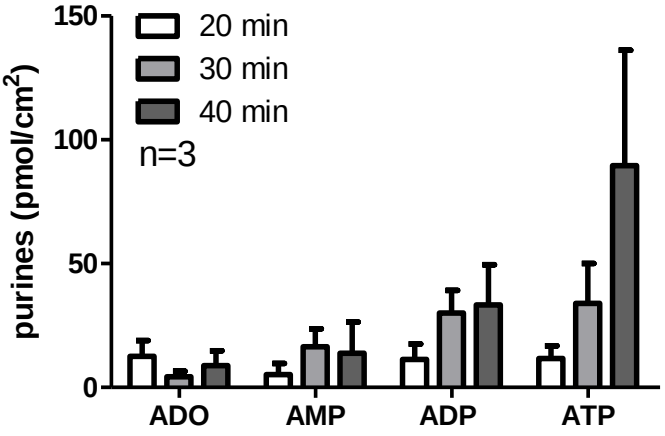
5.1.2 eATP levels are present in basal conditions in primary cultures from mouse trachea, human adenoid and rat oviduct

In undisturbed culture medium of primary cultures from human adenoids, eATP levels were 11.7 ± 5.1 pmol/cm^2 at 20 min; 34.0 ±16.1 pmol/cm^2 at 30 min; and 89.6 ±46.5 pmol/cm^2 at 40 min (Figure 3A). These results showed accumulation of ATP over time, however not significant differences in eATP levels were observed at different times. ADO, AMP, ADP were also measured at the same time as ATP, without obtaining significant differences over time (Figure 3A).

In primary cultures from mouse trachea and rat oviduct eATP levels, after 20 min of undisturbed cultures were 15.2 ± 7.6 and 21.5 ± 5.8 pmol/cm^2 in mouse trachea and rat oviduct, respectively (Figure 3B). eATP levels obtained in primary cultures from human adenoids at 20 min are shown in Figure 3A and Figure 3B.

e[ATP] measured in ALI cultures showed a basal [ATP] of 15.9 ± 5.1, 15.4 ± 6.6, 7.7 ± 5.8 and 3.2 ± 1.4 pmol at 15 minutes of undisturbed culture in mouse septum, etmoides and trachea and human seno-nasal respectively (n=9, 5, 6 and 12, respectively) (data not shown).

A



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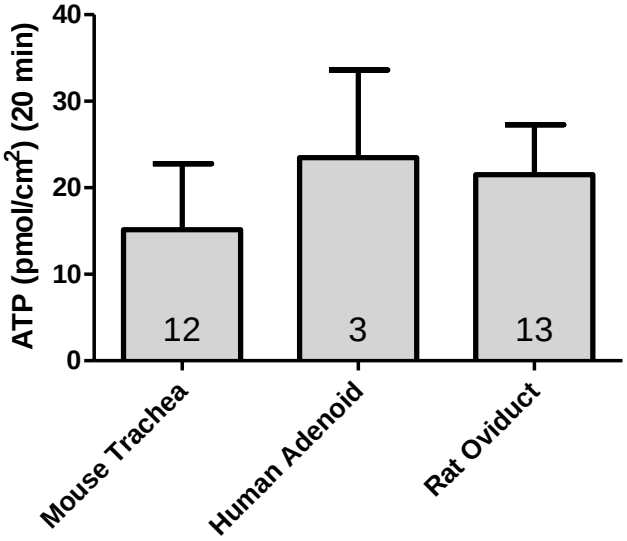


Figure 3. Quantification of basal eATP levels in primary cultures from human adenoid, mouse trachea and rat oviduct.

A. ADO, AMP, ADP and ATP levels were determined by a HPLC separation and fluorometric detection in samples of culture medium obtained from undisturbed human adenoid primary cultures at 20, 30 and 40 min (n=3). eATP levels are expressed per cm^2, measured as the surface culture area occupied by the epithelial cells. No significant differences were observed in the extracellular purines levels over time.

B. Basal ATP levels detected by luminometric technique in culture medium samples at 20 min of undisturbed primary cultures from mouse trachea and rat oviduct. Human adenoid measurement corresponds to the value shown in (A) at 20 min No significant differences were observed in the ATP levels between mouse trachea, human adenoid and rat oviduct.

5.1.3 eATP hydrolysis reduces the CBF in ciliated cells of epithelial primary cultures

To assess whether basal e[ATP] affect basal CBF, apyrase (APY), an ectonucleotidase that hydrolyzes ATP, was added to the primary cultures from mouse trachea. Figure 4A shows representative time courses of CBF change with APY. APY (20 U/mL) reduced CBF by a 31.4% from baseline, returning to baseline after 7 min. A higher concentration of APY (100 U/mL) reduced CBF by a 53% from baseline. This effect had a slight recovery but did not return to baseline during 20 min of recording. Figure 4B shows a concentration dependent decrease effect in CBF with APY. The percentage of CBF reduction was 3.8 ± 1.0 %, 31.4 ± 3.5 %, 45.5 ± 2.3 % and 53.2 ± 2.5 % at vehicle, 20, 50 and 100 U/mL of APY, respectively (n=7, 5, 4 and 6, respectively). This result suggests that eATP levels contribute to the control of ciliary activity in basal conditions.

To confirm the effect of APY on eATP levels, APY (50 U/mL) added to primary cultures of mouse trachea, significantly lowered eATP levels compared to vehicle (3.8 ± 1.4 versus 8.1 ± 0.8 pmol/cm^2 after 1 min of treatment and 1.8 ± 0.4 versus 8.7 ± 0.2 pmol/cm^2 after 20 min, $p<0.05$, n= 3) (Figure 5). This data indicates that APY (50 U/mL) reduced eATP levels at least in a 50% after 1 min. The slight recovery on CBF after 20 min, with a higher reduction on e[ATP], suggest that a compensatory mechanism is involved in CBF regulation.

To assess whether Ca^{2+}_i levels were affected with APY, Ca^{2+}_i level changes were measured in the presence of APY. Figure 6A shows a time course of the changes in Ca^{2+}_i level in cells after APY treatment (10 U/mL). APY (10 U/mL) decreased Ca^{2+}_i levels, which was maintained by 7 min. The maximum change of 340/380 ratio was -0.27 ± 0.03 and -0.28 ± 0.02 at 10 and 50 U/mL of APY respectively (n=3 and 4, $p<0.05$ compared with the basal Ca^{2+}_i level for each experiment) (Figure

6B). This result indicates that the mechanism associated with basal CBF involves the maintaining of Ca^{2+}_i levels.

To confirm the effect of ADO on ciliary activity, ADO was added on primary cultures of mouse trachea. ADO 1 and 100 μM increased CBF by 72% and 32%, respectively (data not shown). To assess the contribution of ADO in the control of basal CBF, 8-(p-Sulfophenyl)theophylline (8-SPT) (100 μM), an antagonist of ADO receptors, was added in cultures. 8-SPT did not modify the basal CBF, however, preincubation with 8-SPT (100 μM) for 5 min followed by APY (50 U/mL) showed a higher reduction on CBF than cultures incubated with APY alone (60.1 ± 4.1 % 8-SPT/APY and 45.5 ± 2.3 % Vehicle/APY, $p<0.05$, n=4) (Figure 7). This result suggests that ADO prevents the reduction of CBF induced by APY.

Application of ADP (10 μM), a metabolite of ATP hydrolysis, to primary cultures from mouse trachea induced an increase in CBF by 50% (data not shown). This result suggests that metabolites produced by the hydrolysis of ATP with APY prevent a higher reduction in basal CBF.

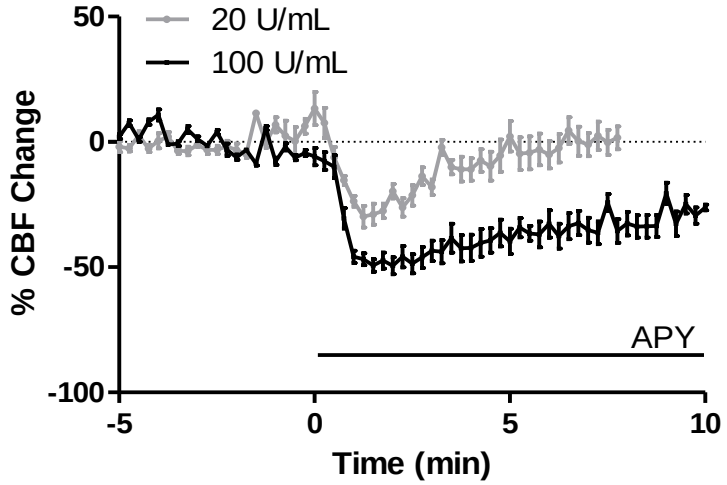
Similar results on CBF were obtained using APY in primary cultures of human adenoid. Primary cultures of human adenoid responded to APY by reducing CBF in -6.1 ± 3.2 %, -19.8 ± 3.7 %, -68.8 ± 10.6 % and -71.8 ± 4.0 % with 5, 10, 20 and 50 U/mL of APY (n=4) (Figure 8). A preliminary study in primary cultures from rat oviduct shows that APY (50 U/mL) also reduced the basal CBF in a 43.8 % and this reduction lasted over 20 min (Figure 8).

To determine if ectonucleotidases of ATP present in epithelium had an effect on basal CBF, an ectonucleotidase inhibitor mix, including AMP-PCP (300 μM), levamisole (10 mM) and ebselen (30μM), was added to primary cultures from human adenoids. The mix of these ectonucleotidase inhibitors did not cause a significant increase in CBF (11.8% versus 17.6%, n=17 and n=5 to

vehicle and ectonucleotidase inhibitor mix, respectively) (data not shown). This result suggests that basal hydrolysis of ATP is not relevant in the control mechanism of basal CBF.

Taken together, our data indicate that constitutive eATP levels participate in the control of basal ciliary activity in mouse trachea, human adenoid and rat oviduct primary cultures.

A



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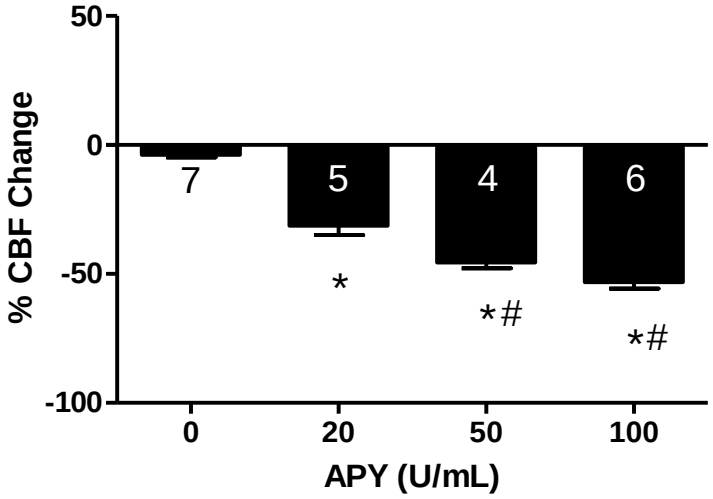

Figure 4. APY, an ectonucleotidase of eATP, decreases the basal CBF in primary culture from mouse trachea.

A. Time course of the CBF change in ciliated cells treated with APY (20 and 100 U/mL). Basal CBF was measured during 5 min. After that, APY was added to the culture (black line) and CBF was recorded during 20 min. Maximum CBF reduction was of 53% from the baseline (0%).

B. CBF change was determined after addition of different concentration of APY. A concentration dependent effect was observed with a significant CBF change with 20, 50 and 100 U/mL of APY compared with vehicle (*, $p<0.05$ v. Vehicle; #, $p<0.05$ v. 20 U/mL).

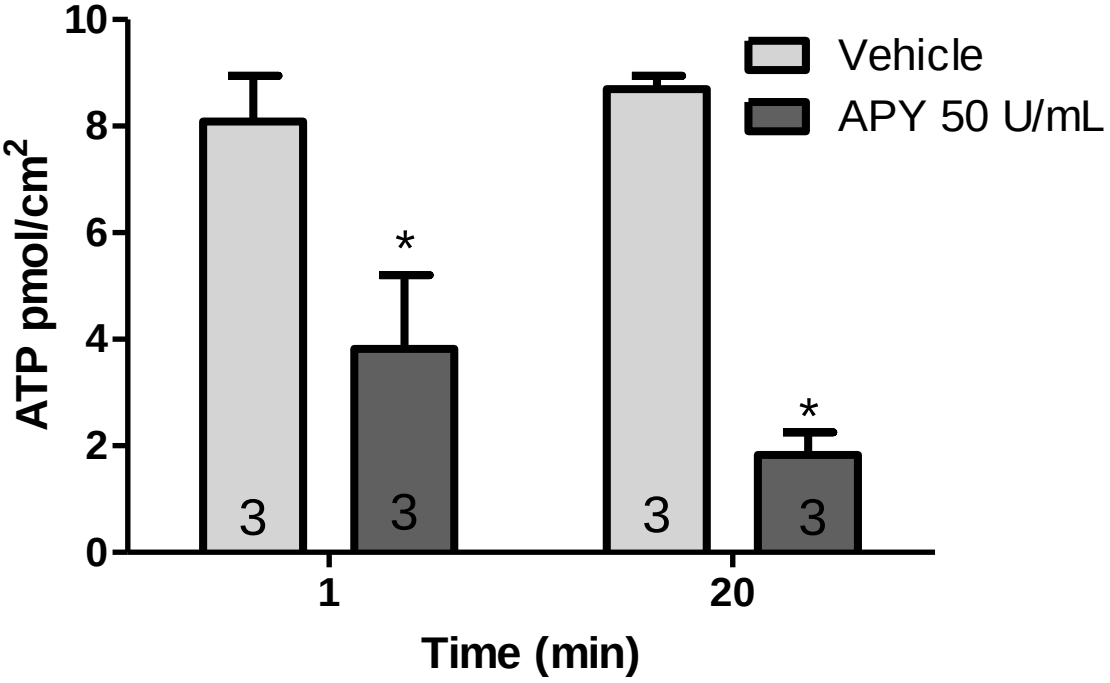

Figure 5. APY decreases eATP levels in primary culture from mouse trachea.

Quantification of ATP levels in culture medium samples of primary cultures from mouse trachea after 1 and 20 min of vehicle or APY (50 U/mL). eATP levels were reduced following APY at 1 and 20 min (*, $p<0.05$ v. Vehicle).

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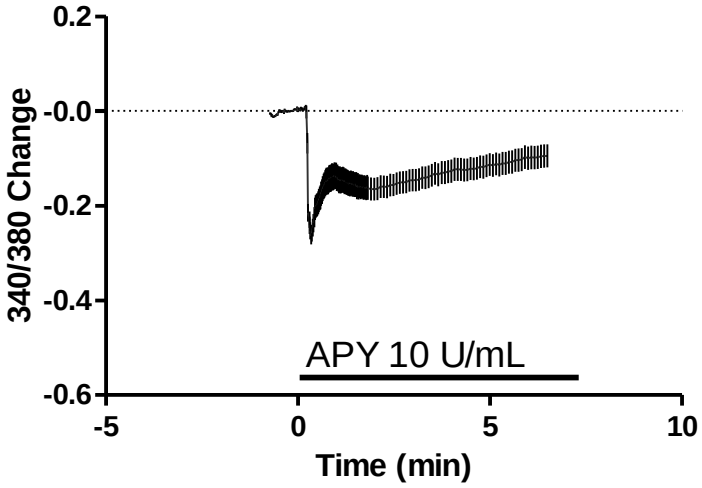

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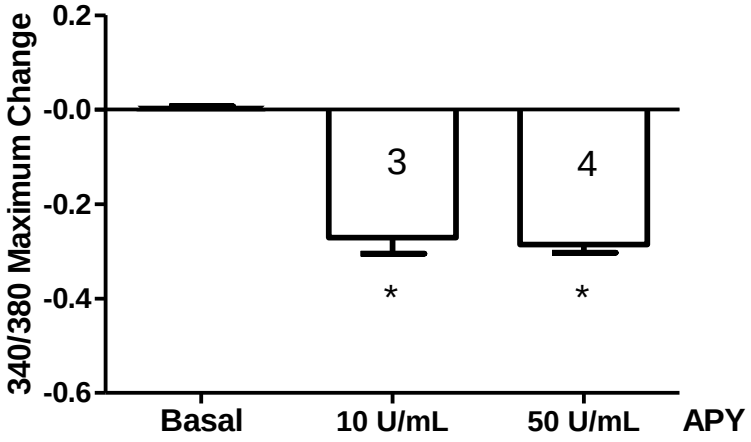


Figure 6. Hydrolysis of e[ATP] decreases $[Ca^{2+}]_i$ in primary cultures from mouse trachea.

A. Time course of $Ca^{2+}{}_i$ level changes treated with APY (10 U/mL) (black line). APY decreased $Ca^{2+}{}_i$ levels.
B. 340/380 change in cells treated with APY (10 and 50 U/mL). APY decrease $Ca^{2+}{}_i$ levels compared with the basal level for each experiment.

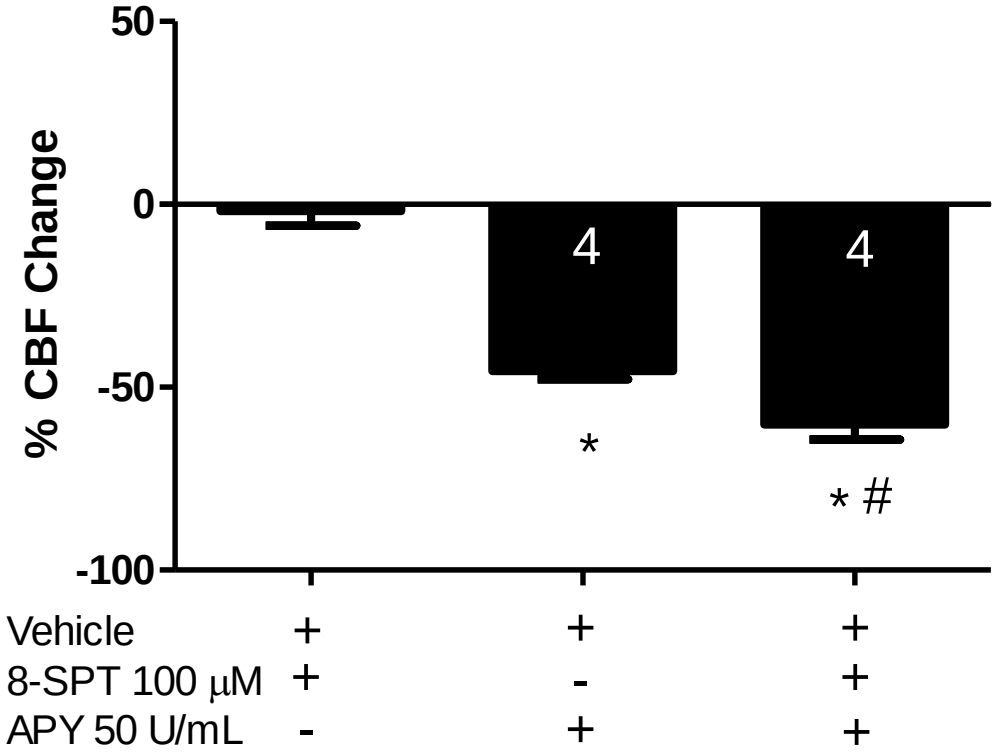


Figure 7. 8-SPT, an ADO receptor antagonist, potentiates the CBF decrease induced by APY in primary cultures from mouse trachea.

CBF change was determined in cultures incubated with 8-SPT (100 μM) and APY (50 U/mL) added in the same culture. 8-SPT (100 μM), was added to the culture 5 min before APY. Significant differences were observed between 8-SPT/APY compared to vehicle/APY (*, $p<0.05$ v. 8-SPT,#, $p<0.05$ v vehicle/APY, n=4).

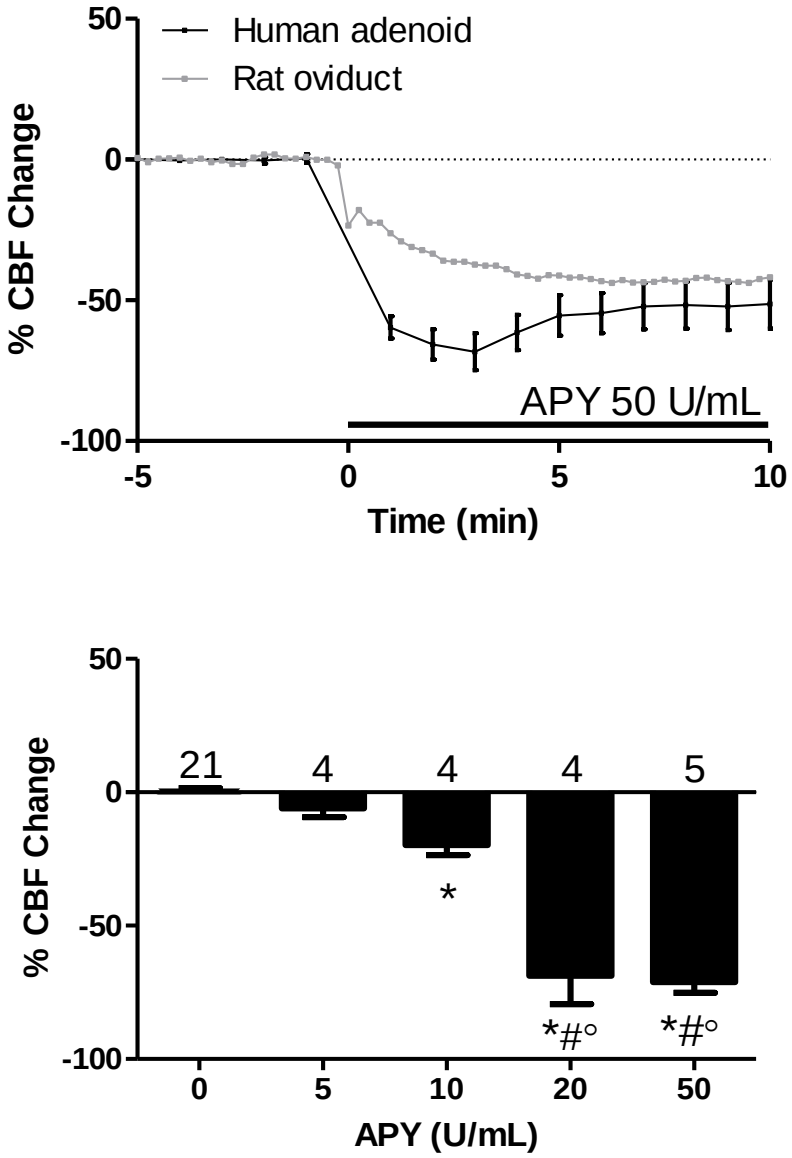


Figure 8. APY decreases the basal CBF in primary cultures from human adenoid and rat oviduct.

A. Time course of the CBF in ciliated cells treated with APY (50 U/mL) in primary culture of human adenoid and rat oviduct. Basal CBF was recorded during 5 min and APY was added to the culture.

B. CBF change was determined after APY addition in primary cultures from human adenoid. APY reduced basal CBF (10, 20 and 50 U/mL) in human adenoid (*, $p<0.05$ v. Vehicle, #, $p<0.05$ v. 5 U/mL; °, $p<0.05$ v. 10 U/mL).

5.1.4 Participation of HCs and purinergic receptors in the control mechanism of basal CBF

5.1.4.1 Expression of HCs and purinergic receptors in epithelial primary cultures

The presence of HCs (Panx1, Panx2, Panx3, and Cx43) and purinergic receptors (P2X7-R) was determined in mouse trachea epithelial cells. Immunohistochemistry of tissue sections showed immunoreactivity to anti-Panx1, anti-Panx2 and anti-Panx3 antibodies in epithelial cells (Figure 9). Immunocytochemistry in primary cultures showed immunoreactivity to anti-Panx1, anti-Panx2 and anti-Panx3 (Figure 10), and to anti-P2X7-R antibody in epithelial cells (Figure 11).

Similar results were obtained in primary cultures from human adenoids. Immunocytochemistry detected reactivity to anti-Panx1 (Figure 12A). In addition, Western blot analysis showed Panx1 expression with a characteristic band over 40 KDa and second band over 55 KDa in human adenoids. Western blot analysis for Cx43 showed an expected band over 40 KDa and the dripping in the upper zones of the gel in primary cultures from human adenoid. Western blot analysis for P2Y2-R showed a band over 40 KDa and a second band over 60 KDa in human adenoids (Figure 12B).

These results indicate that tissue sections and primary cultures from mouse trachea express Panx1, Panx2, Panx3 and P2X7-R, and human adenoid express Panx1, Cx43 and P2Y2-R.

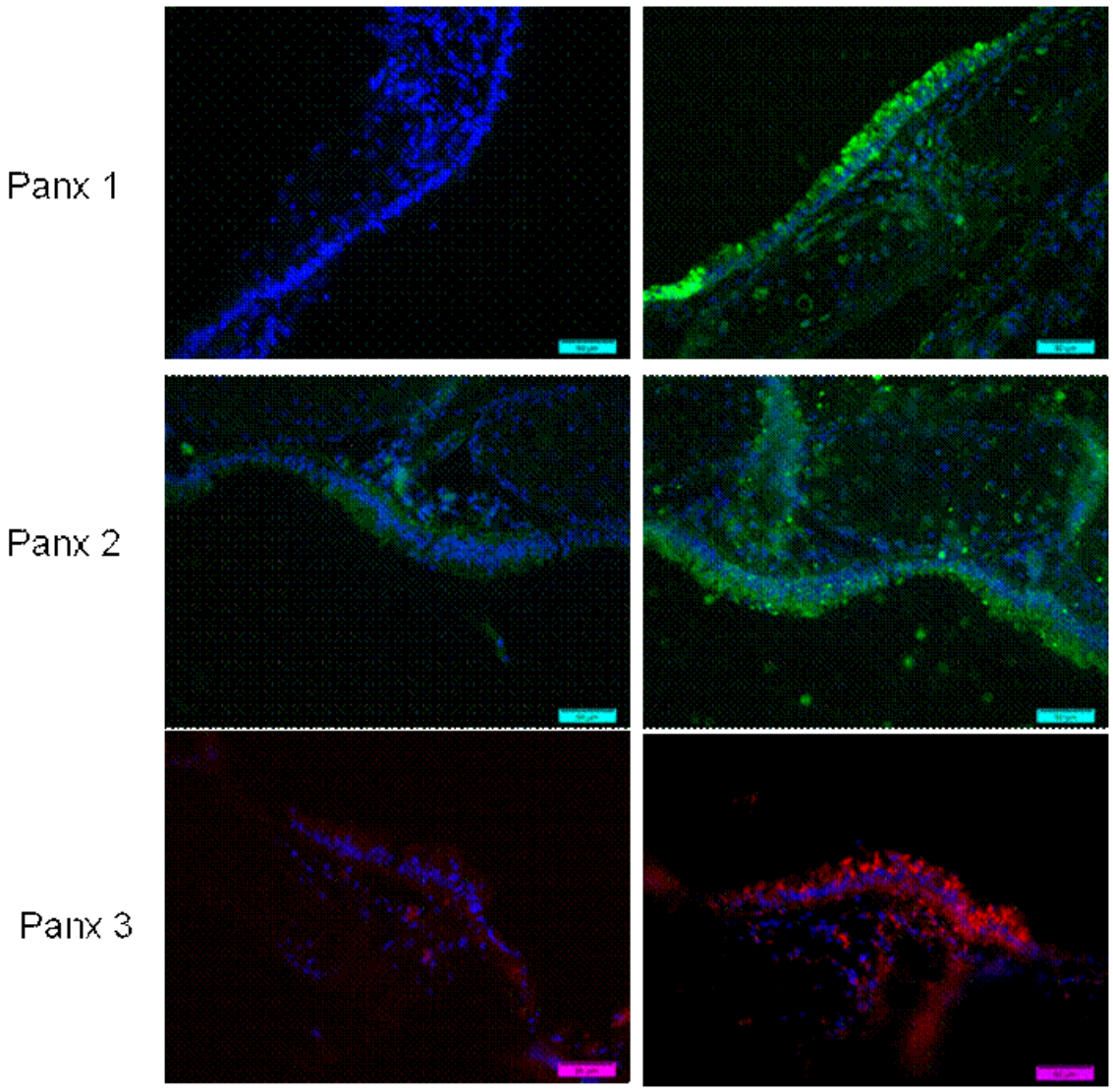


Figure 9. Detection of panx 1, 2 and 3 in tissue sections of mouse trachea.

Inmunohistochemistry of histological sections from mouse trachea processed to visualize the binding of anti-panx1 (green), anti-panx2 (green) and anti-panx3 (red) polyclonal antibodies. DAPI was used to stain nuclei (blue). Left panels correspond to control group (without primary antibodies). Bar = 50 µm. Positive immunoreactivity for Panx1, Panx2 and Panx3 was observed in mouse trachea epithelium.

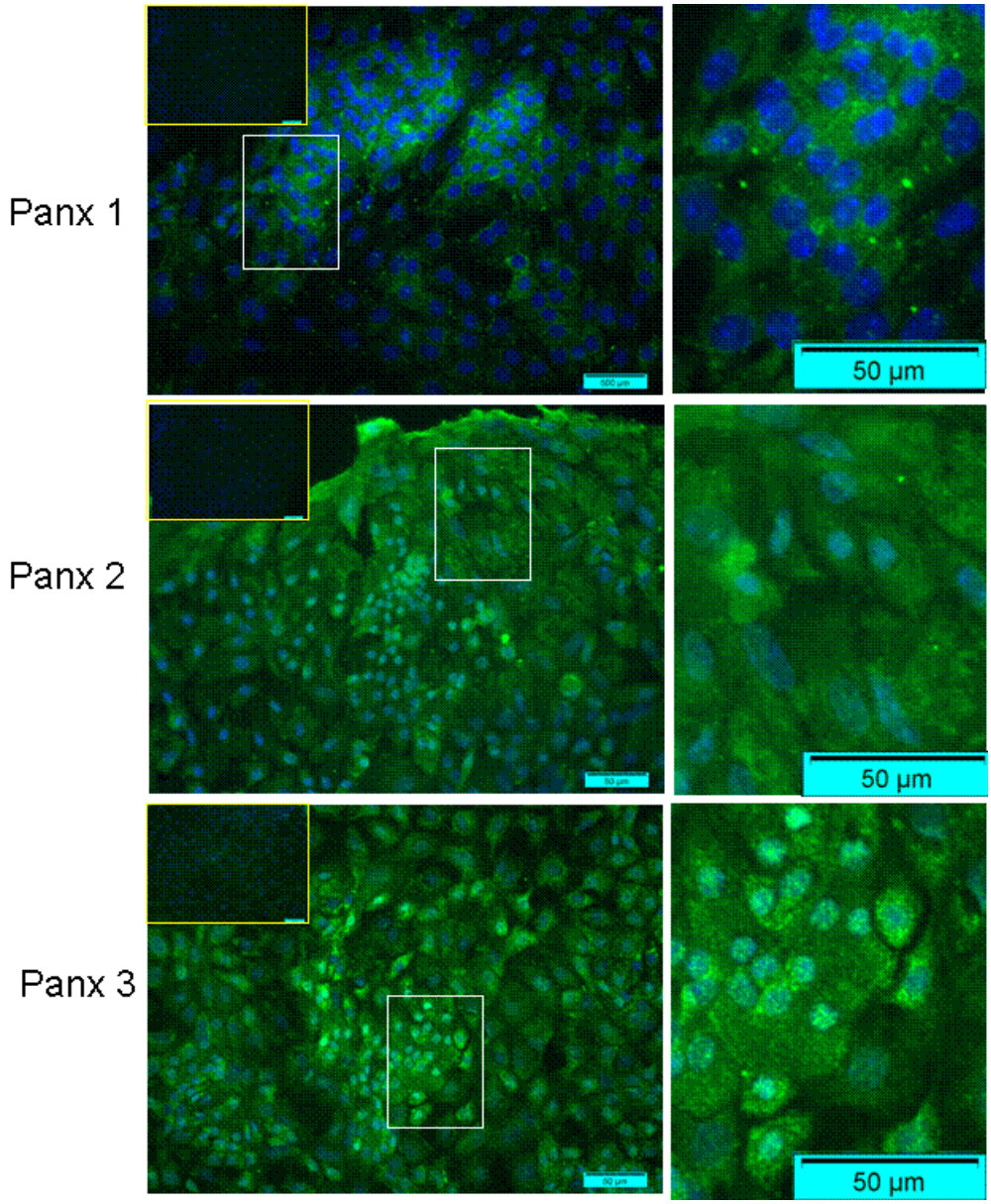


Figure 10. Detection of panx1, panx2 and panx3 in primary culture from mouse trachea.

Representative immunofluorescences developed with anti-panx1, anti-panx2 and anti-panx3 (green). DAPI was used to stain nuclei (blue). The negative control corresponds to primary culture without primary antibody (up left yellow square). Right panels show an enlarged image of the zone surrounded with a white square. Bar = 50 µm. Positive immunoreactivity for Panx1, Panx2 and Panx3 was observed in mouse trachea primary cultures.

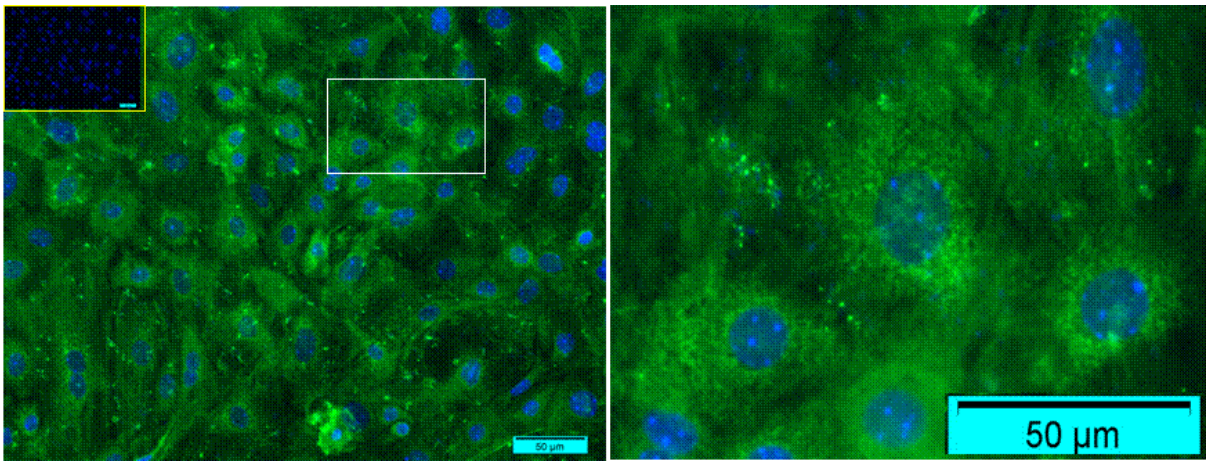
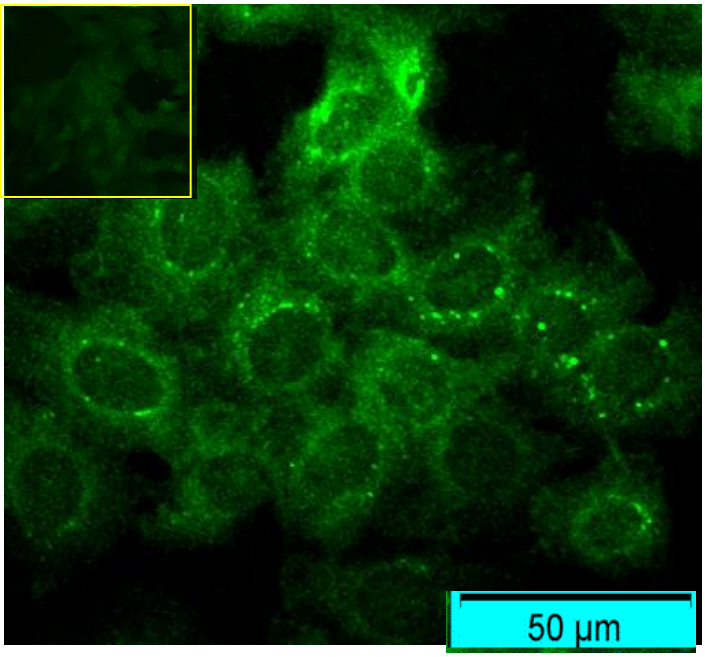


Figure 11. Immunocytochemistry of P2X7-R in primary culture from mouse trachea.

Representative immunofluorescence with anti-P2X7-R (green), a polyclonal antibody, in mouse trachea primary cultures. DAPI was used to stain nuclei (blue). Negative control without primary antibody is showed in the yellow square in upper left. In the right an enlarged image of the zone surrounded with a white square. Bar = 50 µm. Positive immunoreactivity for P2X7-R was observed.

A



B

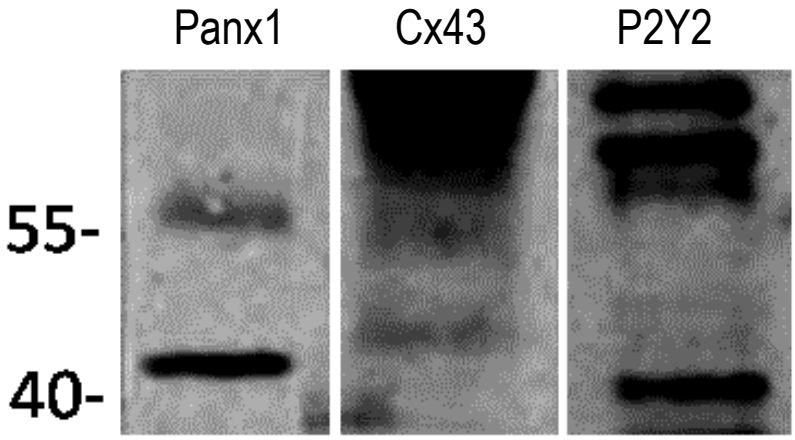


Figure 12. Expression of Panx1, Cx43 and P2Y2-R in human adenoid.

A. Indirect immunofluorescence using anti-panx1 antibody (green, left) in human adenoids primary cultures. Negative control without primary antibody is showed in the left square. Bar = 50 µm. Positive immunoreativity for Panx1 was observed in human adenoids primary cultures.

B. Western blot analysis using polyclonal anti-panx1, anti-Cx43 and anti-P2Y2-R antibodies. Cx43 was determined in primary cultures; however Panx1 and P2Y2-R were determined in adenoid epithelium. A band for Panx1 was found over 40 KDa. Dripping in the upper zones and a band over 40 KDa are showed for Cx43. Bands over 40 KDa and over 60 KDa are showed for P2Y2-R.

5.1.4.2 Inhibition of HCs and P2X7-R reduce the basal CBF in ciliated cells of epithelial primary culture

In primary cultures of mouse trachea, suramin (SUR) (200 μM), a non-specific antagonist of purinergic receptors or oATP (1, 10 and 100 μM), an antagonist of P2X7-R receptor, had no effect on basal CBF (Figure 13). A similar response was observed with KN-62 (10 μM), which inhibits P2X7-R (data not shown). Gd^{3+} and La^{3+} (200 μM), which block Ca^{2+} permeable channels, including CxHCs (Garré et al., 2010), decreased the CBF reaching the maximum CBF decrease at 1 min. Thepercentage of CBF reduction was -49.1 ± 2.5 % and -48.7 ± 4.2 %, (n=4 and 5,$p<0.05$) for Gd^{3+} and La^{3+} respectively. CBF was recovered at 5 min. Carbenoxolone (CBX) (100 μM), an inhibitor of Cx and Panx1 HCs, decreased transiently the basal CBF by 24.9 ± 6.8 % (n=12) $p<0.05$) during 15 sec (Figure 13). CBX (1 and 10 μM), in concentrations that only inhibits panx1, had no effect on basal CBF (data not shown). Probenecid (PROB) (1000 μM), a panx1 inhibitor (Hayoz et al., 2012), had no effect on basal CBF (Figure 13). These results suggest that CxHCs could participate in the control of basal CBF.

oATP (100 μM) and CBX (100 μM) added simultaneously to primary culture of mouse trachea decreased CBF reaching a reduction of 57.5 ± 3.0 % at 5 min and returned to baseline after 20 min. This reduction was significantly different to vehicle (-3.8 ± 1.0 %, $p<0.05$) and oATP alone (-6.8 ± 1.7 %, $p<0.05$) (Figure 14). oATP (200 μM) and CBX (200 μM) added at the same time, reduced CBF in 74.3 ± 1.7 %, this was significantly different to vehicle, but not respect to oATP (100 μM) and CBX (100 μM) added at the same time (data not shown). oATP (10 μM) and CBX (10 μM) together had no effect on CBF (-2.6 ± 3.0 %, n=3)(data not shown). CBX (100 μM) and APY (50 U/mL) added simultaneously showed a reduction compared to vehicle (-51.7 ± 1.6 %, $p<0.05$, n=3) but did not show difference compared with APY alone (Figure 14). Concomitant treatment with CBX (100 μM), oATP (100 μM) and APY (50 U/mL) further reduced the basal CBF

in 85.2± 4.8 % (n=10), that was lower than those obtained when oATP, CBX and APY were added separately (n=7, 3, 12 and 4 for vehicle, oATP, CBX and APY, respectively, $p<0.05$) (Figure 14). In some cultures when oATP, CBX and APY were added together, ciliary activity was reduced by 100% for approximately two minutes. These results suggest that eATP levels and the molecular components of ATP release (CxHCs) and/or ATP receptor activation (P2X7-R) are necessary to maintain basal ciliary activity in ciliated epithelia.

To assess whether HCs and P2X7-R participate in the regulation of e[ATP], mouse trachea primary cultures were exposed to blockers and eATP levels were measured. eATP levels measured 5 min following addition of oATP (100 μM), CBX (100 μM) or oATP plus CBX, did not show significant differences compared with vehicle (19.9 ± 9.5 pmol/cm^2 for vehicle, 24.7 ± 4.5 pmol/cm^2 for oATP, 8.4 ± 3.6 pmol/cm^2 for CBX and 95.5 ± 56.5 pmol/cm^2 for oATP/CBX, n=11, 4, 4 and 6, respectively). When APY (50 U/mL), oATP and CBX were added simultaneously ATP levels showed a significant reduction (2.6 ± 0.1 pmol/cm^2, n=4, $p<0.05$) (Figure 15). e[ATP] was also measured 10 and 20 min after the addition of oATP, CBX and APY. e[ATP] did not vary over time as no differences were observed compared to e[ATP] obtained after 5 min of treatment (data not shown).

In addition, preliminary results of changes in Ca^{2+}_i levels were assessed using inhibitors of HCs and P2X7-R. oATP (100 μM), produced a transiently fast increase in the Ca^{2+}_i levels. The recovery from the rise in Ca^{2+}_i levels was observed approximately after 2 minutes (data not shown). CBX (100 μM) did not modify Ca^{2+}_i levels (data not shown). Concomitantly, oATP and CBX produced a slightly reduction in Ca^{2+}_i levels (change in 340/480 was -0.2) (data not shown). The combined treatment with APY (50 U/mL), oATP and CBX caused a rapid reduction in Ca^{2+}_i levels (340/380 change was -0.6) maintaining this response during 10 min of measurement (data not shown).

These results are consistent with those obtained in CBF, which supports that CBF depends on Ca^{2+}_i levels.

Similar results were obtained in primary cultures from human adenoids. In these cultures, SUR (100 μM), TNP-ATP (100 μM), an antagonist of P2X-R, and oATP (100 μM), did not modify basal CBF, however La^{3+} (100 μM) and CBX (200 μM) reduced the CBF in 21.7 ± 4.3 % and 24.0 ± 4.6 % n=4 and 9, respectively) (Figure 16). La^{3+} (200 μM) had similar effect as a lower concentration of La^{3+} (100 μM) on CBF (21.1 ± 4.4 % for 200 μM, n=3, data not shown). β-GA (50 μM), a gap junction and CxHC inhibitor, decreased the CBF in 34.1± 5.6 % ($p<0.05$ v. ethanol vehicle, n=3) (Figure 16). Concomitant treatment with SUR and CBX showed a similar CBF compared with CBX alone (-26.8 ± 4.4 % CBX/SUR versus -23.9 ± 4.6 % for CBX alone). However, when CBX was added simultaneously with oATP, CBF was reduced respect to oATP or CBX separately (-77.3 ± 12.4 % CBX/oATP versus -23.9 ± 4.6 % for CBX, and -7.6 ± 1.4 % for oATP; $p<0.05$ v. Vehicle; $p<0.05$ v. oATP; $p<0.05$ v. CBX) (Figure 17).

In primary cultures from rat oviduct, oATP reduced basal CBF compared to vehicle (-6.4 ± 1.6 % for vehicle versus -32.9 ± 7.4 % for oATP, $p<0.05$, n=5). CBX did not produce a significant reduction in basal CBF (-15.3 ± 5.1 %). Concomitant treatment with oATP, CBX and APY reduced basal CBF, but these changes were not significantly different compared to oATP alone (-32.4 ± 9.8 % for CBX/oATP and -51.2 ± 19.7 % for APY/CBX/oATP) (Figure 18).

Similar preliminary results were obtained in respiratory ALI cultures. ALI cultures from human seno-nasal epithelium showed a reduction in CBF with oATP (100 μM) and CBX (200 μM) added at the same time, but not with CBX or oATP added alone (-35.0 ± 2.7 % for oATP/CBX versus -5.2 ± 1.5 % for CBX and -3.5 ± 0.8 % for oATP, n=2)(data not shown). Measurements of eATP levels were 80.0 ± 75.9 pmol for vehicle and 9.3 ± 5.80 pmol for CBX (200 μM) (n=3) after 15 min of

treatment (data not shown). ALI cultures from mouse septum showed a reduction in basal CBF with CBX alone and CBX with oATP added together, however no additional decrease was observed with CBX and oATP added together (-58.6 ± 8.0 % for oATP/CBX , -54.2 ± 0.4 % for CBX and -9.9 ± 4.3 % for oATP, n=2) (data not shown). eATP levels were 78.1 ± 56.8 pmol for vehicle and 11.4 ± 2.0 pmol for CBX (n=5) (data not shown). Despite these results, the differenceobserved between CBX and vehicle was not statistically significant due to the large variability in the measurements of eATP levels for vehicle. These results suggest that eATP levels and the molecular components of ATP release (CxHCs) and/or ATP activation (P2X7-R) are necessary to maintain basal ciliary activity in ciliated epithelia. However, there are some differences between models studies. In respiratory cultures, HCs seems to be more important, while P2X7-R have a major role in rat oviduct in the tontrol of basal ciliary activity.

A preliminary result in primary cultures from Panx1-/- knock out mouse tracheas, oATP (100 μM) and CBX (100 μM) added together reduced basal CBF by a 43%. oATP alone had no effect in basal CBF (data not shown). These results were similar to those obtained with primary cultures of mouse trachea, suggesting that HCs, particularly CxHCs, and P2X7-R are molecular components involved in the control mechanism of basal CBF.

EGTA (2 mM), a chelant of extracellular Ca^{2+}, was added to primary cultures from human adenoid, without removing Ca^{2+} of the culture medium. EGTA caused a reduction in CBF, stopping ciliary activity completely after 5 min of treatment. Ciliary activity was restored by replacing culture medium (data not shown), suggesting that extracellular Ca^{2+} is important to maintain normal ciliar activity in these cells.

In summary, our data indicated that eATP levels are constitutively released to the extracellular compartment in primary ciliated cell cultures from mouse trachea in resting conditions.

Treatment with APY significantly reduced eATP levels and basal CBF (-45%), suggesting that eATP participates in the control of basal ciliary activity. Treatment with La^{3+} and Gd^{3+} reduced basal CBF, suggesting that Ca^{2+} channels participates in the control of basal ciliary activity. Concomitant treatment with APY, CBX and oATP further reduced basal CBF (-85%). These results suggest that eATP concentration and the molecular components involved in ATP release (Cx/Panx HCs) and/or ATP receptor activation (P2X7-R) are necessary to maintain basal ciliary activity in ciliated epithelia. Furthermore, basal CBF reduction by ATP hydrolysis was associated with decreases in $Ca^{2+}{}_i$ levels suggesting that $Ca^{2+}{}_i$ levels are regulated by e[ATP] to maintain basal CBF. In addition, treatment with 8-SPT further enhanced the decrease in CBF induced by APY, suggesting that hydrolysis of ATP to ADO is involved in the control of basal ciliary activity. Figure 19 shows a model that integrates these results with previous studies of literature (Morales et al., 2000).

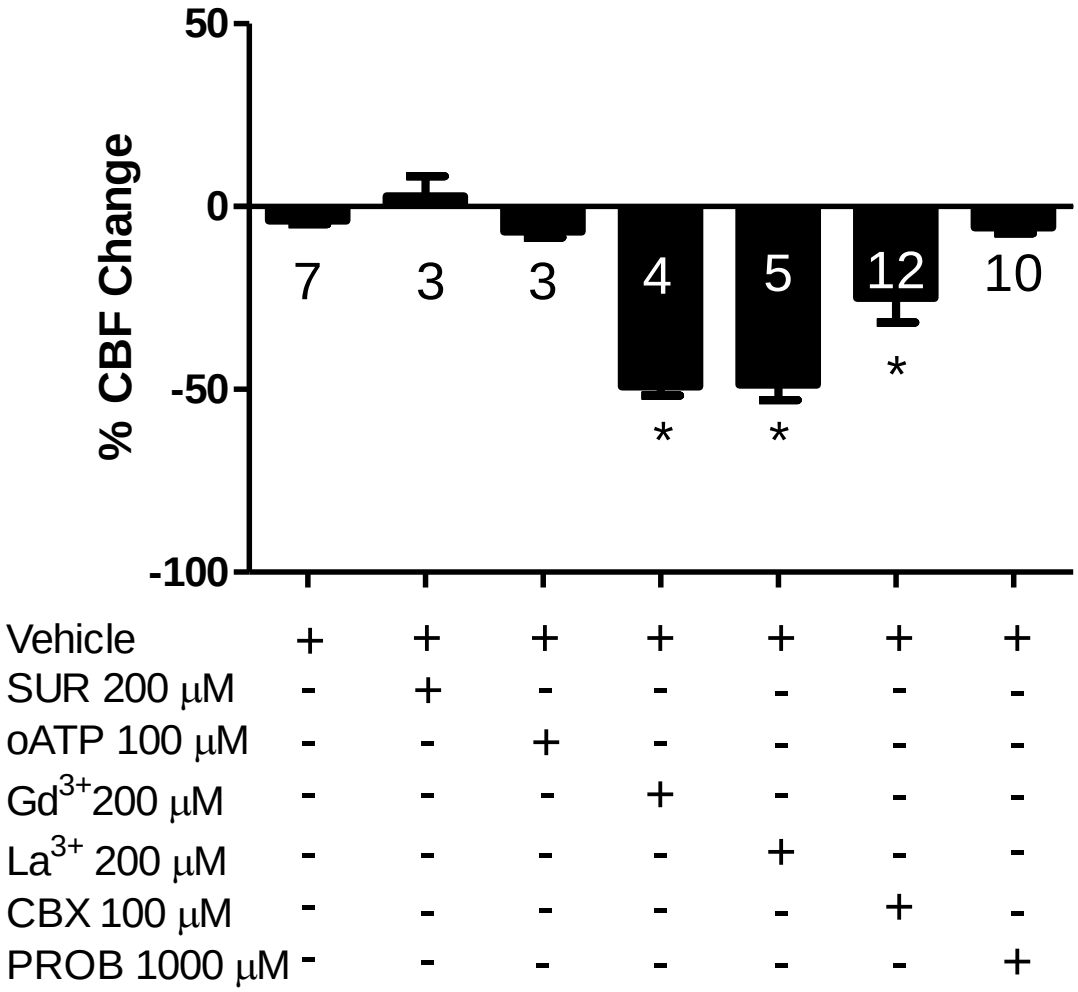


Figure 13. HC inhibition decreases the basal CBF in primary culture from mouse trachea.

CBF change was determined after the treatment with purinergic receptor antagonists (SUR, oATP) and HC inhibitors (Gd^{3+}, La^{3+}, CBX and PROB). Basal CBF was measured during 5 min, afterward blockers were added to the cultures and CBF was measured for 10 min. Gd^{3+}, La^{3+} and CBX transiently reduced the CBF. Bars represent the maximal reduction attained in each culture following the addition of antagonist (*, $p<0.05$ v. Vehicle).

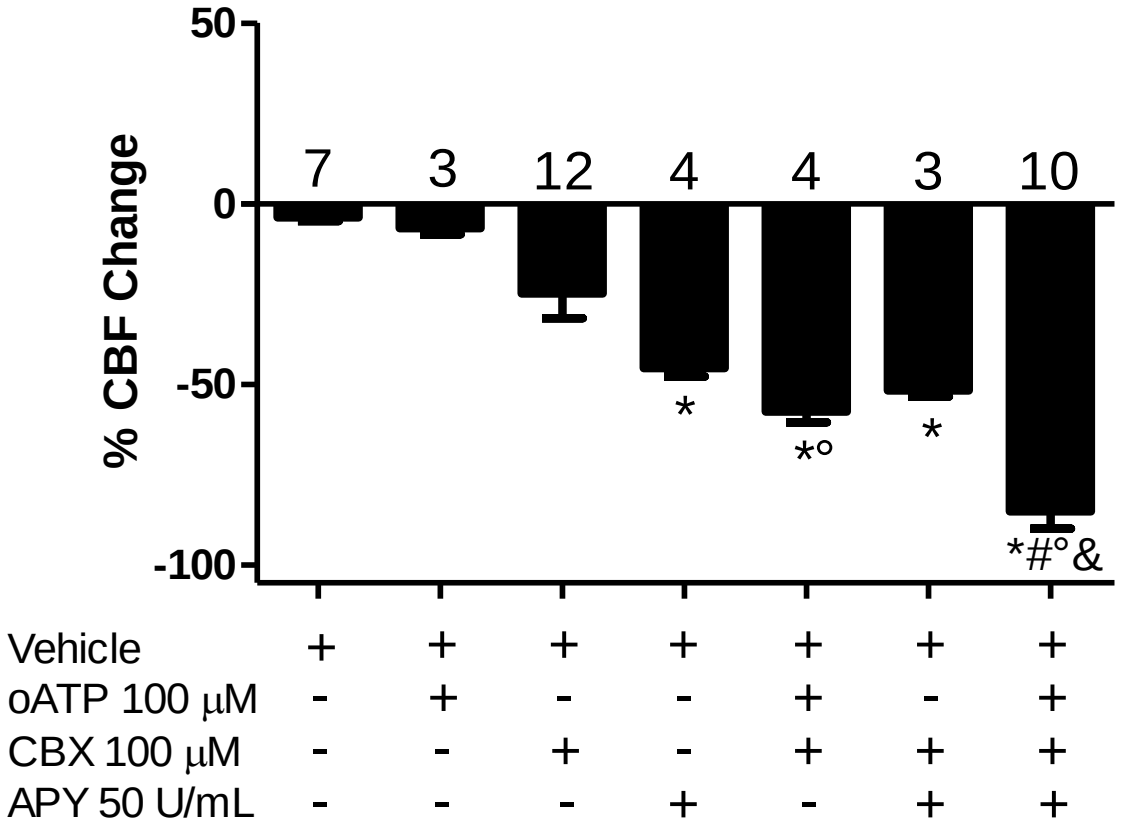


Figure 14. CBX, oATP and APY decrease basal CBF in primary culture from mouse trachea.

CBF change was determined after the treatment with oATP, CBX, and APY. These inhibitors were added alone or in different combinations to the culture. Bars represent the maximal reduction in CBF observed in each culture. oATP and CBX added simultaneously reduced significantly basal CBF compared with CBX and oATP alone. CBX alone reduced transiently CBX for 15 sec. However, oATP and CBX added simultaneously reduced basal CBF by 20 min (*, $p<0.05$ v. Vehicle; #, $p<0.05$ v. CBX; °, $p<0.05$ v. oATP; &, $p<0.05$ v. APY).

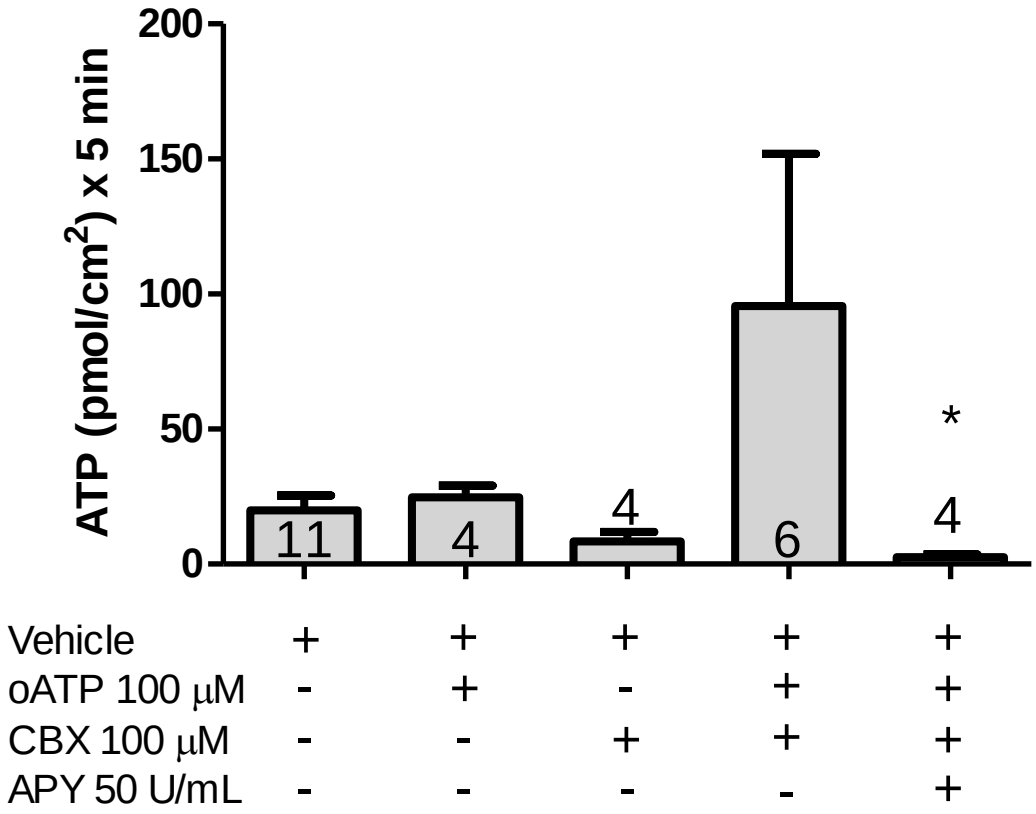


Figure 15. Effect of oATP, CBX and APY on the eATP levels in primary cultures from mouse trachea.

Quantification of eATP levels 5 min after addition of vehicle, oATP (100 μM), CBX (100 μM) and APY (APY) (50 U/mL). Not significant differences were observed with oATP, CBX and oATP plus CBX. However, concomitant treatment with APY, oATP and CBX decreased the eATP levels (*, $p<0.05$ v. Vehicle).

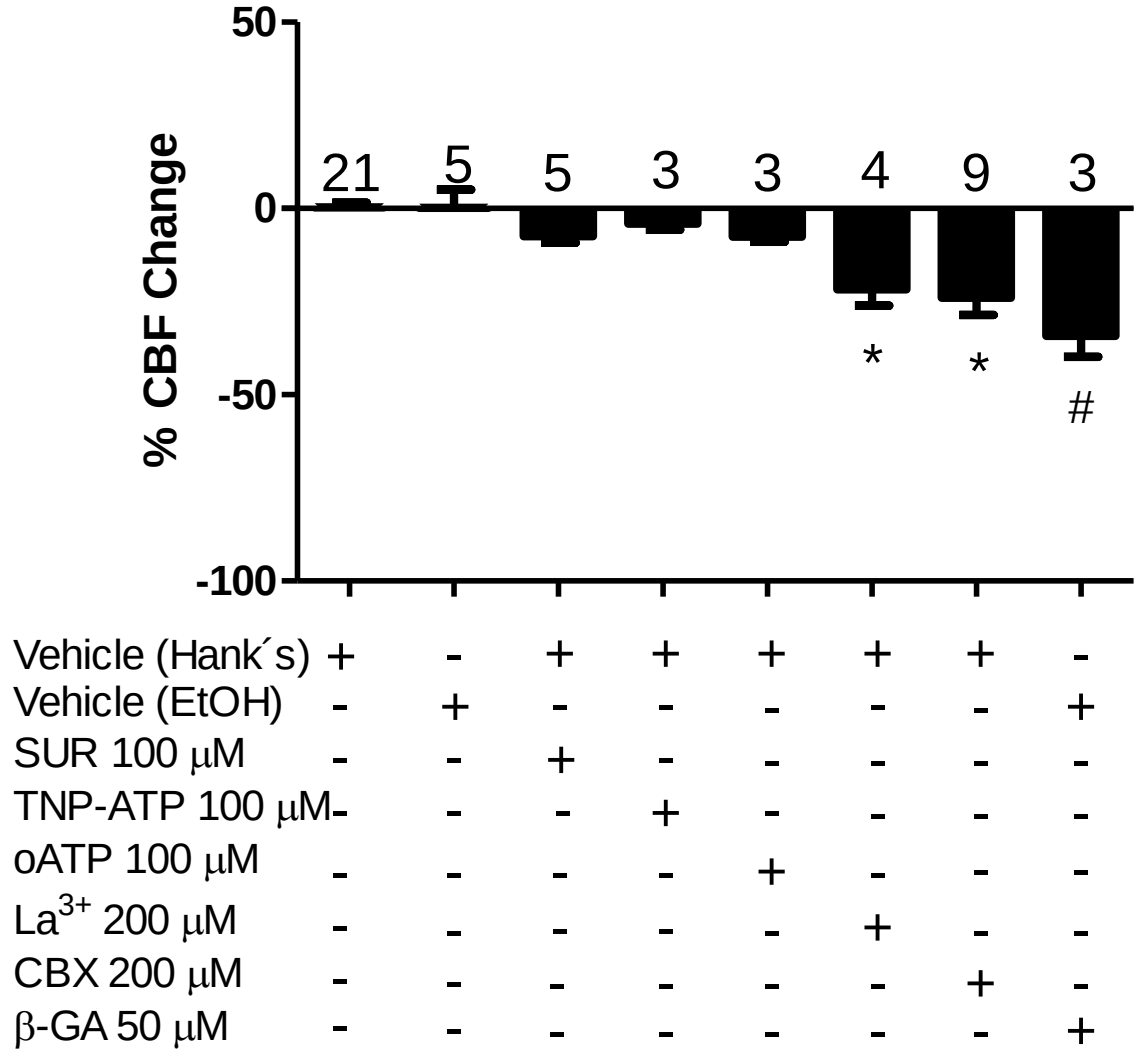


Figure 16. HC inhibition reduces basal CBF in primary culture from human adenoid.

CBF change was determined after the treatment with SUR, TNP-ATP, oATP, La^{3+}, CBX and β-GA. La^{3+}, CBX and β-GA reduced significantly basal CBF (*, $p<0.05$ v. Vehicle; # , $p<0.05$ v. Vehicle (EtOH).

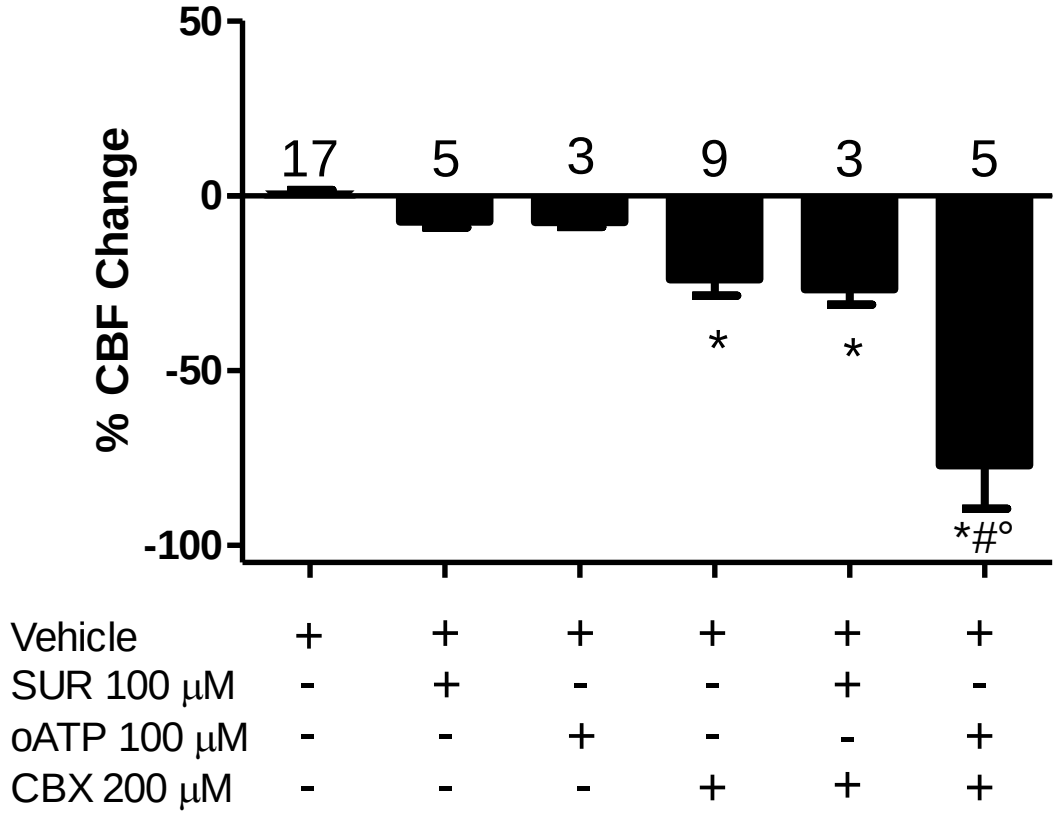


Figure 17. Inhibition of HCs and P2X7-R decreases basal CBF in primary culture from human adenoid.

CBF change was determined after the addition of SUR, oATP and CBX. CBX decreased the basal CBF, effect that was enhanced with the addition of oATP (*, $p<0.05$ v. Vehicle; #, $p<0.05$ v. oATP; °, $p<0.05$ v. CBX).

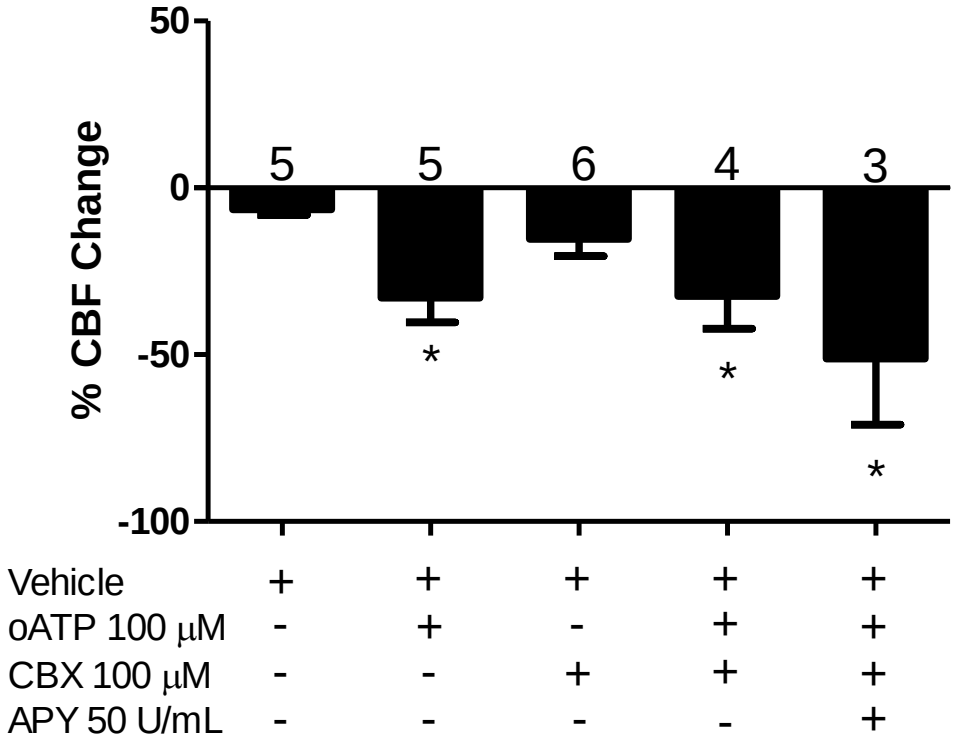


Figure 18. oATP, P2X7-R antagonist, decreases the basal CBF in primary culture from rat oviduct.

CBF change was determined after the addition of oATP, CBX and APY separately or together. oATP significantly reduced basal CBF (*, $p<0.05$ v. Vehicle).

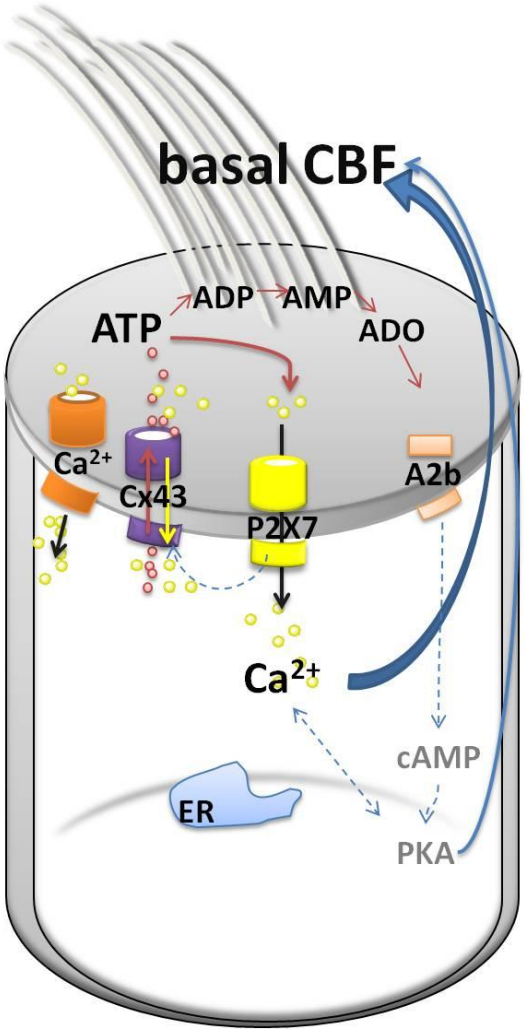


Figure 19. Control mechanism of basal CBF in epithelial ciliated cells.

eATP interacts with P2X7-R inducing Ca^{2+} entry. $Ca^{2+}{}_i$ maintains basal CBF. P2X7-R promotes ATP release by CxHCs. Other, non identified Ca^{2+} channels, participate in Ca^{2+} entry. ATP is hydrolyzed to ADO, which activates A2b-R, regulating cAMP and PKA and contributing to maintain the basal CBF mediating a cross talk with $Ca^{2+}{}_i$ pathway.

5.2 Mechanical and chemical stimulation increases CBF, effect that depends on ATP release

5.2.1 CBF increase induced by a mechanical stimulation is associated with an e[ATP] increase

A mechanical stimulus (MS) applied to primary cultures from human adenoid increased the CBF in 11% from the baseline (Figure 20A). The levels of ADO, AMP, ADP and ATP, pre and post MS (1, 3, 5 and 10 min) are shown in Figure 20B. eATP levels increase following 1 min of the MS from 11.7 ± 5.1 in basal conditions up to 58.9 ± 27.8 pmol/cm2 (n=3, $p<0.05$). Afterwards, e[ATP] decayed gradually over time. Changes in ADO, AMP and ADP did not show any significant differences, but AMP and ADP had a similar response when compared to ATP.

In addition, a MS in a single cell of the culture increased the $[Ca^{2+}]_i$ and this increase was detected in neighboring cells and subsequent cells with less intensity, corresponding to a ICW. Figure 21A shows pseudocolor consecutive images that represent changes in $Ca^{2+}{}_i$ levels. Figure 21B shows $Ca^{2+}{}_i$ levels in function of the time and distance from the stimulated cell. ICW had a propagation velocity of 6.6 ± 0.9 μm/sec calculated from 0 to 50 μm (n=11). Pre-incubation with APY (50 U/mL) for 10 min reduced $[Ca^{2+}]_i$ increase in the stimulated cell. Change of 340/380 ratio was 1.132 ± 0.007 for vehicle and 0.039 ± 0.005 for APY (n=4 and 3, $p<0.05$) (Figure 21C). In addition, APY also reduced the ICW propagation distance from 102.5 ± 30.1 μm in the vehicle group to 16.6 ± 3.3 μm with APY ($p<0.05$, n=4 and 3, respectively, Figure 21D). These results suggest that increase in $Ca^{2+}{}_i$ levels and propagation of ICW induced by MS depend on ATP release.

Similar preliminary result was obtained in primary cultures from mouse trachea. MS induced an ICW with a propagation distance of 180 μm and a propagation velocity of 4.3 μm/sec. APY (50

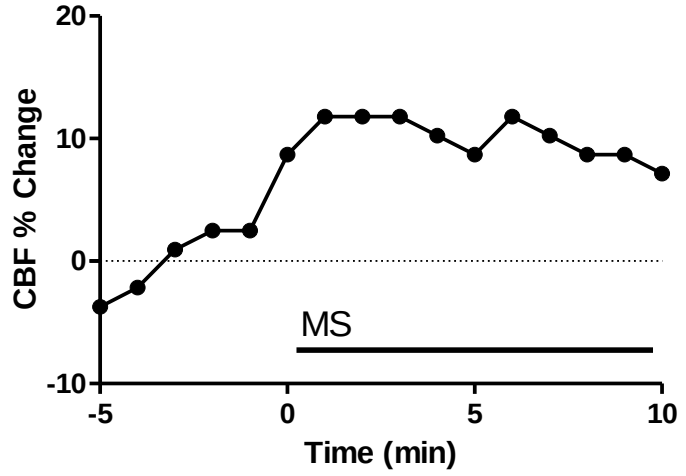
U/mL) abolished the ICW propagation (Figure 22). In addition, in ALI cultures from mouse septum, etmoides and trachea, stimulation with a pulse of air pressure (50 mmHg/50 msec) increased the CBF 1 min after the application of the stimulus (5.5 ± 0.5 to 9.7 ± 0.7 Hz for septum, 4.1 ± 0.4 to 9.5 ± 1.1 Hz for etmoides and 15.4 ± 1.1 to 19.1 ± 0.9 Hz for trachea, n=9, 5 and 6, $p<0.05$) (Figure 23A). MS also increase e[ATP] (17.0 ± 4.5 pmol to 42.3 ± 7.3 pmol for septum, 21.0 ± 7.4 pmol to 48.5 ± 7.0 pmol for etmoides, and 12.3 ± 4.9 pmol to 55.6 ± 12.0 pmol for trachea, n=12, 9, 9, $p<0.05$) (Figure 23B). These results are in agreement with human adenoid cultures, suggesting that the increases in CBF observed after the MS with a pulse of air are associated with an increase in e[ATP].

In septum ALI cultures, APY (50 U/mL) prevented the CBF increase in response to a MS with an air pulse (12.0 ± 4.53 % for APY versus 80.0 ± 18.32 % for vehicle, n=6, $p<0.05$) (data not shown and published in Zhao et al., 2012). SUR (1 mM), also blocked the increase in CBF after a MS (9.5 ± 3.3% for SUR versus 62.3 ± 6.2 % for vehicle, n=8, $p<0.05$) (data not shown and publish in Zhao et al., 2012). In addition, MS increased the intensity of fluorescence of Fluo4, an indicator of Ca^{2+}_i levels in 304 ± 49% above baseline. This effect was inhibited with APY (50 U/mL) (168 ± 13% for APY versus 321 ± 35% for vehicle, n=6, $p=0.05$) (data not shown and published in Zhao et al., 2012). To determine whether the release of ATP was dependent on Ca^{2+}_i increases, either from intra or extracellular, intracellular and extracellular Ca^{2+} was chelated in the same experiments using ethylene glycol tetraacetic acid (EGTA 0.5 mM, 10 min) and BAPTA-AM (10 μM, 30 min). The CBF change induced by a MS was reduced in cultures that were exposed to EGTA and BAPTA-AM (64 ± 8.8 % for vehicle versus 11 ± 2.1 % for EGTA/BAPTA, n=6, $p<0.05$). Cultures exposed to EGTA and BAPTA, also reduced the effect of ATP on CBF (100 μM) (91 ± 15 % for vehicle versus 5.0 ± 4.0 % for EGTA/BAPTA, n=6, $p<0.05$). Treatment with EGTA and BAPTA did not affect the e[ATP] following MS, as no difference was found between the control group (before

MS: 342.9 ± 42.39 nM; after MS: 1556.00 ± 95.15 nM, n=3) versus the experimental group (before MS: 530.8 ± 52.1 nM; after MS 1514.0 ± 281.4 nM; n=5) (data not shown and published in Zhao et al., 2012). These results suggest that the release of ATP is not dependent of $[Ca^{2+}]_i$ changes.

In summary, MS of ciliated epithelia produced increases in CBF, which is an effect associated with increments of $Ca^{2+}{}_i$ levels, propagation of intercellular calcium waves (ICWs) and elevated e[ATP]. All these changes were prevented with APY, which reduced e[ATP]. These results suggest that an increase in eATP release by the ciliated epithelium is required to propagate mechanical signals in ciliated cells. Figure 24 shows a model that integrates these results with previous studies (Bao et al., 2004; Andrade et al., 2005).

A



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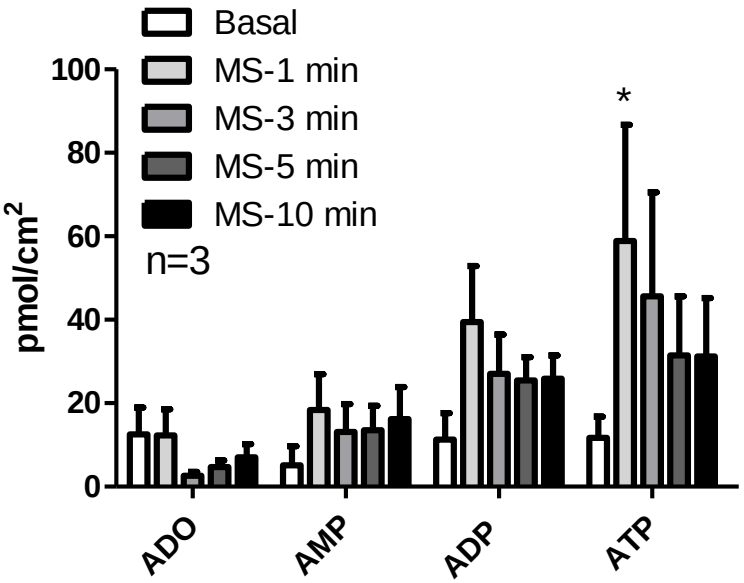


Figure 20. Mechanical stimulation (MS) increases CBF and e[ATP] in primary cultures from human adenoid.

A. Representative time course of CBF previous and after a MS (black line). An increase on CBF was observed in the human adenoid primary culture.
B. e[Purines] (ADO, AMP, ADP and ATP) were measured in medium cultures obtained previously (basal) and after MS (1, 3 5 and 10 min). An increase in e[ATP] was observed 1 min following MS (*, $p<0.05$ v. Basal, n=3).

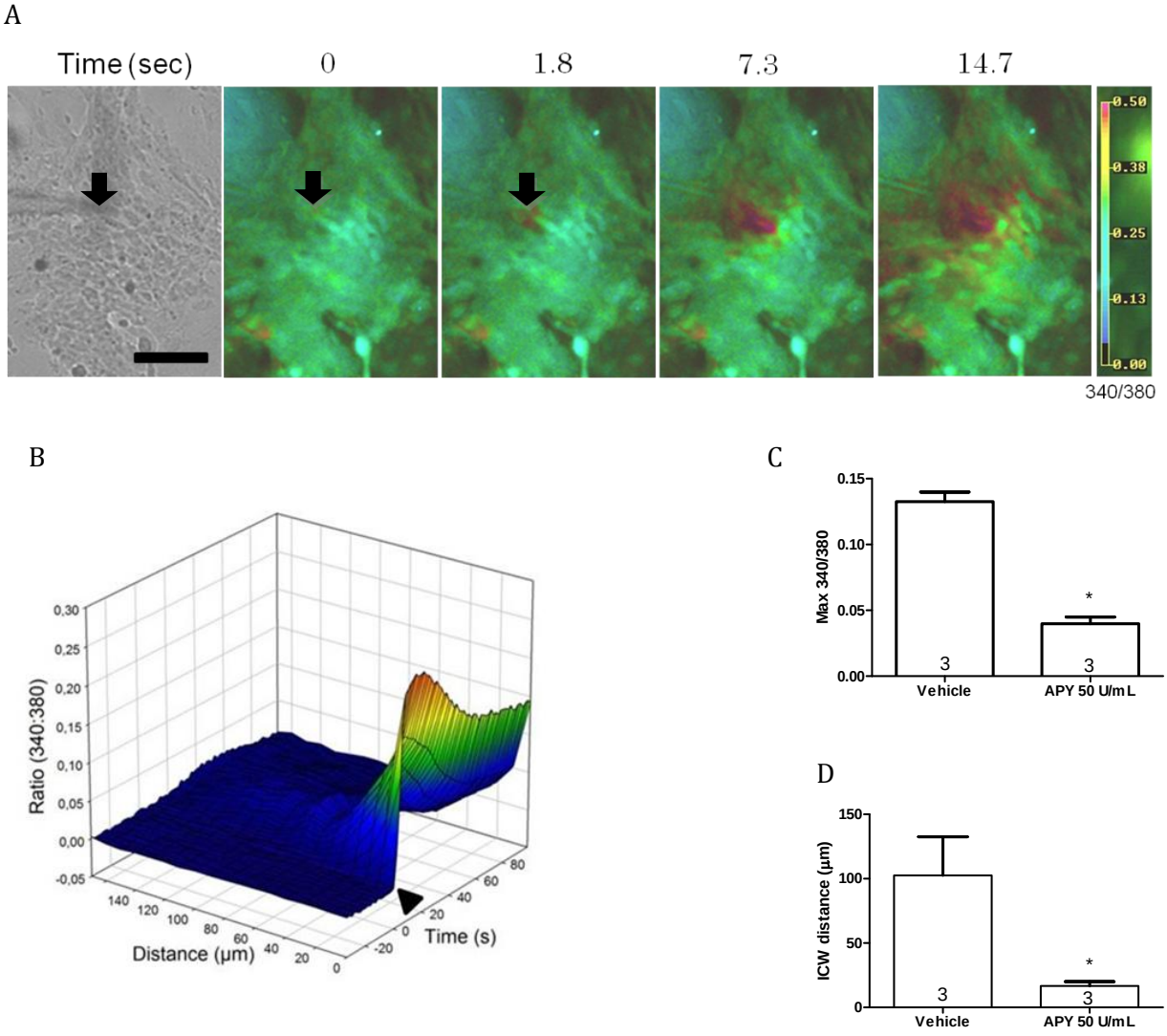


Figure 21. APY decreases intercellular calcium wave (ICW) propagation induced by a MS in primary cultures from human adenoid.

A. Image sequence of $[Ca^{2+}]_i$ (expressed as 340/380) in primary cultures from human adenoids stimulated in a single cell with a micropipette (arrow). Color scale of Ca^{2+} levels (340/380) are showed in right. Bar = 50 µm. The propagation velocity of ICW was 4.5 µm/sec.
B. Change of 340/380 ratio in function of time (sec) and distance (µm) from the stimulated cell (arrow) (representative of n=11). 340/380 ratio dependson the distance from the stimulated cell (0 µm). Propagation distance of ICW was 102.5 µm.
C. Maximum 340/380 change of stimulated cell in cultures pre-incubated with vehicle or APY (50 U/mL). APY decreased Ca^{2+}_i increase compared with vehicle (*, $p<0.05$ v. Vehicle, n=3).
D. Propagation distance (µm) of ICW from the stimulated cell in cultures pre-incubated with vehicle or APY (50 U/mL). APY decreased the ICW distance compared with vehicle (*, $p<0.05$ v. Vehicle, n=3).

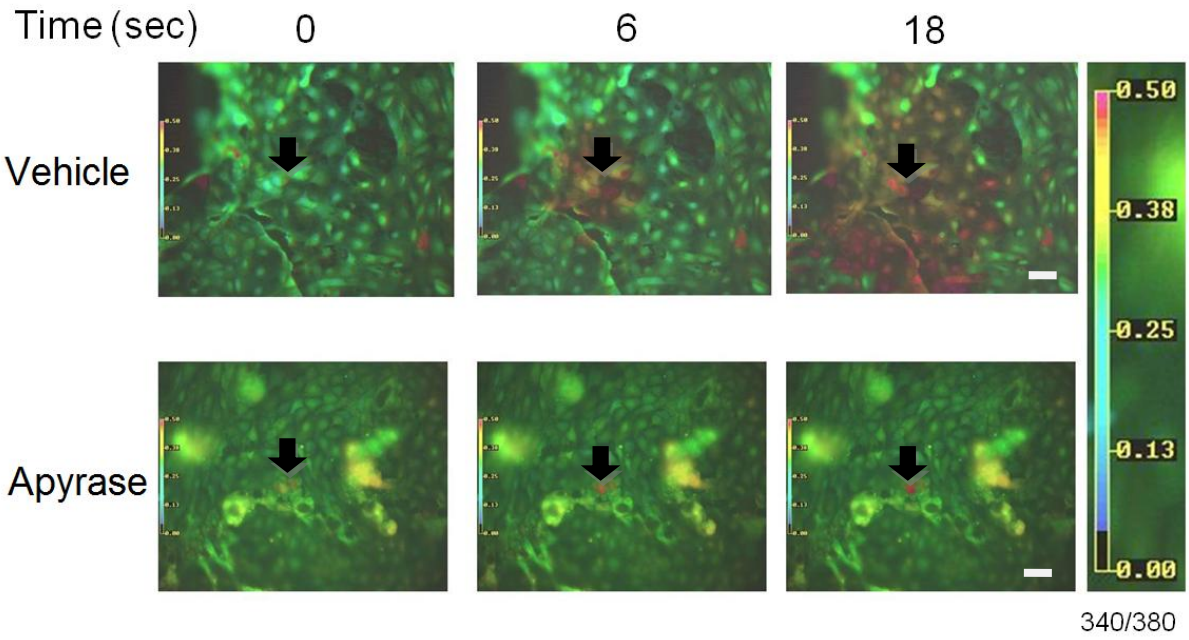
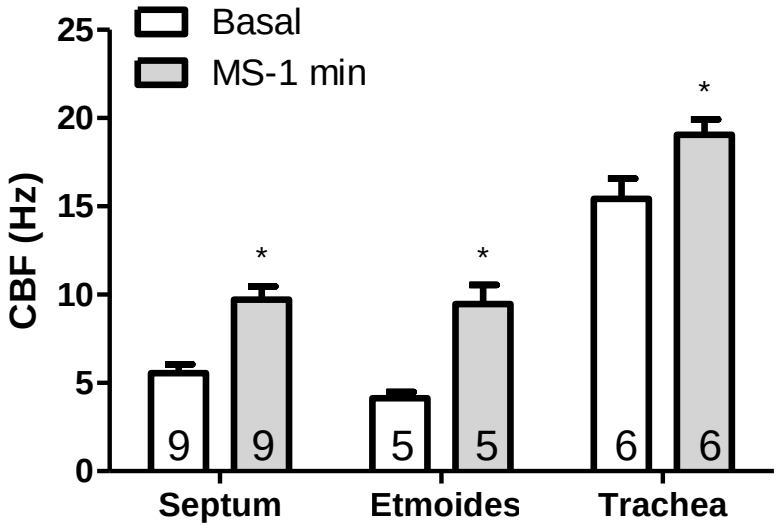


Figure 22. APY decreases the propagation of ICW induced by a MS in primary cultures from mouse trachea.

Image sequence of fluorescence (340/380) in mouse trachea primary cultures. Cultures were stimulated with a MS in a single cell (black arrow). In right, the scale of the 340/380 ratio. Calibration bar = 50 μm. Red correspond to high Ca^{2+}_i levels and blue correspond to low Ca^{2+}_i levels. Vehicle or APY (50 U/mL) were added to the cultures 10 min before the stimulation. APY inhibited the ICW propagation.

A



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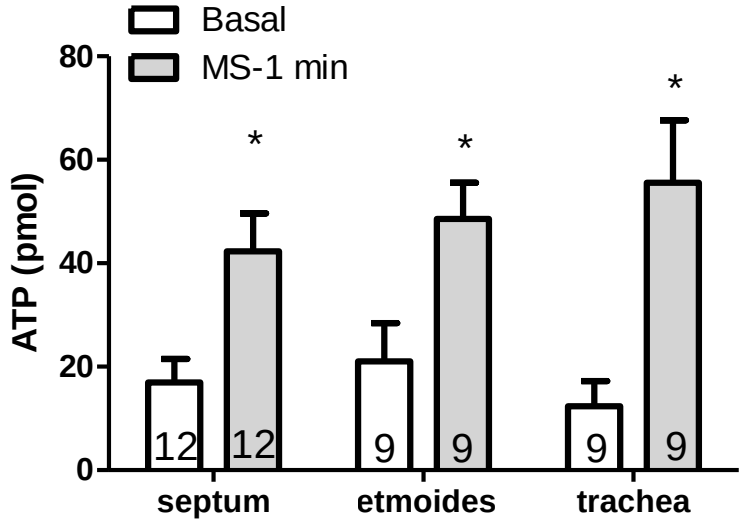


Figure 23. MS increases CBF and e[ATP] in ALI cultures from mouse septum, etmoides and trachea.

A. CBF (Hz) was measured in ALI cultures previously (Basal) and after aMS (55 mmHg/50 ms). MS increased CBF in ALI cultures from septum, etmoides and trachea (*, $p<0.05$ v. Basal).
B. e[ATP] wasmeasured in the culture medium obtained from confluent ALI cultures previously and after aMS (55 mmHg/50 ms). MS increases e[ATP] in ALI cultures from septum etmoides and trachea (*, $p<0.05$ v. Basal).

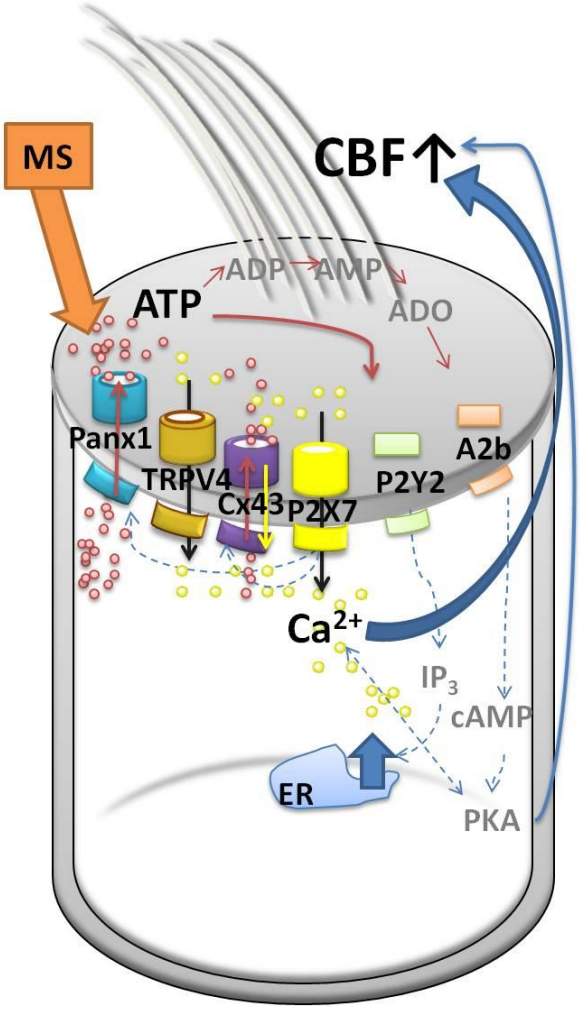


Figure 24. CBF increase induced by mechanical stimulation depends on ATP release in ciliated epithelium.

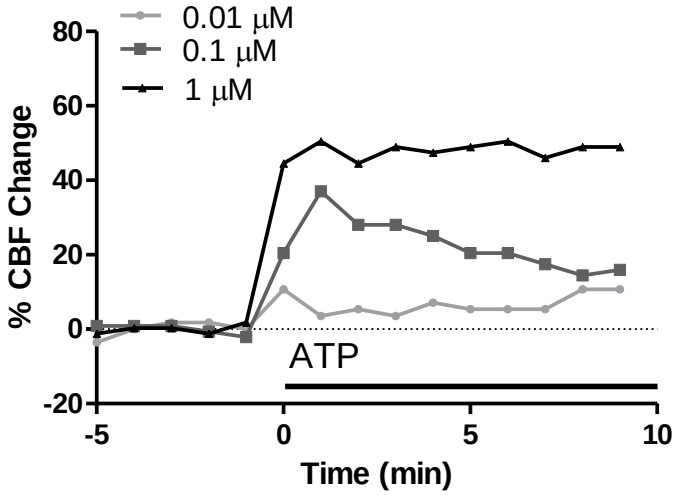
MS induces ATP release mediating a mechanism independent of Ca^{2+}_i level changes, possibly through Panx1 or Cx43 which are mechanosensitive channels. eATP activates purinergic receptors, possibly P2Y2-R and P2X7-R, which leads to an increase in Ca^{2+}_i levels and CBF. MS also can induce the entry of Ca^{2+} through the activation of mechanosensitive TRPV4 channels.

5.2.2 Chemical stimulation with ATP increases CBF and $[Ca^{2+}]_i$

In primary cultures from mouse trachea, addition of different concentrations of ATP (0.01 μM to 1000 μM) increased CBF. Figure 25A shows the effect of ATP (0.01, 0.1 and 1 μM) on CBF. ATP 0.01 μM induced a small increase in CBF (10%), ATP 0.1 μM induced a 37% increase in CBF with a subsequent fall. ATP 1 μM induced a rapid increase in CBF (50%) but did not decay during the time recorded (10 min), maintaining a sustained response. Ten and 100 μM of ATP showed similar effect as 1 μM, however, ATP 1000 μM induced a reduction in CBF (-36% from baseline, n=4) (data not shown). Non linear fit analysis of CBF change induced by ATP showed an EC50 of 0.04 μM of ATP (Figure 25B). CBF increase was 7.2 ± 2.4 % with 0.001 μM, 13.6 ± 4.7 % with 0.01 μM, 39.3 ± 6.5 % with 0.1 μM, 47.5 ± 7.8 % with 1 μM, 53.1 ± 2.8 % with 10 μM and 51.6 ± 11.2 % with 100 μM of ATP.

In primary cultures of human adenoids CBF increase induced by ATP was 28.7% with 10 μM, 33% with 50 μM and 29.4% with 100 μM of ATP (n=9, 3 and 6, respectively) (data not shown and published in González et al., 2013). Addition of ATP (10 μM) also increases $Ca^{2+}{}_i$ levels in primary cultures from human adenoids. The increase of $[Ca^{2+}]_i$ reached a peak of 4-fold higher than the baseline within 10 sec. This increase was followed by a slower decline that almost reached the baseline at 2.5 min (n=6 cells) (Figure 26). These results are in concordance with previous studies that ATP increases CBF and $[Ca^{2+}]_i$.

A



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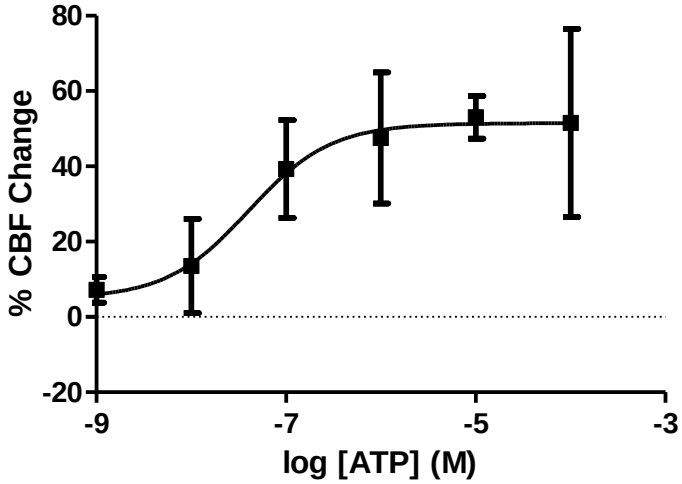


Figure 25. ATP increases CBF in primary cultures from mouse trachea.

A. Representative time course of CBF in response to ATP 0.01 μM, 0.1 μM and 1 μM (representative of n= 7, 4, 5). ATP 0.1 μM induced an increase in CBF with a subsequent fall. ATP 1 μM induced a rapid increase on CBF maintaining a sustain response.
B. CBF change was measured following the addition of different ATP concentration. Calculated EC50 was 0.04 μM (nonlinear fit).

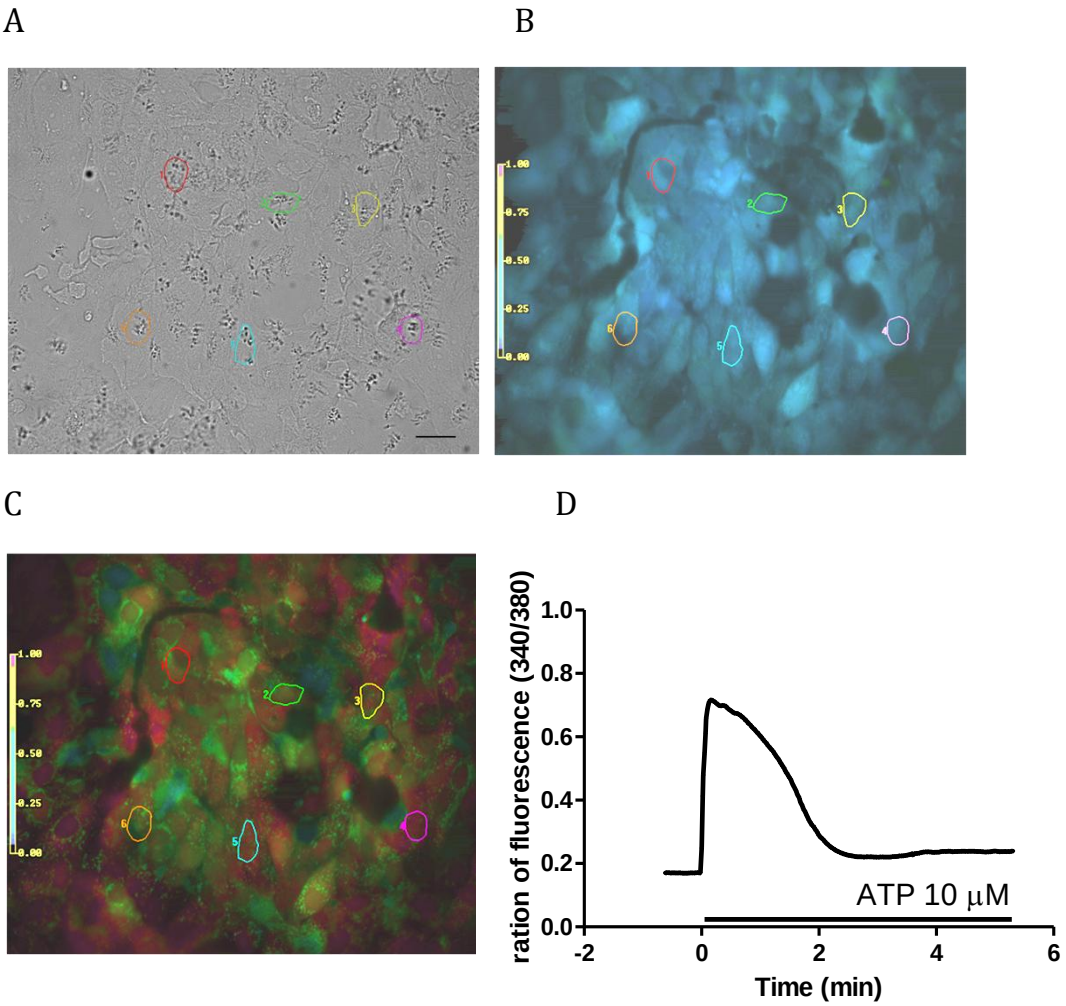


Figure 26. ATP increases Ca^{2+}_i levels in primary cultures from human adenoid.

A. Microphotography of primary cultures from human adenoid showing 6 region of interest (ROI), corresponding to ciliated cells. Bar = 50 µm.
B. and C. images of fluorescence 340/380 of cultures previously (B) and following the addition of ATP (C). Color scale of 340/380 ratio is showed in left, blue correspond to low Ca^{2+}_i levels and red correspond to high Ca^{2+}_i levels. ATP (10 µM) increased Ca^{2+}_i levels.
D. Representative time course response of Ca^{2+}_i levels (340/380) corresponding to mean of selected ROI (n_{ROI}=6). Addition of ATP (10 µM) induced a rapid increase in the Ca^{2+}_i levels, followed by a slower decline.

5.2.3 Effect of HCs and P2X7-R blockers on CBF and e[ATP] increase induced by a chemical stimulation with ATP

Primary cultures from mouse trachea were pre-incubated with HC and P2X7-R blockers to evaluate the CBF response to ATP. As some of these blockers had an effect on basal CBF, ATP was added to the medium cultures only when the basal CBF was reestablished and remained stable for 2 min. oATP (10 and 100 μM) and CBX (10 μM), did not modify the CBF increase induced by ATP (10 μM), however CBX (100 μM) inhibited the CBF increase induced by ATP (51 ± 6.1 % for Vh/ATP versus 9.8 ± 1.5 % for CBX100/ATP, $p<0.05$, n=3) (Figure 27). Concomitant preincubation with oATP (10 μM) and CBX (10 μM) did not modify the effect on CBF induced by ATP, however, CBX (100 μM) plus oATP (100 μM) lowered CBF following ATP (-61.5 ± 3.6 %, n=3) (Figure 27). PROB (1000 μM) reduced the CBF increase induced by ATP (51.5 ± 6.0 % for vehicle versus 26.2 ± 1.4 % for PROB, $p=0.05$, n=8) (Figure 27). These results suggest that HCs (Panx1/Cx) are involved in the CBF response induced by ATP.

To determine if CBX affected the sustained component of the CBF increase induced by ATP, primary cultures of mouse trachea were stimulated with ATP (1 μM) for 1 min to induce the increase in CBF, and then, were treated with vehicle or CBX (100 μM). CBX induced a faster fall in the increased CBF induced by ATP when compared to control, maximum differences in the CBF were observed 4 min after ATP addition (36.6% ± 8.4 for vehicle and 3.1% ± 10.88 for CBX, $p<0.05$, n=4) (Figure 28A). In order to determine if ATP induces the ATP release through HCs, eATP levels were measured in the same conditions as described in figure 28A. e[ATP] measured in culture following ATP addition (1 μM) was 1.01 ± 0,09 μM at 4 min and 1.12 ± 0.057 μM at 20 min (n=6). This result suggests that e[ATP] are stable over time of measurement, indicating that the rate of release is similar to degradation of ATP by ectonucleotidases. CBX reduced the eATP levels measured at 4 min (10.3 ± 1.0 nmol/cm^2 for vehicle versus 3.8 ± 0.5 nmol/cm^2 for CBX,

n=6 $p<0.05$) (Figure 28B) and 20 min after ATP addition (7.7 ± 0.9 nmol/cm^2 for vehicle versus 4.9 ± 0.6 nmol/cm^2 for CBX, $p<0.05$, n=6) (data not shown). This result suggests that ATP is released by HCs following an exogenous stimulus of ATP. Measurement of eATP levels following the addition of vehicle or CBX alone were 0.0199 ± 0.0095 nmol/cm^2 and 0.0084 ± 0.0036 nmol/cm^2 at 5 min respectively (Data in Figure 15). These results suggest that a chemical stimulation with ATP induces ATP release to mediate the increase in CBF.

Primary cultures from rat oviduct incubated with oATP (100 μM) showed a reduction in the CBF increase induced by ATP (100 μM) (54.7% ± 7.0 for vehicle versus 25.9 % ± 7.5 for oATP, $p<0.05$, n=6 and 5 respectively) (Figure 29). CBX (100 μM) completely blocked the increase in CBF in response to ATP (100 μM) (54.7% ± 7.0 for vehicle/ATP versus -34.1 % ± 5.5 for CBX/ATP, $p<0.05$, n= 6) (Figure 30 A and B). In addition, the increase in $[Ca^{2+}]_i$ induced by ATP was lower in cultures incubated with CBX (100 μM) (Change 340/380 was 0.7 ± 0.16 for vehicle and 0.20 ± 0.08 for CBX, n=3, $p<0.05$) (Figure 30 C and D). These results suggest that HCs are an important component in the $[Ca^{2+}]_i$ increase induced by ATP, possibly by the influx of Ca^{2+} from the extracellular compartment in primary cultures from rat oviduct.

In summary, stimulation with ATP increased CBF and $[Ca^{2+}]_i$ in respiratory primary cultures. The increase in CBF induced by ATP was reduced by PROB and CBX. CBX also reduced e[ATP] following the addition of ATP, suggesting that the chemical stimulation with ATP affects CBF by inducing the release of ATP via HCs in ciliated epithelial cells (Figure 31).

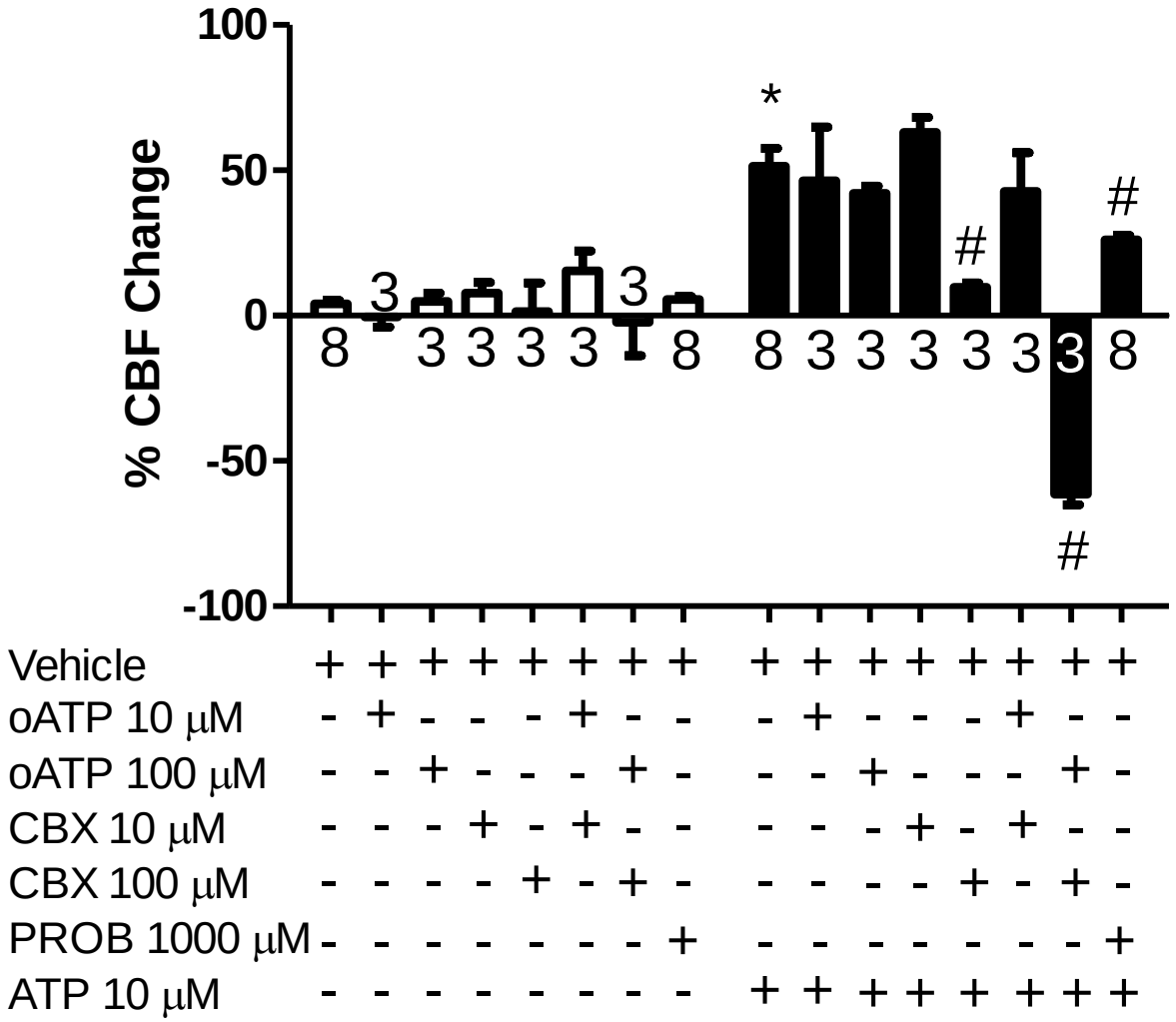
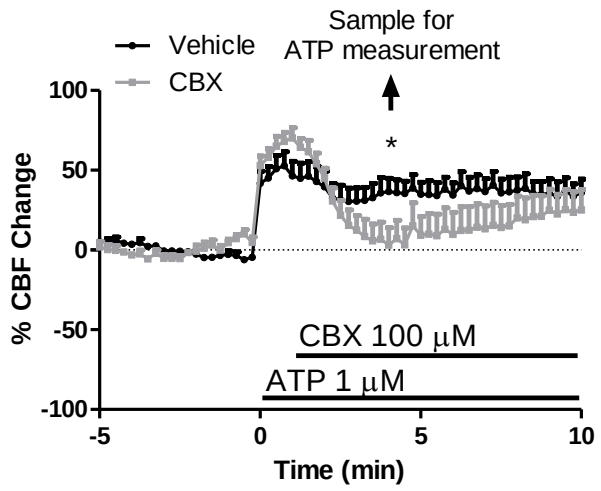


Figure 27. Effect of HC and P2X7-R inhibition on CBF increase induced by ATP in primary cultures from mouse trachea.

CBF change was measured following the addition of ATP (10 μM) in cultures pre-incubated with oATP, CBX and PROB (black). Basal CBF correspond to CBF change previous to ATP addition (white). ATP increased CBF in cultures pre-incubated with vehicle, however, a pre-incubation with CBX prevented the increase in CBF induced by ATP. In addition, ATP reduced the CBF in cultures pre-incubated with oATP and CBX added together in the culture (*, $p<0.05$ v. Vh; #, $p<0.05$ v. Vh/ATP).

A



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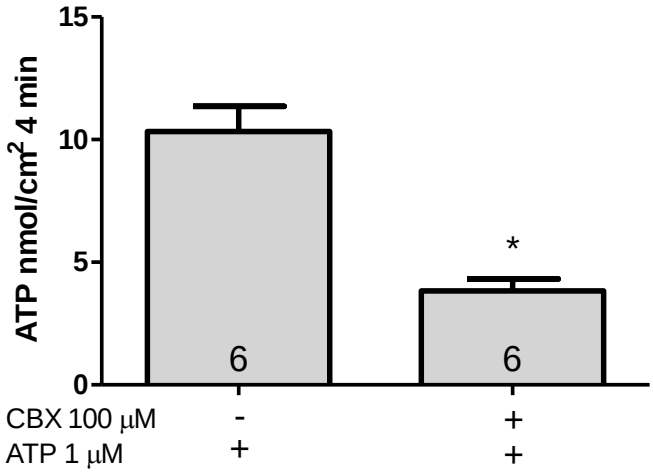

Figure 28. Inhibition of HC with CBX reduces the increase of e[ATP] following the addition of ATP in primary cultures from mouse trachea.

A. Time course of CBF change in response to ATP (1 μM) and CBX (100 μM) added 1 minute after ATP (black line). CBX reduced the effect of ATP on CBF 4 min after ATP addition (*, $p>0.05$).

B. e[ATP] was measured 4 min following the addition of ATP in cultures treated with CBX or vehicle 1 min after ATP addition. CBX decreased the e[ATP] following the addition of ATP (*, $p<0.05$). Measurement of eATP levels following the addition of vehicle or CBX alone were 0.0199 ± 0.0095 nmol/cm^2 and 0.0084 ± 0.0036 nmol/cm^2 at 5 min respectively (Data in Figure 15).

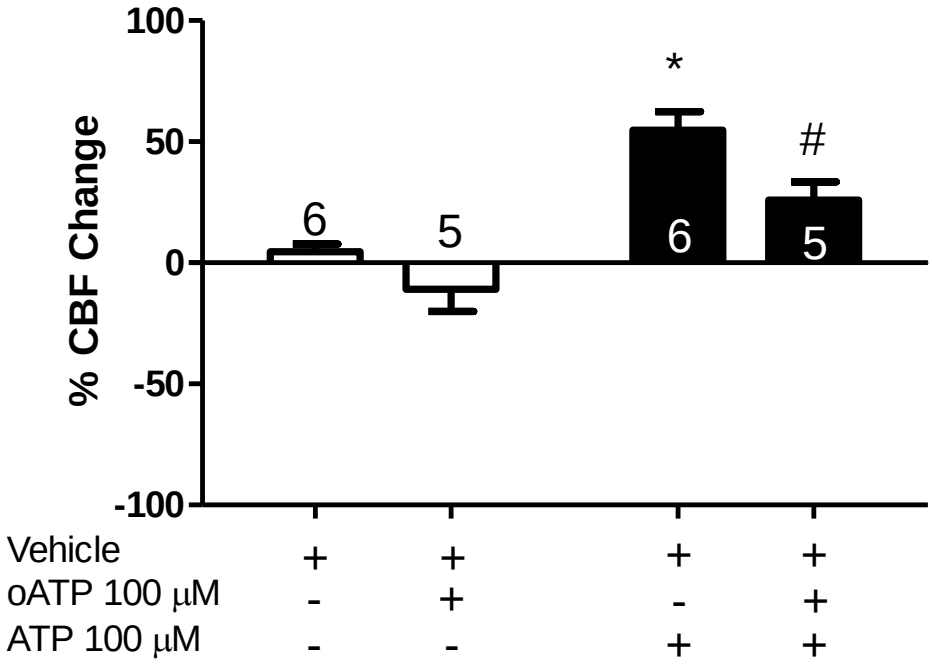

Figure 29. Blockade of P2X7-R with oATP decreases CBF increase induced by ATP in primary cultures from rat oviduct.

CBF change was measured following the addition of ATP (10 μM) in cultures pre-incubated with oATP (100 μM) (black). Basal CBF correspond to CBF change previous to ATP addition (white). oATP decreased the CBF increase induced by ATP (*, $p<0.05$ v. vehicle; #, $p<0.05$ v. PROB; °, $p<0.05$ v. vehicle/ATP).

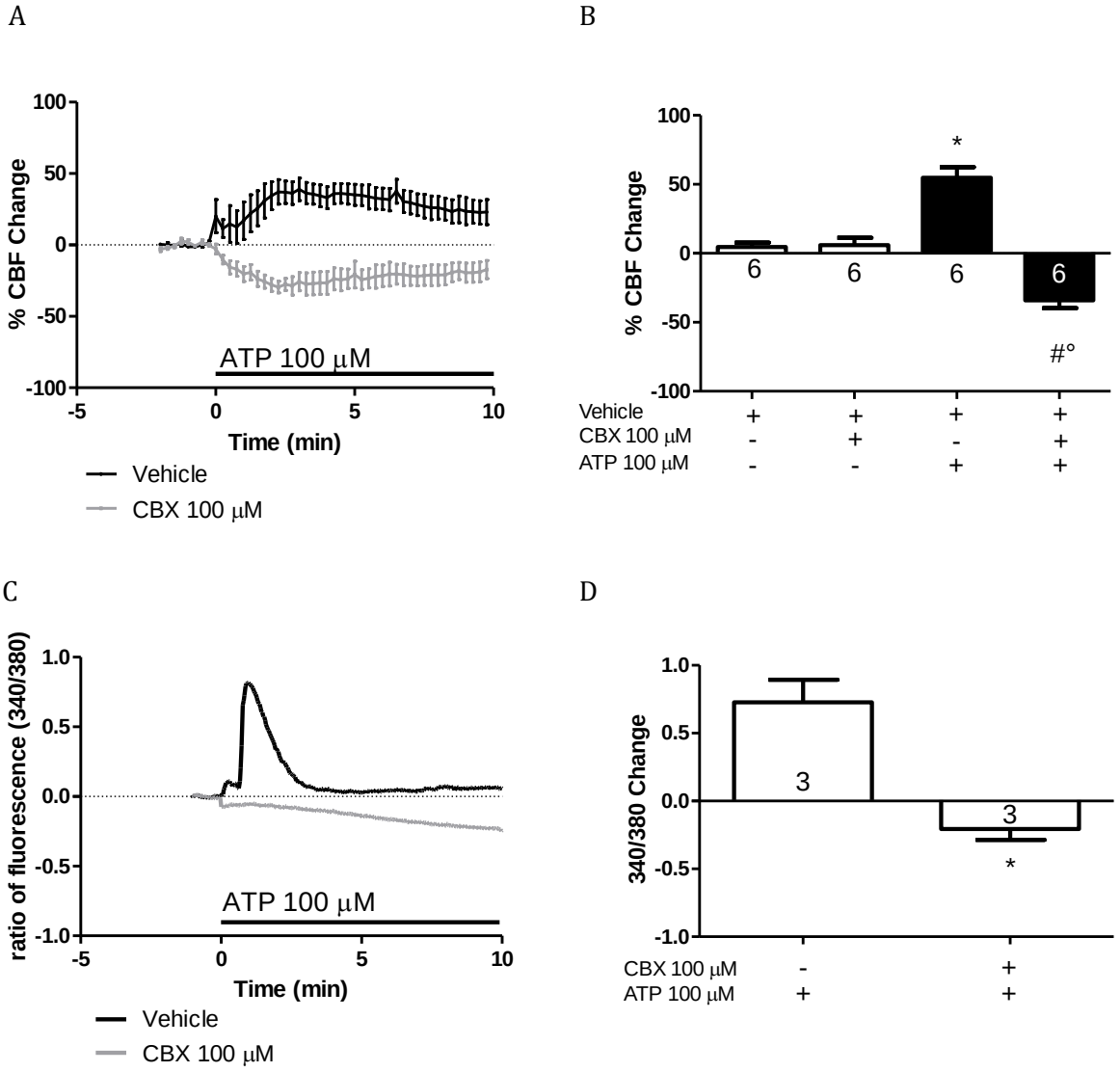

Figure 30. HC inhibition with CBX decreases $[Ca^{2+}]_i$ increase induced by ATP in primary cultures from rat oviduct.

ATP (100 μM) response was evaluated on CBF and Ca^{2+}_i levels in the presence of CBX (100 μM). (A) Time course of CBF in response to ATP in cultures pre-incubated with CBX or vehicle by 10 min. (B) Maximum CBF change in response to ATP (100 μM) in cultures treated with CBX. It was observed a reduction in the CBF increase induced by ATP in cultures treated with CBX (*, $p<0.05$ v. Vehicle). (C) Representative time course of Ca^{2+}_i levels (340/380 ratio) in response to ATP in cultures pre-incubated with vehicle or CBX. (D) Maximal change of 340/380 ratio in cultures treated with ATP following the addition of CBX. CBX prevented the $[Ca^{2+}]_i$ increase induced by ATP (*, $p<0.05$ v. Vehicle).

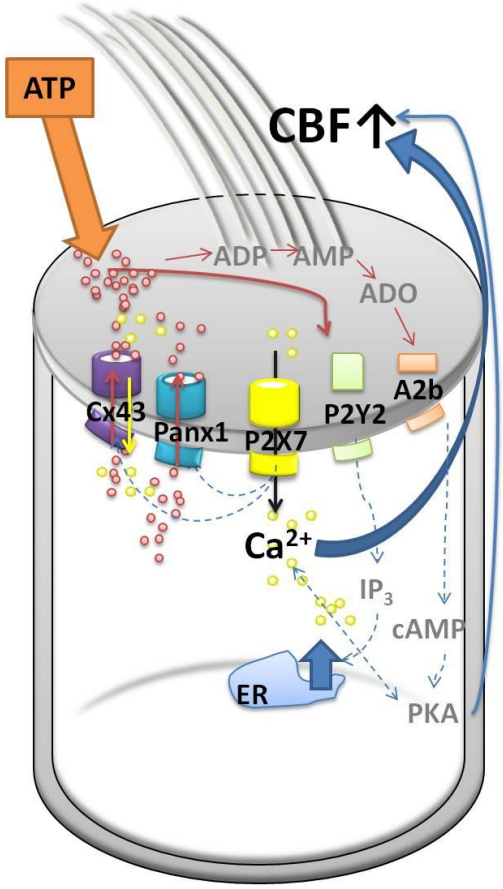

Figure 31. ATP release mediates the increase in CBF induced by ATP in ciliated epithelium.

A chemical stimulation with exogenous ATP increases CBF through the activation of purinergic receptors. Also, P2X7-R, associated to Ca^{2+} entry and the release of ATP through HCs (Cx or Panx1), participate in the increase of CBF induced by ATP.

Discussion

Mouse trachea ciliated cells were used to study the control mechanisms of ciliary activity in mucociliated respiratory epithelium. In addition, some experiments were performed in characterized ciliated cellular model of human adenoid, respiratory ALI cultures and oviductal ciliated cells, in order to complement the observations about the control mechanism of ciliary activity. Treatment with APY reduced e[ATP] and basal CBF in primary cultures of mouse trachea, human adenoid. In addition, concomitant treatment with APY, CBX and oATP further reduced basal CBF, suggesting that eATP and the molecular components of ATP release (HCs) and ATP activation (P2X7-R) contribute in the control mechanism of basal ciliary activity.

Chemical and mechanical stimuli of the ciliated epithelium caused a CBF increase associated to $[Ca^{2+}]_i$ and e[ATP]. Hydrolysis of e[ATP] decreased the CBF and $Ca^{2+}{}_i$ levels observed in response to MS. Blockade of HCs prevented the increase in CBF and $[Ca^{2+}]_i$ and reduced e[ATP] in response to ATP addition, suggesting that ATP release mediates the CBF increase induced by mechanical and chemical stimulation in ciliated epithelial cells.

7.1 Cellular models of mucociliary epithelium

Respiratory and reproductive models of mucociliated epithelium were used, including primary cultures from mouse trachea, human adenoid and rat oviduct, as well as interface liquid air (ALI) cultures from mouse septum, etmoides and trachea and human seno-nasal epithelium. All these cultures presented ciliated cells with a spontaneous ciliary beat in our experimental conditions, allowing the study of mechanism that control basal ciliary activity in respiratory and reproductive epithelia.

The technique used to prepare primary cultures obtained from human adenoid biopsies was published by González et al. (2013). These cultures presented an enriched monolayer of ciliated cells that contained a reduced number of secreting cells due to the fact that other growing conditions are necessary to obtain these cells. Adenoids are usually obtained from non-allergic patients whose epithelium has not been under chronic treatment or infections and obtaining the sample does not add any extra morbidity to patients. Sample availability of human adenoid depends on the medical center, patient conditions and signed informed consent, so a regular sample supply is not guaranteed. These cultures responded to ATP similar to previous reports (Villalón et al., 1989; Morales et al., 2000; Kerr, 2004; Jiao et al., 2012; Kawakami et al., 2004; Korngreen and Priel, 1996; Morse et al., 2001) and constitute a reproducible and feasible experimental model to understand ciliary activity control mechanisms.

ALI cultures from mouse septum, etmoides and trachea and from human sinonasal epithelia were obtained using a standardized technique in Dr. Cohen´s laboratory, where part of this thesis was carried out. ALI cultures are composed of a confluent monolayer of respiratory epithelial cells, where approximately 30% and 90% are ciliated cells in cultures of trachea and septum respectively, whereas the rest are non-ciliated and clara cells. The advantage of this experimental model is that ALI cultures are morphologically similar to the respiratory epithelium *in vivo*, maintaining the polarity of epithelial cells and facilitating the study of both sides of the cells, basal and apical (Woodworth et al., 2007; Antunes et al., 2007). The time to observe ciliated cells (21 days) and the large number of mice required to obtain a significant number of cultures (2 trachea = 1 culture) are some of the limitations of this type of cultures versus primary cultures (5-7 days; 1 trachea = 3-6 cultures) (Antunes et al., 2007). Nevertheless, ALI cultures constitute a useful experimental model to study the control mechanisms that regulate ciliary activity (Woodworth et al., 2007; Antunes et al., 2007).

The technique used to prepare primary cultures from mouse trachea was similar to that described to prepare primary cultures from human adenoids. Primary cultures from mouse trachea showed the characteristic monolayer with beating ciliated cells from the fourth day of culture. These cultures presented ~25% of ciliated cells and were used to carry out most of the experimental protocols of this study. Primary cultures of rat oviduct were used to study the control mechanism of basal ciliary activity in the reproductive tract. This model has been used to study the regulation of ciliary activity in response to ATP, progesterone and estradiol (Kerr, 2004; Sanchez, 2009). All these cultures have the advantage of avoiding the immediate influence of inflammatory mediators released during the removal of the tissue sample. Inflammatory mediators, such as TNF-α, modify CBF in the short term (Pérez, 2011).

Taken in account the advantages and limitations of culture preparation, the main model used in this thesis to study ciliary activity was primary cultures from mouse trachea, since they provide a reproducible and feasible experimental model. In addition, some experiments were performed in primary cultures from human adenoid and oviductal cells, characterized models of our laboratory, in order to assess whether a similar control mechanism of ciliary activity was present in human upper respiratory tract and reproductive epithelial ciliated cells.

7.2 eATP contributes to the control mechanism of basal ciliary activity

Basal CBF was measured in primary cultures from mouse trachea, human adenoids and rat oviducts. They showed a mean basal CBF of 13.3, 10.3 and 11.5 Hz, respectively, which is in the range of previous reports of respiratory and reproductive tract epithelia (Cohen, 2006; Nakahari, 2007; Zhang et al., 2004). In ALI cultures from mouse septum, etmoides and trachea as well as

human senonasal epithelium, basal CBFs were 4.7, 4.4, 13.2 and 5.1 Hz, respectively, which are concordant with the basal CBF obtained in previous studies (Antunes et al., 2007; Woodworth et al., 2007).

e[ATP] was measured in resting mouse trachea, human adenoid and rat oviduct primary cultures (~20 pmol/cm^2 or 30 nM/cm^2). These levels were similar to previous reports in primary cultures from human respiratory epithelia, suggesting that ATP is released constitutively from respiratory and oviductal epithelial cells (Lazarowski et al., 2004).

APY, a commercial ecto-nucleotidase which hydrolyzes ATP and ADP (Zimmermann & Braun, 1996), decreased basal CBF in primary cultures of mouse trachea. This reduction of basal CBF was concentration-dependent, showing a ~53% decrease with 100 U/mL of APY. Since an increase in CBF (16%) is associated with a high change in mucociliary clearance (MCC) velocity (56%) (Seybold et al., 1990), the reduction of basal CBF observed following APY treatment may have important implications for MCC. APY also reduced basal e[ATP] by ~50% from the baseline at 1 min, increasing this reduction over time. These results did not directly provide a causal link between e[ATP] and basal CBF, but suggest that eATP contributes with at least ~50% in the control mechanism of basal CBF. Additionally, ATP hydrolysis with APY reduced $[Ca^{2+}]_i$ in these cultures. $[Ca^{2+}]_i$ has been recurrently associated to CBF changes, since increases in CBF with eATP are related to $[Ca^{2+}]_i$ increases (Villalón et al., 1989) and depletion of Ca^{2+} from intracellular stores and chelation of extracellular Ca^{2+} prevent the increase in $[Ca^{2+}]_i$ and CBF (Kerr, 2004). These observations are in agreement with our results, which suggest that e[ATP] contributes to the control mechanism of basal ciliary activity regulating $[Ca^{2+}]_i$ to maintain basal CBF.

Kur & Newman (2014) demonstrated that APY (500 U/mL) dilated retinal arterioles in control conditions, demonstrating the importance of ATP in the control of basal vascular tone. This study

together to our results, suggest that physiological basal conditions may be controlled by constitutive ATP release. The large amount of apyrase used in resting conditions compared to the lower amount required to inhibit the effect of ATP released after a stimulus (Kawakami et al., 2004) suggest that the local effect of eATP in basal conditions is difficult to be modified.

A similar effect on basal CBF was observed in primary cultures from human adenoids (-72%) and rat oviducts (-44%) treated with APY (50 U/mL), suggesting that contribution of eATP on basal ciliary activity is conserved in different species and organs.

Extracellular [ADO], [AMP] and [ADP] were also detected in the culture medium of undisturbed primary cultures from human adenoids. These nucleotides derive from ATP hydrolysis by ecto-nucleotidases expressed in the respiratory epithelium: ecto-5´-nucleotidase –(NTPDase3, CD39L3-, E-NPP and non-specific alkaline phosphatases (Lazarowski et al., 2004; Zuo et al., 2008). ADP and ADO increased CBF in primary cultures of mouse trachea, in line with previous reports (Zhang et al., 2004). It was described that ADP affects CBF after its hydrolysis to ADO (Morse et al., 2001), which activates A2b-R, increasing CBF (Morales et al., 2000; Morse et al., 2001). Primary cultures of mouse trachea treated with the A2b-R blocker 8-SPT did not show significant differences in basal CBF, however, the addition of APY in cultures preincubated with 8-SPT reduced basal CBF to a greater extent than in cultures pretreated with a vehicle. This result suggests that ADO participates reducing the fall of basal CBF observed with APY treatment. In a previous study, it was demonstrated that the concomitant addition of ATP and ADO to oviductal epithelial cultures increases CBF in concentrations that by themselves do not have any effect, hence suggesting a synergic mechanism of CBF regulation by ATP and ADO (Morales et al., 2000). Our results suggest that e[ATP] participates in the control mechanism of basal CBF both directly (~45%) and after its hydrolysis to ADO (~15%).

7.3 Participation of HCs and purinergic receptors in the control mechanism of basal CBF through ATP release

Molecular techniques were used to detect HCs (Panx1, Panx2, Panx3, Cx43) and purinergic receptors (P2X7-R and P2Y2-R) in ciliated cells. Expression of Panx1, Panx2 and Panx3 was detected by immunofluorescence in tissue section and in primary cultures from mouse trachea. Previously, Panx1, Panx2 and Panx3 have been detected in respiratory epithelial cells (Bruzzone et al., 2003; Ohbuchi et al., 2013). Cx43 was detected in primary cultures from human adenoids. Previously, expressions of Cx23, Cx43 and Cx46 have been demonstrated in epithelial cultures of rabbit trachea (Isakson et al., 2006), however, only Cx43 was found in nasal mucosa and cultured epithelial cells (Yeh, 2003).

Detection of purinergic receptors, P2Y2 and P2X7 was performed using Western blot analysis and immunofluorescence. P2Y2-R was expressed in human adenoids and P2X7-R in primary cultures of mouse trachea. Previously, P2X3, P2X4, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 and P2Y12 receptors have been identified in human nasal epithelial cells (Kim et al., 2004). However, only P2Y2 and P2X7 receptors have been associated to a CBF increase induced by ATP (Morse et al., 2001; Hayashi et al., 2005; Ma et al., 1999; Ma et al., 2002; Ma et al., 2006; Kim et al., 2004).

In primary cultures of mouse trachea, Gd^{3+} and La^{3+}, inhibitors of Ca^{2+} channel including CxHCs and TRP channels (Garrea, 2010; Gkoumassi et al., 2009; Lipski et al., 2006), decreased CBF by a ~50% . This reduction in CBF was recovered at 5 min. CBX and probenecid has been used to determine the contribution of Cx and Panx1 in the release of ATP in airways epithelium exposed to hypotonic stress (Ransford et al., 2009). Using CBX (100 μM), inhibitor of Cx and Panx1, CBF was reduced by a 25% during 15 sec, while PROB, which inhibits Panx1 but not CxHCs (Hayoz et al., 2012), had no effect on basal CBF. As Cx43 has been found in cultured epithelial cells (Yeh et

al., 2003), and Cx43 is the main Cx related to ATP release, our results suggest that Cx43 HCs would be involved in the control of basal CBF. As CBX is a nonspecific blocker, additionally studies, using specific blocking peptides, are necessary to establish the participation of Cx43 in the control of basal CBF. In addition, the transiently response in CBF following CBX treatment suggests a compensatory mechanism to regulate CBF, where Cx controls partially CBF by ATP release or Ca^{2+} entry. When Cx is blocked, probably other channels are activated to regulate CBF. Lorenzo et al. (2008) demonstrated that TRPV4 contributes to an ATP-induced increase in CBF through Ca^{2+} entry. As Gd^{3+} and La^{3+} decreased CBF during 5 min, it suggests that TRPV4 channels also participate in regulate basal CBF.

In primary cultures of mouse trachea, SUR and oATP did not affect basal CBF. In order to assess whether compensatory mechanisms control basal CBF, concomitant treatment with CBX and oATP was used in primary culture of mouse trachea. CBX plus oATP reduced basal CBF by a ~57% at 5 min and returned to baseline after 20 min. These results suggest that HCs and P2X7-R participate in the control mechanism of basal CBF as parallel mechanisms. Simultaneously treatment with APY, CBX and oATP further reduced basal CBF by a ~85% in primary culture of mouse trachea. This treatment also stopped the ciliary movement in a 40% of the experiments. This result suggests that e[ATP] and the molecular components of ATP release (HCs) and/or ATP activation (P2X7-R) all contribute additively in the control mechanism of basal ciliary activity in ciliated epithelia. The hypothesis that P2X7-R participates in the control mechanism of basal CBF is supported by the finding that the sustained component of CBF increase in response to ATP depends on P2X7-R (Surprenant and North, 2009; Ma et al., 1999 and 2006; Li et al., 2010), .

Basal eATP levels did not change following the addition of CBX and oATP at 5, 10 and 20 minprobably the technique used in this study is unable to detect low changes in local eATP levels. eATP levels showed a high variability following CBX plus oATP addition at 5 min. As the recovery

of the reduction induced by oATP plus CBX in basal CBF began at 5 min, the high variability in eATP levels could be explained by a compensatory mechanism of ATP release in some cultures. eATP levels only showed a significantly reduction when APY, oATP and CBX were added at the same time in the culture, indicating that the concomitant treatment with APY, oATP and CBX significantly reduces eATP levels and basal CBF.

Preliminary studies show that oATP induced a transitory fast increase in $[Ca^{2+}]_i$, which could be explained because oATP is an irreversible antagonist of P2X7-R, but it acts briefly as an agonist, allowing for a short entry of Ca^{2+} before the inhibition of P2X7-R takes place (Donnelly and Jarvis, 2007). Despite the shortly effect of CBX on basal CBF, addition of this blocker did not change $[Ca^{2+}]_i$. It is possible that the technique used to measured $[Ca^{2+}]_i$ did not detect local changes in $[Ca^{2+}]_i$. In addition, the hypothesized microdomains described in the plasmatic membrane (Parekh, 2008; Castillo et al., 2007) suggest that local changes in $[Ca^{2+}]_i$ are able to control basal CBF.

CBX plus oATP produced a slight reduction on $[Ca^{2+}]_i$. Furthermore, $[Ca^{2+}]_i$ showed a higher reduction with APY, oATP and CBX added at the same time (data not show), suggesting that $[Ca^{2+}]_i$ is regulated by eATP, HCs and P2X7-R. Previous reports indicate that P2X7-R could have a physical association with Panx1 and Cx43. P2X7-R activation by eATP promotes the gradually opening of HCs which leads to ATP release (Suadicani et al., 2006; Faria et al., 2005). Possible mechanisms described for the interaction between P2X7-R and HCs are tyrosine kinase phosphorilation, Rho kinase activation, actin cytoskeleton, reactive oxygen species (ROS) and $Ca^{2+}{}_i$ movements (D´hondt et al., 2011; Dolmatova et al., 2012; Ohshima et al., 2012). Further studies are necessary to establish a relation between P2X7-R and HCs on $Ca^{2+}{}_i$ and its contribution to basal ciliary activity.

In primary cultures of rat oviduct, oATP decreased basal CBF (~33%), while CBX had any additional effect in the decrease of CBF. These results suggest that P2X7-R, without interacting with HCs, participates in the control mechanism of basal CBF in rat oviductal epithelial cells, differently to results obtained in primary cultures of mouse trachea.

In summary, concomitant treatment with APY, CBX and oATP reduced basal CBF (-85%) and e[ATP] in primary cultures of mouse trachea. These results suggest that eATP and the molecular components of ATP release (Cx/Panx HCs) and/or ATP activation (P2X7-R) are necessary to maintain basal ciliary activity in respiratory ciliated epithelia.

7.4 ATP release mediates CBF increase induced by mechanical and chemical stimulation

MS increased CBF and e[ATP] in primary cultures of human adenoids. Similar results were observed in ALI cultures from septum, etmoides and trachea after a MS using a pulse of air (55 mmHg/50 msec). Parameters used in the pulse of air are consistent with intranasal pressure measured during human sneezing (Gwaltney et al., 2000). Previous studies showed that MS, like cyclic compressive stress, shear stress and osmotic stress, releasesATP in respiratory epithelial cells (Lazarowski et al., 2000; Ramsingh et al., 2011; Bodin and Burnstock, 2001; Yegutkin et al., 2000; Tarran et al., 2005). However, these studies indicate that the result of releasing ATP is to increase e[ADO], which regulates respiratory surface hydration mediating modifications in the ionic interchange (Lazarowski et al., 2004). We did not observe changes in e[ADO] following MS in primary cultures of human adenoids. APY and SUR prevented CBF increase induced by MS in mouse septum ALI cultures (data not shown, but published in Zhao et al., 2012), suggesting that the increase in CBF induced by a mechanical stimulus depends on ATP release, through the

activation of purinergic receptors, in primary cultures of ciliated cells. MS on a single cell induced an increase in $[Ca^{2+}]_i$ and the propagation of ICW in primary cultures of human adenoids. ICW presented a propagation velocity of 6.6 μm/sec. This velocity was lower compared to previous studies in rabbit tracheal epithelium (Sanderson and Dirksen, 1989; Sanderson et al., 1990), possibly due to the different temperatures used, being lower in this study. APY reduced $[Ca^{2+}]_i$ increase induced by MS and prevented ICW propagation in primary cultures of human adenoids and mouse trachea, suggesting that ATP release is necessary to propagate $[Ca^{2+}]_i$ signals to neighboring cells in respiratory epithelial cells.

Studies using flourescent dye have demonstrated that tracheal airway epithelial cells grown in matrices of collagen are poorly coupled (Isakson et al., 2006). In addition, mathematical modeling have proposed a regenerative release of ATP from the stimulated cell as the mechanism of Ca^{2+} wave propagation in airway epithelium (Warren et al., 2010). These results support our model, in which mechanical stimulation induces release of ATP that diffuses in the extracellular environment and binds purinergic receptors on adjacent cells, activation cellular signals that lead to increases in $[Ca^{2+}]_i$.

In ALI cultures from mouse septum, EGTA and BAPTA-AM prevented CBF increase induced by MS. Nevertheless, the measure of e[ATP] did not show changes compared with the vehicle (data not shown, but published in Zhao et al., 2012). These results suggest that the release of ATP induced by a pulse of air was independent of Ca^{2+} release from intracellular compartments as well as from Ca^{2+} entry. A previous study in a cell line of human bronchial epithelium indicated that the vesicular secretion of ATP induced by MS is dependent on $[Ca^{2+}]_i$ (Ramsingh et al. 2011), suggesting two ATP release mechanisms in mucociliated epithelium following MS: vesicular secretion dependent on $[Ca^{2+}]_i$ in secreting cells (Ramsingh et al., 2011), and a mechanism independent of Ca^{2+} mediated by HCs.

It has been described that Panx1, Cx43 and TRPV4 are mechanosensitive channels that can be opened by mechanical stress (Bao et al., 2004; Batra et al., 2013; Andrade et al., 2005). In hamster oviductal ciliated cells, changes in the fluid viscosity generate Ca^{2+} signals associated with the activation of TRPV4 (Andrade et al., 2005). Similar results were observed in mouse trachea epithelium, where hypotonic stress activates TRPV4 (Lorenzo et al., 2008). TRPV4 induces Rho activation and evokes ATP release through Panx1 (Seminario Vidal et al., 2011). TRPV4 was also described to mediate the release of ATP through Cx43HCs in human esophageal epithelial cells (Ueda et al., 2011). Panx1 and Cx43 also can be directly activated by mechanical stress through a mechanism independent of $[Ca^{2+}]$ (Bao et al., 2004; Batra et al., 2013). As Zhao et al (2012) suggests that the release of ATP induced by a pulse of air was independent of Ca^{2+} release from intracellular compartments as well as from Ca^{2+} entry, it is possible suggest that HCs (Panx1 or Cx43) could be the channels that facilitate the ATP release in response to MS in mouse septum ALI cultures without directly participation of TRPV4. However, TRPV4 could contribute in the increase of CBF through the $[Ca^{2+}]$ increase and not in ATP release.

In primary cultures of mouse trachea, adding ATP increased CBF with an EC50 of 0.04 μM. In primary cultures of human adenoids, the concentration of ATP used to stimulate CBF and Ca^{2+}_i was in the range of 1 μM and higher (published in Gonzalez et al, 2013). Previously, it was demonstrated in our laboratory that the ATP concentrations utilized to modify CBF and Ca^{2+}_i levels in rat oviduct ciliated cells were in the range of μM, where 10 μM showed the maximum increases in CBF and 0.1 μM had no effect on CBF (Kerr, 2004; Sanchez, 2009). These results suggest that primary cultures of mouse trachea are more sensitive to ATP than primary cultures of human adenoids and rat oviduct in affecting CBF.

In primary cultures from mouse trachea, CBX prevented CBF increase induced by ATP, suggesting that HCs participate in the regulation of this response. PROB also reduced CBF

increase induced by ATP, suggesting the participation of Panx1. As ABC transporter also can be blocked by PROB, their participation cannot be discarded. As the blockage in CBF increase induced by ATP was higher with CBX compared with PROB suggests that Cx and Panx participate in the CBF increase induced by ATP. oATP had no effect on CBF increase induced by ATP, but concomitant treatment with oATP and CBX reduced CBF in response to ATP (∼-61%). This result indicates that concomitant inhibition of P2X7-R and HCs (Panx1/Cx) disrupts the ATP effect on CBF, however P2X7-R alone seems not participate in the CBF increase induced by ATP (10 μM). Additionally studies, using a lower concentration of ATP could be required to determine the participation P2X7-R, associated to a CBF increase induced by ATP. The concentration of ATP used in this study was a saturating concentration to increase CBF. A previous study showed that LPS (a bacterial wall component) induced a CBF increase through a mechanism dependent on ATP release via Panx1 and P2X7-R in primary cultures of mouse trachea (Carreño, 2013). CBX also reduced the increments in eATP levels following the ATP addition, suggesting that ATP affects CBF by inducing the release of ATP from ciliated epithelium of mouse trachea. In neonatal mouse olfactory epithelium, it was demonstrated that activation of purinergic receptors by ATP evokes ATP release through HCs (Hayoz et al., 2012). These results suggest a positive feedback in which ATP induces its own release and could explain a control mechanism of basal ciliary activity and the increase in CBF induced by chemical and mechanical signals, however, by some yet unidentified mechanism, the released ATP seems not interact with P2X7-R. We may speculate on some pharmacological artifact in the concomitant blockade of HCs and P2X7-R which reduced CBF following ATP addition.

Similar results were obtained in primary cultures of rat oviducts. CBX prevented increases in CBF and $[Ca^{2+}]_i$ following addition of ATP, suggesting that HCs could be responsible for the Ca^{2+} entry induced by ATP that regulates CBF increase. It has been previously demonstrated that increases

in CBF induced by ATP are dependent on Ca^{2+} movements from the intracellular store and extracellular compartment (Kerr, 2004; Barrera et al., 2004; Morales et al., 2000).

In summary, our results suggest that ATP release from epithelial ciliated cells is necessary to maintain the basal ciliary activity and mediates the CBF increase induced by mechanical and chemical stimulation. The underlying molecular mechanism that mediates this response might involve the release of ATP via HCs (Cx and Panx1) and P2X7-R activation by ATP in epithelial ciliated cells.

7.5 Projections

The results of this thesis offer a first insights into the molecular basis involved in the control mechanism of basal ciliary activity by eATP. This basal control mechanism of CBF was associated to constitutive ATP release through CxHCs and P2X7-R activation. ATP release through HCs is an accepted mechanism in the literature. However, more studies are necessary to determine if Cx43 or Panx1 are responsible for the ATP release that regulates ciliary activity. In addition, interactions between P2X7-R, Panx1 and CxHCs in the cilium system could be further investigated. The hypothesized microdomains described in the plasmatic membrane (Parekh, 2008; Castillo et al., 2007) and the ATP release restricted to a point source (Arcuino et al., 2002) suggest a local regulation mechanism of CBF through ATP release and $[Ca^{2+}]_i$ increase.

Lack of ciliary response to a pulse of air was described in ALI cultures obtained from patients with chronic rhinosinusitis (Zaho et al. 2012). Furthermore, there are high risks of infections and complications like sinusitis and obstructive bronchitis in patients with asthma, where the viscosity of the mucus increases. In this thesis, it was proposed that ATP is an

autocrine/paracrine molecule required to propagate Ca^{2+} signals to neighboring cells following a MS. In addition, HCs, such as Panx1 and Cx, and P2X7-R participated in CBF increase through ATP release. Moreover, P2X7-R activation has been frequently associated with inflammation where e[ATP] is elevated (Bours et al., 2006; Burnstock, 2006). Accordingly, the study of these molecules is an interesting topicto pursue in the field ofresponses to MS in respiratory pathologies.

Likewise, the results obtained in ciliated epithelial cells of rat oviduct with regards to CBF regulation through ATP release may be of relevance in the studies of pathologies related to infertility, like ectopic pregnancy, obesity and other inflammatory diseases associated with mucociliary transport in the oviduct, since progesterone and estrogen affect ATP regulation mechanisms in the presence of CBF changes (Kerr, 2004; Sanchez, 2011).

The results of this thesis provide a valuable contribution to elucide the control mechanisms of basal ciliary activity and CBF increase induced by mechanical and chemical signals.

Model

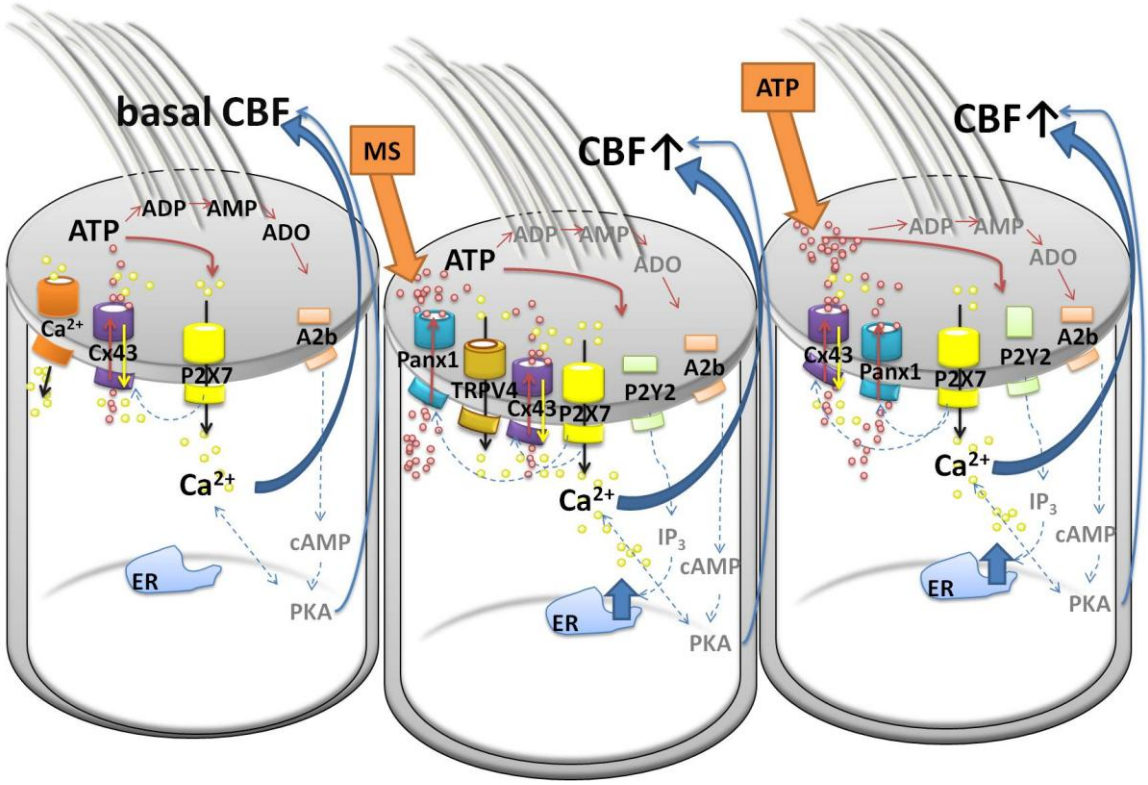

Figure 32. Control mechanisms of basal CBF and increased CBF induced by mechanical and chemical stimulation in ciliated epithelium.

eATP interacts with P2X7-R inducing Ca^{2+} entry. $Ca^{2+}{}_i$ maintains basal CBF (CBF). P2X7-R promotes ATP release by Cx43HCs. Other no identified Ca^{2+} channels participate in Ca^{2+} entry, possibly CxHCs and/or TRPV4. ATP is hydrolyzed to ADO, which activates A2b-R, regulating cAMP and PKA and helping to maintain the basal CBF mediating a cross talk with $Ca^{2+}{}_i$ pathway.

MS induces ATP release, mediating a mechanism independent of $Ca^{2+}{}_i$ levels, possibly through Panx1 or Cx43 which are mechanosensitive channels. eATP activates P2Y2-R, which via IP3 and release of Ca^{2+} from intracellular stores (endoplasmic reticulum (ER)) increase the CBF. eATP induced the propagation of ICW.

A chemical stimulus like ATP induces the Ca^{2+} entry through HCs and activates P2Y2-R to increase the CBF. Also, P2X7-R is associated to Ca^{2+} entry and release of ATP through HCs (Cx43 or Panx1) and participates in the increase of CBF induced by ATP.

Conclusion

eATP in nM concentration was measured in primary ciliated cell cultures from mouse trachea in resting conditions. Treatment with APY significantly reduced e[ATP] and basal CBF (-45%), suggesting that eATP is a chemical signal that participates in the control of basal ciliary activity. Concomitant treatment with APY, CBX and oATP further reduced basal CBF (-85%). These results suggest that eATP concentration and the molecular components of ATP release (HCs) and/or ATP activation (P2X7-R) are necessary to maintain basal ciliary activity in ciliated epithelia. Furthermore, basal CBF reduction by ATP hydrolysis was associated with decreases in $[Ca^{2+}]_i$, suggesting that $Ca^{2+}{}_i$ levels are regulated by e[ATP] to maintain basal CBF.

MS of ciliated epithelia produced increases in CBF, which is an effect associated with increments of $Ca^{2+}{}_i$ levels, propagation of intercellular calcium waves (ICWs) and an increase in e[ATP]. All these changes were prevented with APY, which reduced e[ATP]. These results suggest that an increase in eATP release by the ciliated epithelium is required to propagate mechanical signals in ciliated cells.

Application of ATP increased CBF and $[Ca^{2+}]_i$, which was prevented with CBX. CBX also reduced e[ATP] following the addition of ATP, suggesting that the stimulation with ATP affects CBF by inducing the release of ATP from ciliated epithelium.

These results suggest that ATP is released from ciliated epithelial cells, contributing to an autocrine/paracrine control mechanism of basal ciliary activity and mediating increases in the CBF induced by mechanical and chemical signals.

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