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Pharmacological analysis of epithelial chloride secretion mechanisms in adult murine airways

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ABSTRACT

Defective epithelial chloride secretion occurs in humans with cystic fibrosis (CF), a genetic defect due to loss of function of CFTR, a cAMP-activated chloride channel. In the airways, absence of an active CFTR causes a severe lung disease. In mice, genetic ablation of CFTR function does not result in similar lung pathology. This may be due to the expression of an alternative chloride channel which is activated by calcium. The most probable protein performing this function is TMEM16A, a calcium-activated chloride channel (CaCC). Our aim was to assess the relative contribution of CFTR and TMEM16A to chloride secretion in adult mouse trachea. For this purpose we tested pharmacological inhibitors of chloride channels in normal and CF mice. The amplitude of the cAMP-activated current was similar in both types of animals and was not affected by a selective CFTR inhibitor. In contrast, a CaCC inhibitor ($CaCC_{inh}$-A01) strongly blocked the cAMP-activated current as well as the calcium-activated chloride secretion triggered by apical UTP. Although control experiments revealed that CaCCinh-A01 also shows inhibitory activity on CFTR, our results indicate that transepithelial chloride secretion in adult mouse trachea is independent of CFTR and that another channel, possibly TMEM16A, performs both cAMP- and calcium-activated chloride transport. The prevalent function of a non-CFTR channel may explain the absence of a defect in chloride transport in CF mice.

1. Introduction

Chloride exit towards the lumen moves osmotically-obliged water, essential for the maintenance of hydrated mucus and a normal volume of periciliary liquid, both necessary for efficient airway mucociliary clearance (Matsui et al., 1998; Tarran et al., 2002, 2005). Two pathways for airway epithelium chloride secretion have been identified. One activated by cAMP is mediated by CFTR, the chloride channel mutated in cystic fibrosis (CF), a human disease that severely affects lung function (Matsui et al., 1998). The second is based on TMEM16A, a calcium-activated chloride channel, CaCC (Pedemonte and Galietta, 2014).

Studies in mice where *Cftr* gene expression was impaired showed that the CFTR protein plays a less important role in airway function compared to human. CF patients are affected by a severe and often lethal pulmonary disease due to complete disappearance of cAMP-dependent chloride secretion in the airways (Blouquit et al., 2006). On the other hand, CF mouse models showed inflammatory lung phenotype associated with progressive tissue remodeling but the contribution of CFTR to chloride transport in mouse airways appears minimal (Scholte et al., 2004; Grubb and Boucher, 1999). More specifically, when cAMP-dependent chloride secretion was measured in the airways of CFTR-defective mice, a slight reduction (Colledge et al., 1996) or no changes (Grubb et al., 1994) were observed. Some differences are explained by the age of mice used in the experiments. CF animals in their first days of life showed reductions in the cAMP-induced currents while adults did not. Phenotype differences can be also influenced by genetic background or strain of animals used, as is the case for intestinal epithelium, where differences in activation and magnitude of anion secretion might determine the survival of the *Cftr*-null mice (Flores et al., 2010; Rozmahel et al., 1996).

Importantly, mice show a robust calcium-activated chloride secretion in the airways. The presence of such alternative chloride conductive pathway has been claimed as a reason for the lack of pulmonary disease in CF mice (Grubb and Boucher, 1999). Such observations strongly suggest that the activation of a non-CFTR chloride secretion might be a promising therapeutic strategy in CF. This approach would be beneficial for all CF patients because it is

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independent of CFTR genotype (Sondo et al., 2014). Early death of *Tmem16a*-null mice has unfortunately made it impossible to study how the absence of TMEM16A can affect epithelial airway function in adult mice (Rock et al., 2008). Nevertheless, studies using tracheas from newborn *Tmem16A*$^{-/-}$ mice demonstrated that nearly 60% of the calcium-activated anion current was absent (Rock et al., 2009; Ousingsawat et al., 2009). However, if such a lack of calcium-activated chloride secretion is maintained and if affects lung function are still open questions. In the current scenario, the availability of novel TMEM16A pharmacological inhibitors CaCCinh-A01 and T16Ainh-A01 (Namkung et al., 2011) could help unveil the function of CaCC in adult mice tissues.

In this study, we aimed to increase our understanding of transepithelial chloride transport in mouse trachea by using normal and CFTR-defective animals plus novel pharmacological tools for CaCCs, never tested before in freshly isolated mouse trachea. Although our experiments revealed that TMEM16A/CaCC inhibitors are not perfectly selective, we can conclude that CFTR plays a negligible role in airway anion secretion and that another channel, possibly TMEM16A, is instead a major component in the trachea of adult mice.

2. Material and methods

2.1. Animals

Animals were maintained in the Specific Pathogen Free mouse facility of the Centro de Estudios Cientificos (CECs) with access to food and water ad libitum. The *Cftr*^{tm1EUR} (*Cftr*$^{\Delta F508/\Delta F508}$) mouse was generously provided by Dr. B. J. Scholte from Erasmus MC Rotterdam, the Netherlands (Van Doorninck et al., 1995; French et al., 1995). 2-5-month-old male mice were used, bred in the C57BL/6J strain. All experimental procedures were approved by the Centro de Estudios Científicos (CECs) Institutional Animal Care and Use Committee.

2.2. Ussing chamber experiments in mice

To characterize ion transport properties of tracheal epithelium from normal and CF mice, tracheas were excised, mounted in Ussing chambers, and basal properties and responses to pharmacological agents were measured.

Tracheae were placed in P2307 of 0.032 cm^2 tissue holders, placed in modified Ussing chambers (Physiologic Instruments Inc., San Diego, CA, USA) bathed with bicarbonate-buffered solution (pH 7.4), of the following composition (in mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$ and 10 D-Glucose, gassed with 5% CO_2–95% O_2 and kept at 37 °C throughout the experiment.

The transepithelial potential difference referred to the serosal side was measured using a VCC MC2 amplifier (Physiologic Instruments Inc., San Diego, CA, USA). The current was clamped to 0 μA, and 200 ms pulses of ± 10 μA were passed across the tissues at 1 s intervals using the Acquire & Analyze 2.3v software and a DI-720 interface (DataQ instruments, Akron, OH, USA). Measurement of the potential difference between the mucosal and the serosal compartment (V_{te}) allowed the calculation of tissue resistance (R_{te}) and equivalent short-circuit current (I_{sc}). Tissues with R_{te} values above 50 Ω cm^2 were considered for analysis.

Net cation absorption and anion secretion are reported as positive I_{sc} values. To determine the amplitude of CFTR currents, cAMP-dependent chloride secretion was induced by adding 100 μM 3-isobutyl-1-methylxanthine (IBMX) plus 1 μM forskolin (FSK). The magnitude (ΔI_{sc}) of this current was calculated as the difference between the current after and before stimulation.

To establish the amplitude of calcium-dependent chloride secretion, 100 μM uridine-5′-triphosphate (UTP) was added to the apical chamber. ΔI_{sc} was calculated at two time points, first the peak response and then five minutes after the addition of UTP.

We used two TMEM16A/CaCC inhibitors: i) 6-t-butyl-2-(furan-2-carboxamido)-4,5,6,7-tetrahydrobenzo[*b*] thiophene-3-carboxylic acid ($CaCC_{inh}$-A01) (De La Fuente et al., 2008) ii) 2-[(5-ethyl-1,6-dihydro-4-methyl-6-oxo-2-pyrimidinyl)thio]-N-[4-(4-methoxyphenyl)-2-thiazolyl]acetamide ($T16A_{inh}$-A01) (Namkung et al., 2011). These compounds were obtained from Calbiochem.

All experiments were performed in the presence of 100 μM apical amiloride to avoid interference of sodium absorptive currents.

2.3. Tissue isolation and PCR

Epithelial tissue was obtained by scraping the open trachea or bronchi with a microbrush and pooled from 3 different animals. Complete lung samples and colon epithelium were also collected and stored in Trizol® (Invitrogen) at −80 °C until further use. cDNA was produced through the ImProm-II™ Reverse Transcription System (Promega) with a final concentration of 2 μg/μL. The PCR amplification primers used for *Tmem16A*, *Tmem16B* and Cyclophilin (used as a housekeeping gene) were: *Tmem16A* forward, 5′-AGG AAT ATG AGG GCA ACC TG-3′ *Tmem16A* reverse, 5′-CGA CAC CAT GGA TTT TGG TA-3′. *Tmem16B* forward, 5′-ATC CAG CCA CCG TCT TCT T-3′ *Tmem16B* reverse, 5′-TTT TCC AGA AAC ATG GTA GCC-3′. Cyclophilin forward, 5′- GGC AAT GCT GGA CCA AAC ACA A-3′ Cyclophilin reverse, 5′-GTA AAA TGC CCG CAA GTC AAA AG-3′. RT-negative controls were obtained by omitting Reverse Transcriptase enzyme from the reaction.

2.4. Cell culture

Isolation and culture of human bronchial epithelial cells were done as previously described (Scudieri et al., 2012). Briefly, cells were initially cultured on plastic in proliferative serum-free medium, containing 1:1 mixture of RPMI 1640 and LHC basal medium (Life Technologies, Monza, Italy) supplemented with various hormones and supplements, 100 U/ml penicillin and 100 μg/ml streptomycin and then cultured on porous membranes (12 mm Snapwell inserts, Corning, code 3801) for 10–12 days with a differentiating medium. The entire procedure, also involving informed consent from patients, was approved by the Ethical Committee of Gaslini Institute under the supervision of the Italian Ministry of Health.

Differentiation of cells into tight epithelium was checked by measuring transepithelial electrical resistance and potential difference with an epithelial voltohmmeter (EVOM1, World Precision Instruments, Sarasota, FL, USA).

The immortalized bronchial epithelial cell line CFBE41o–, with overexpression of wild type human CFTR, was cultured in minimal essential (MEM) medium.

Fischer rat thyroid (FRT) cells with stable expression of wild type CFTR, were cultured in Coon's modified Ham's F-12 medium. Both media were supplemented with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA), 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.5. Short-circuit current recordings

Snapwell supports carrying differentiated bronchial epithelia were mounted in a vertical diffusion chamber resembling a Ussing chamber with internal fluid circulation. Both apical and basolateral hemichambers were filled with 5 ml of a Kreb's bicarbonate solution (in mM): 126 NaCl, 0.38 KH_2PO_4, 2.13 K_2HPO_4, 1 $MgSO_4$, 1