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Inhaled PDE5 inhibitors restore chloride transport in cystic fibrosis mice

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Abstract

Sildenafil and vardenafil, two selective inhibitors of phosphodiesterase type 5 (PDE5) are able, when applied by intraperitoneal injection, to activate chloride transport in cystic fibrosis (CF) mice homozygous for the F508del mutation. Oral treatment with the drugs may be associated with adverse hemodynamic effects. We hypothesized that inhaled PDE5 inhibitors are able to restore ion transport in F508del-CF airway epithelium.

We developed a restraint-free mouse chamber for inhalation studies. PDE5 inhibitors were nebulized for 15 minutes at concentrations adjusted from recommended therapeutic oral doses for male erectile dysfunction. We measured *in vivo* nasal transepithelial potential difference 1 hour after a single inhalation of sildenafil, vardenafil or tadalafil in F508del-CF and in normal homozygous mice.

After nebulization with the drugs in F508del mice, chloride transport, evaluated by perfusing the nasal mucosa with chloride-free buffer containing amiloride followed by forskolin, was normalized; the forskolin response was increased with the largest values being observed with tadalafil and intermediate values with vardenafil. No detectable effect was observed on sodium conductance.

Our results confirm the role of PDE5 inhibitors for restoring chloride transport function of F508del-CFTR protein and highlight the potential of inhaled sildenafil, vardenafil and tadalafil as a therapy for CF.

Keywords: cystic fibrosis; inhalation; nasal potential difference; sildenafil; tadalafil; vardenafil

Running head: inhaled PDE5 inhibitors in CF

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Introduction

The steadily growing understanding of molecular and biological mechanisms underlying cystic fibrosis (CF) lung disease has resulted in new exciting targets for treatment. Fundamental research focusing on correcting F508del mutant protein can be considered as disease-modifying approaches and would be beneficial for most CF patients. Indeed, the F508del mutation, a misfolded protein that fails to escape the endoplasmic reticulum [1], represents the most common *CFTR* mutation worldwide.

Inhalation drug therapy has several potential advantages over oral and intravenous routes, including rapid onset of pharmacological action, minimized systemic adverse effects and reduced effective drug doses compared to the same drug delivered orally [2]. Nebulization of topically active drugs has become increasingly common for treatment in CF [3]. Novel drug formulations acting through distinct mechanisms, including restoration of airway surface liquid or mucus, anti-inflammatory or anti-infective agents, and immunosuppressive agents, have been currently tested by inhalation therapy in CF [4].

Sildenafil, vardenafil and tadalafil are highly selective inhibitors of cyclic guanosine monophosphate (cGMP)-dependent phosphodiesterase type 5 (PDE5) [5] commonly used for improving erectile dysfunction. Moreover, oral therapy with sildenafil promotes nitric oxide mediated pulmonary vasodilation with dose-dependent decrease of pulmonary artery pressure and vascular resistance [6]. These features provide the rationale behind its use for the treatment of pulmonary arterial hypertension. We have previously shown [7] that intraperitoneal injection of sildenafil and vardenafil to F508del-CF mice corrects CFTR-dependent chloride transport. However, systemic hemodynamics, including flushing, headache and other cardiovascular effects [8,9] could compromise their potential in CF. Therefore, we hypothesized that *in vivo* nebulisation of PDE5 inhibitors restore chloride transport through F508del-CFTR. In the present study, we developed a low stress mouse

chamber for inhalation studies and we sought to investigate efficacy of inhaled PDE5 inhibitors on ion transport across the nasal mucosa of F508del-CF mice. Our results provide clear evidence that inhalation of PDE5 inhibitors restores chloride transport across the respiratory epithelium of F508del-CF mice.

Methods

Animal model

Young adult CF mice homozygous for the F508del-CFTR mutation in the 129/FVB outbred background ($cftr^{tm1Eur}$)[10] and their normal homozygous wild-type littermates were studied. Mouse age ranged from 4-5 months and their weights ranged from 20-30g. Animals were housed at the Animal Care Facility of our University following recommendations of the Federation of European Laboratory Animal Science Associations [11]. To prevent intestinal obstruction in CF animals, Movicol (55,24g/L; Norgine, Heverlee, Belgium) was administered in acidified drinking water. The mice were genotyped at 21 days of age by using Taqman quantitative PCR, as previously described [12]. These studies and procedures were approved by the local Ethics Committee for Animal Welfare and conformed to the European Community regulations for animal use in research (CEE n° 86/609).

Preparation of PDE5 inhibitors

Stock nebulizer solutions were prepared within the limit of water solubility for each PDE5 inhibitor, *i.e*. 3.5 mg/ml for sildenafil citrate; 1.1 mg/ml for vardenafil and 0.002 mg/ml for tadalafil. Nebulizer solutions were used at concentrations adjusted from recommended initial oral doses for improving erectile dysfunction, *i.e.* 0.7 mg/kg sildenafil, 0.14 mg/kg vardenafil or tadalafil. For each animal (~25 g body weight), the required amount of drugs corresponding to 0.0175 mg sildenafil citrate, 0.0035 mg vardenafil HCl or tadalafil was

formulated in 3 ml saline, stored at 4°C and used within the 3 days after preparation. Saline was nebulized in placebo control experiments. Sildenafil citrate was obtained from Pfizer (Sandwich, UK); vardenafil, from Bayer (West Haven, Germany) and tadalafil, from Lilly (Brussels, Belgium).

Nebulization

A small, transparent mouse chamber for drug inhalation studies was designed for this study. The inhalation chamber consists in a cylindrical polymethylmethacrylate box with an expiratory gate, a floor area of 113 cm^2 and a volume of 960 cm^3; it was designed to accommodate a single non-restrained, conscious and spontaneously breathing mouse. A nebulizer with standardized aerodynamic properties (Sidestream, Medic-Aid, West Sussex, UK), driven by its own compressor (Portaneb, Medic-Aid), was connected to the top cover of the inhalation chamber. Nebulizer experiments were conducted using the nebulizer reservoir filled with 3 ml of nebulizer solutions. Experiments were performed under dynamic conditions, the airflow being introduced continuously and vertically at the top of the chamber. Nebulizer exposure lasted for about 15 min. At the end of the exposure, the animals stayed in the chamber for 5 minutes and then returned to their cages until nasal potential difference (PD) measurements were performed.

Nasal potential difference measurements

A nasal PD, performed as previously described [7,13,14], was undertaken at different time points, as indicated, after a single nebulizer exposure containing PDE5 inhibitors. Transepithelial sodium conductance was evaluated by the PDmax, representing the most negative baseline value, and by the amiloride response, evaluated by the change in PDmax after perfusion with Ringer's saline solution containing 10^{-4} M amiloride. Chloride

conductance (SumCl) was evaluated by nasal perfusions with a chloride-free solution of sodium gluconate containing amiloride, followed by addition of 10^{-5} M forskolin, an adenylate cyclase agonist. Forskolin response was evaluated by the fractional component of the global chloride transport, and corresponded to the changes after perfusion of the nasal mucosa with 10^{-5} M forskolin. To verify the proportion of respiratory ciliated tissue at the site of nasal PD measurement, the nasal mucosa of wild-type non-treated animals was excised at the end of NPD experiments and tissue was fixed in 4% buffered paraformaldehyde for histological studies. Sections processed at 5 μm thickness for light microscopy were stained with hematoxylin and eosin.

Histological control of the site of nasal PD measurement

Differences in the distribution of types of epithelia across the anatomical regions of the mouse nasal cavity were observed; these regional differences were not influenced by the CF genotype (data not shown). From the vestibulum nasi to the mid-proximal portion along the dorsal nasal concha (Supplemental Figure 1A), the epithelium was predominantly olfactory (Supplemental Figure 1B), with small regions of lightly keratinized squamous epithelium restricted to the nostril opening regions (Supplemental Figure 1C), and transitional epithelium lining the lateral wall (Supplemental Figure 1D). Proceeding caudally to the middle nasal concha, the phylogenetic equivalent of the human middle turbinate [15], progressive transition from olfactory to respiratory ciliated epithelia was found (Supplemental Figure 1E). The nasal PD catheter was usually located at 4-6 mm distance from the mouse nostril in the middle nasal concha. When, at the end of nasal PD measurements, a focal nasal lesion (~0.25 mm; corresponding to the OD of the nasal PD probe) was intentionally produced by exerting a light pressure of the probe against the distal wall of the middle concha, a respiratory epithelium with ciliated cells was found at this site (Figure 1A). In the posterior region lining the

perpendicular plate of the ethmoidal bone and corresponding to the ethmoidal concha, the phylogenetic equivalent of the human superior turbinate [15], ciliated respiratory epithelium was mostly found (Figure 1B). In the most distal portions of the nasal cavity, close to the olfactory bulb, regions with predominant olfactory epithelium were also found.

Statistics

Descriptive statistics (mean and SEM) and tests of statistical significance were performed using SAS-JMP software (SAS Institute, Cary, NC, USA). Number of mice was selected based on sample size calculations when setting a two sided α-level at 0.05 and a (1-β) level at 0.8. Between-group comparisons were evaluated using one-way analysis of variance. Posthoc comparisons were made using Student's *t* test or Tukey-Kramer honestly significant difference (HSD) test for 2 or more *x* levels, as needed. Null hypothesis was rejected at $p < 0.05$.

Results

Baseline and stimulated PD values in nontreated mice

The nasal PD of the CF mouse is very different from that of the wild-type mouse. In CF mice [7,14], as in CF patients [16,17], the nasal PD is characterized by increased sodium conductance and reduced chloride conductance across the epithelium. Baseline hyperpolarization (i.e. more negative basal values) and increased depolarizing amiloride response, both reflecting increased ENaC-dependent sodium transport activity, were found in the nasal mucosa of the F508del mouse (Figure 2). In addition, cumulated voltage changes after nasal perfusion with chloride-free buffer and with forskolin reliably discriminated

between CF and wild-type animals. As illustrated in Figure 2, mean SumCl in F508del mutant animals was reduced to approximately one-third of that found in controls.

Nebulization treatment

Nebulizer experiments were performed in non-anesthetized, spontaneously breathing animals, at room temperature, in a quiet exposure environment at the animal house, under specific pathogen free conditions [11] with respect to clean air supply, noise, vibration, lighting and freedom of movement.

Nebulizer drug and saline treatment was well tolerated and no adverse effect was observed. Inhalation of saline, used as placebo, did not influence any nasal PD value. Indeed, irrespective of the genotype, mean nasal PD parameters, recorded 1 hour after inhaled saline were similar to those monitored in the corresponding non-treated animals (data from treated wild-type group not shown in Figure 3).

Aerosolized sildenafil and vardenafil

In our previously published work [7], we have shown that *in vivo* intraperitoneal administration of therapeutic doses of sildenafil and vardenafil to F508del-CF mice was able to correct CFTR-dependent chloride transport across the nasal mucosa. In this work, we tested, in the same CF mouse model, the potential of the inhaled drugs. Mean SumCl values obtained from CF mice 1 hour after nebulization with sildenafil or vardenafil were significantly increased, reaching values comparable to those recorded in placebo-treated wild-type mice (Figure 3).

Sildenafil and vardenafil did not influence transepithelial sodium transport. Indeed, basal PDmax values and amiloride response were not modified 1 hour after nebulization with the drugs as compared to placebo (Figure 3). The correcting effect of vardenafil lasted at least 8

hours while that of sildenafil was progressively lost over time and was completely absent 8 hours after inhalation (Figure 4).

Aerosolized tadalafil

In our previous intraperitoneal data [7], the effect of tadalafil, a highly selective PDE5 inhibitor acting through a similar mechanism as sildenafil and vardenafil, on CFTR function could not be investigated due its extremely poor water solubility. As illustrated in Figure 5, chloride transport in F508del mice was completely restored 1 hour after nebulization with tadalafil as compared to placebo. Mean SumCl values after nebulization in CF animals reached values similar to those recorded in the placebo wild-type group (Figure 3). As for the two other PDE5 inhibitors, no effect was observed with taladafil on sodium transport (Figure 3).

Comparative effects of the PDE5 inhibitors

We next compared, in CF mice, the degree of responses to inhaled PDE5 inhibitors by analyzing the contribution of each component of the global chloride transport (*i.e.*, the chloride diffusion potential recorded after nasal perfusions with chloride-free buffer containing amiloride, and the subsequent response to forskolin). No differences were detected on chloride diffusion potential between CF treated groups (data not shown). However, differences among treated groups were observed on the forskolin response. As illustrated in Figure 6, forskolin response was at least 3 times larger in the sildenafil-treated as compared to the placebo group. The largest values were found in the tadalafil group and intermediate values in the vardenafil group (Figure 6).

Discussion

The present work was designed to investigate whether inhalation with PDE5 inhibitors, comprising three drugs approved for non-CF indications, influences transepithelial ion transport defects of the F508del-CF mouse. In our previously published data [7], we have demonstrated that intraperitoneal administration of therapeutic oral doses of sildenafil and vardenafil, two potent and selective PDE5 inhibitors initially developed for treatment of male erectile dysfunction, is followed by complete restoration of CFTR-dependent chloride transport defects across the nasal mucosa of F508del-CF mice and that it does not involve an alternative chloride transport pathway. In the present work, we tested the three orally available clinically used PDE5 inhibitors, sildenafil, vardenafil and tadalafil. We showed that their administration through a single inhalational exposure is able to restore chloride transport across the respiratory epithelium of F598del-CF mice and that the effect of vardenafil lasts at least 8 hours.

Respiratory delivery to mice is technically challenging due to inherent anatomical and physiological animal characteristics and particularly to small animal size [18-20]. The nebulizer setup we developed in the present work comprises a whole-body immersion exposure chamber designed for a single mouse. Major advantages of a whole-body inhalation chamber over other methods of inhalation exposure for live animals, including head-only exposures, nose- or mouth-only methods, lung-only exposures, and partial-lung exposures [18-20], comprise lack of restraint or anesthesia during experiment and controllable low stress environment with respect to clean air supply, noise, vibration and lighting exposure. These practical aspects, while bringing physical comfort for the animal, minimize environmental stresses which may have detrimental effect on data. Moreover, whole-body chambers can generate relatively uniform and stable aerosol drug concentrations throughout the exposure

zone with reduced inter-experiment variability [18-20]. A disadvantage of whole-body exposure chambers is the considerable losses of inhaled test drugs in the animal fur, eyes and mouth, and in the chamber walls. However, no skin, eye or mouth irritation was observed in the present study.

The ideal subject for studies relevant to human is human himself. However, design of clinical trials can advantageously benefit from research validated in preclinical animal models. The nasal mucosa of our F508del mouse model [10] has proven to be valuable for studying novel therapeutic strategies that aim at activating chloride and/or sodium transport function [7,14]. Recently, concerns on the relevance of the murine nasal mucosa as a human equivalent model have emerged [21]. These concerns are based on the presence of differential proportions of various cell types constituting the mouse and the human nasal epithelium [22] and, subsequently, on different functional roles played by each cell population subset. Accordingly, in the mouse, the respiratory ciliated and the olfactory epithelia line approximately 46 and 47% of the nasal cavity, with small regional fractions being covered by squamous and transitional epithelia [22]. In contrast, in human, respiratory epithelium lines the large majority of the nasal cavity with only about 3% of the nasal surface being covered by olfactory epithelium [23]. Therefore, attention has been drawn to the issue that assessing respiratory transepithelial ion transport in the nose of mouse, as during nasal PD measurements, may possibly integrate the contribution of the olfactory neuroepithelium in the generation of bioelectrical signals [21]. We confirmed here that the nasal PD in mouse can be directed at the respiratory epithelium with high density of ciliated epithelial cells found at the site of nasal PD measurement. This finding highlights the nasal PD in mouse as a valuable tool to evaluate efficacy of fundamental pharmacological therapies in CF. As the olfactory epithelium in CF mice shows progressive, age-related morphological and functional losses

[21], the changes we observed in adult CF mice after treatment with PDE5 inhibitors are not expected to reflect olfactory epithelium modifications.

Nasal PD data obtained here confirmed our previously published data [7], identified the inhalational route as a potential therapy for sildenafil and vardenafil in CF, and demonstrated, for the first time, that tadalafil also restores airway transepithelial chloride transport through the F508del-CF protein. We showed here that nebulisation exposure of an amount of drug adjusted from oral therapeutic doses of the three clinical available PDE5 inhibitors was able to stimulate forskolin-dependent chloride conductance across the respiratory epithelium of the F508del-CF mouse. Distinct relative potencies among the drugs may contribute to the different degrees of forskolin response that we observed (in decreasing order: tadalafil $\geq$ vardenafil > sildenafil). Indeed, vardenafil and tadalafil are more potent and more selective than sildenafil at inhibiting PDE5 [5]. As previously reported [7], no significant effect of PDE5 inhibitors was detected on sodium transport. These data might argue against a direct reciprocal relationship between CFTR and ENaC activity. This could reflect a property of the murine nose where sodium hyperabsorption is less pronounced than in the human nose: indeed, basal PD values recorded in CF patients are on average substantially more negative than in F508del-CF mice [16,17].

Local activation of CFTR protein function following airway deposition may be expected to contribute to the pharmacodynamic effects observed. Reminding that mice are obligate nose breathers [23], drug exposure to mouth during nebulization in a whole-body inhalation chamber model, and subsequent licking off fur and chewing drugs deposited in skin/mouth might scarcely contribute to the bioavailability of PDE5 inhibitors during whole-body inhalation exposures. In line with our results, a similar CFTR correcting effect of sildenafil

has been originally shown in culture of nasal epithelial cells harvested from CF patients and attached to an impermeable support, a configuration that allows interaction of drugs through the apical side of epithelia [24]. It has been recently demonstrated that the inhalation route of administration for vardenafil is associated with an acceptable safety profile [25]. Apart from brief coughing on inspiration, no clinically significant changes in blood pressure or heart rate and no serious adverse events were recorded [25].

A recent work has reported that sildenafil therapy in a patient with severe CF lung disease was followed by clinically significant improvements in exercise tolerance and pulmonary hypertension [26]; even though its prevalence is difficult to establish, pulmonary hypertension develops in a significant proportion of adult CF patients. It has been recognized that subclinical pulmonary hypertension correlates with the severity of the disease and is associated with increased mortality in CF [27]. However, further studies designed to examine the beneficial effect of PDE5 inhibitors on pulmonary hypertension in CF patients are needed.

An additional potential interest of PDE5 inhibitors in CF might be raised by the assumption that PDE5 inhibitors have a possible anti-inflammatory action [28-30]. Actually, it has been shown that application of sildenafil for 24 hours to CF bronchial epithelial cultured cells exposed to *Pseudomonas aeruginosa*, a crucial pathogen responsible for the progressive loss of lung function in CF, reduced secretion of interleukin-8, a cytokine abnormally elevated in CF and responsible for neutrophil infiltrate and subsequent inflammatory cascade [29]. These data further support the view of a local airway effect of PDE5 inhibitors. A clinical trial to be performed at our center aiming at investigating the effect of a single nasal topical administration of vardenafil on NPD measurements in CF patients homozygous for the F508del mutation is listed on www.clinicaltrials.gov (Identifier NCT01002534).

In the present work, we developed a low stress setup for inhalation studies in small animals. We confirmed the potential of sildenafil and vardenafil to restore chloride transport defects of the CFTR protein, and we showed for the first time that tadalafil is also able to correct chloride transport defects across the nasal mucosa of F508del-CF mice. The three PDE5 inhibitors, applied by inhalation, restored chloride transport across the respiratory epithelium and the effect of vardenafil lasts at least 8 hours.

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Legend to figures

Figure 1 – Light micrographs of the surface epithelia lining the nasal cavity of a wild-type mouse. Tissue sections stained with hematoxylin and eosin. (A) Region of the middle nasal concha, where the nasal potential difference (PD) probe is usually located, showing transition from olfactory to respiratory epithelium containing numerous ciliated cells. Focal nasal lesion (marked by brace) produced by the PD probe at the site of nasal PD measurement. (B) Respiratory, pseudostratified ciliated columnar epithelium lining the mid-distal portion of the septal wall of the middle concha and the mid-proximal region of the ethmoidal concha. Arrowheads identify olfactory epithelium; arrows identify respiratory epithelium with ciliated cells. Calibration bars correspond to 50μm in panels A and B and to 10 μm in the inset of panel A.

Figure 2 - Maximal baseline difference (PD) values (PDmax), amiloride response (Δamil), and chloride transport (SumCl) in non-treated wild-type homozygous control (open rectangles) and F508del mice (closed rectangles). Amiloride transport was evaluated by changes in nasal PD after perfusion with basal isotonic saline solution containing 10^{-4} M amiloride. Chloride transport was evaluated by the cumulative changes in nasal PD after perfusion with chloride-free solution in the presence of amiloride followed by addition of 10^{-5} M forskolin. Open circles correspond to individual values obtained from 27 wild-type animals. Closed circles correspond to individual values obtained from 26 CF animals. A p value <0.0001 was obtained for comparison of the corresponding mean value (horizontal line in each group of dots) for each of the three parameters vs. that obtained for the same parameter in the wild-type group of mice.

Figure 3 - Maximal baseline difference (PD) values (PDmax), amiloride response (Δamil), and chloride transport (SumCl) in wild-type homozygous control 1 h after nebulisation with placebo and in F508del mice 1 hour after nebulisation with placebo, sildenafil, vardenafil or tadalafil. Amiloride response was evaluated by changes in nasal PD after perfusion with Ringer's saline solution containing 10^{-4} M amiloride. Chloride transport was evaluated by the cumulative changes in nasal PD after perfusion with chloride-free solution in the presence of amiloride followed by addition of 10^{-5} M forskolin. Results are expressed as means ± SEM for 5-8 animals in each group. Levels not connected by same letter are significantly different at a two-sided α level of 0.05 for the corresponding parameter.

Figure 4 – Duration of the effect of inhaled sildenafil and vardenafil on chloride transport in F508del-CF mice. A nasal transepithelial potential difference was performed 1, 4, 6, 8 or 24 hours after a single nebulisation with placebo (closed rectangles), sildenafil (white rectangles) or vardenafil (grey rectangles). Chloride transport (SumCl) was evaluated by the cumulative changes in nasal PD after perfusion with chloride-free solution in the presence of amiloride followed by addition of 10^{-5} M forskolin. Results are expressed as means ± SEM for 3-7 animals in each group. *$p<0.05$ for comparison of the corresponding mean value vs. that obtained for the same parameter in the placebo-treated group of mice.

Figure 5 – Nasal transepithelial potential difference (PD) measurements in F508del-CF mice 1 hour after nebulisation with placebo or tadalafil. Representative tracings show sequential response of the nasal mucosa to perfusion consecutively with Ringer's saline solution, Ringer's solution containing 10^{-4} M amiloride (amil), chloride-free with amiloride (0 Cl) followed by addition of 10^{-5} M forskolin. Arrows indicate changes of solutions

Figure 6 – Effect of PDE5 inhibitors on forskolin response in F508del-CF mice. A nasal transepithelial potential difference (PD) test was performed 1 hour after nebulisation with placebo, sildenafil, vardenafil or tadalafil in F508del-CF mice. Forskolin response was evaluated by the fractional component of the global chloride transport, and corresponded to the changes observed after perfusion of the nasal mucosa with 10^{-5} M forskolin solution. Results are expressed as means ± SEM for 5-8 animals in each group. Levels not connected by same letter are significantly different at a two-sided α level of 0.05 for the corresponding parameter.

Online supporting material

Legend to figures

Supplemental Figure 1 – (A) Midsagittal section of the nasal passages of the mouse with the septum removed to reveal the nasal cavity. (1) Vestibulum nasi. (2) Ventral nasal concha. (3) Dorsal nasal concha. (4) Middle nasal concha. (5-7) Ethmoidal concha (endoturbinate II-IV). (8) Upper incisor tooth. (9) Hard palate. (10) Soft palate. (11) Cartilage of the nasal septum. (12) Perpendicular plate of the ethmoidal bone. (13) Olfactory bulb. (14) Cerebral hemisphere.

(B-E) Light micrographs of the principal types of surface epithelia lining the nasal cavity of a wild-type mouse. Tissue sections stained with hematoxylin and eosin. (B) Olfactory epithelium lining the mid-proximal region of the dorsal nasal concha and the distal portion of the nasal cavity, close to the olfactory bulb. (C) Keratinized, stratified squamous epithelium restricted to the proximal nasal cavity, close to the nostril opening. (D) Transitional, nonciliated columnar epithelium lining the lateral wall of the mid-proximal region of the dorsal concha. (E) Transition from olfactory to respiratory epithelium containing numerous ciliated cells. Arrowheads identify olfactory epithelium; arrows identify respiratory epithelium with ciliated cells. Calibration bars correspond to 50μm.

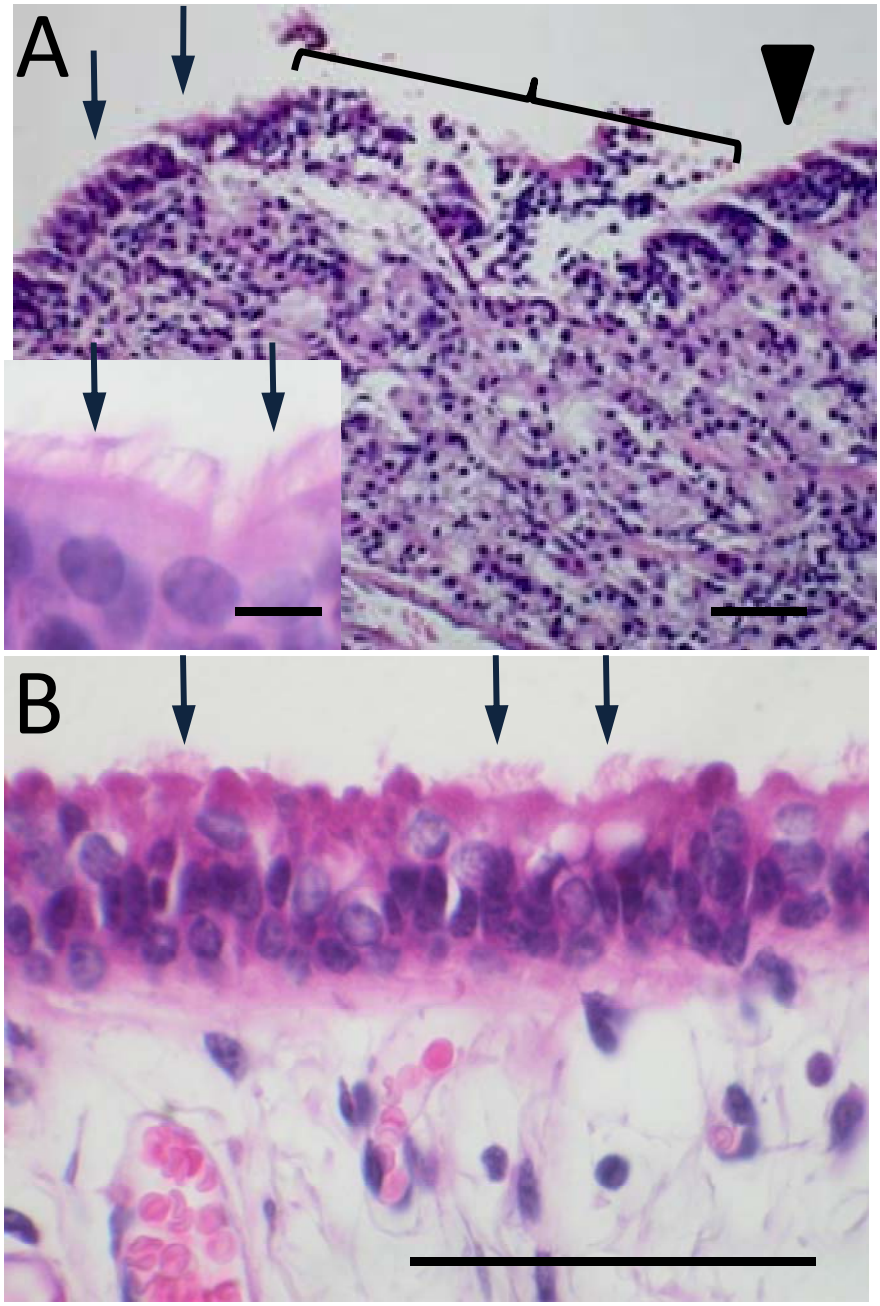

Figure 1

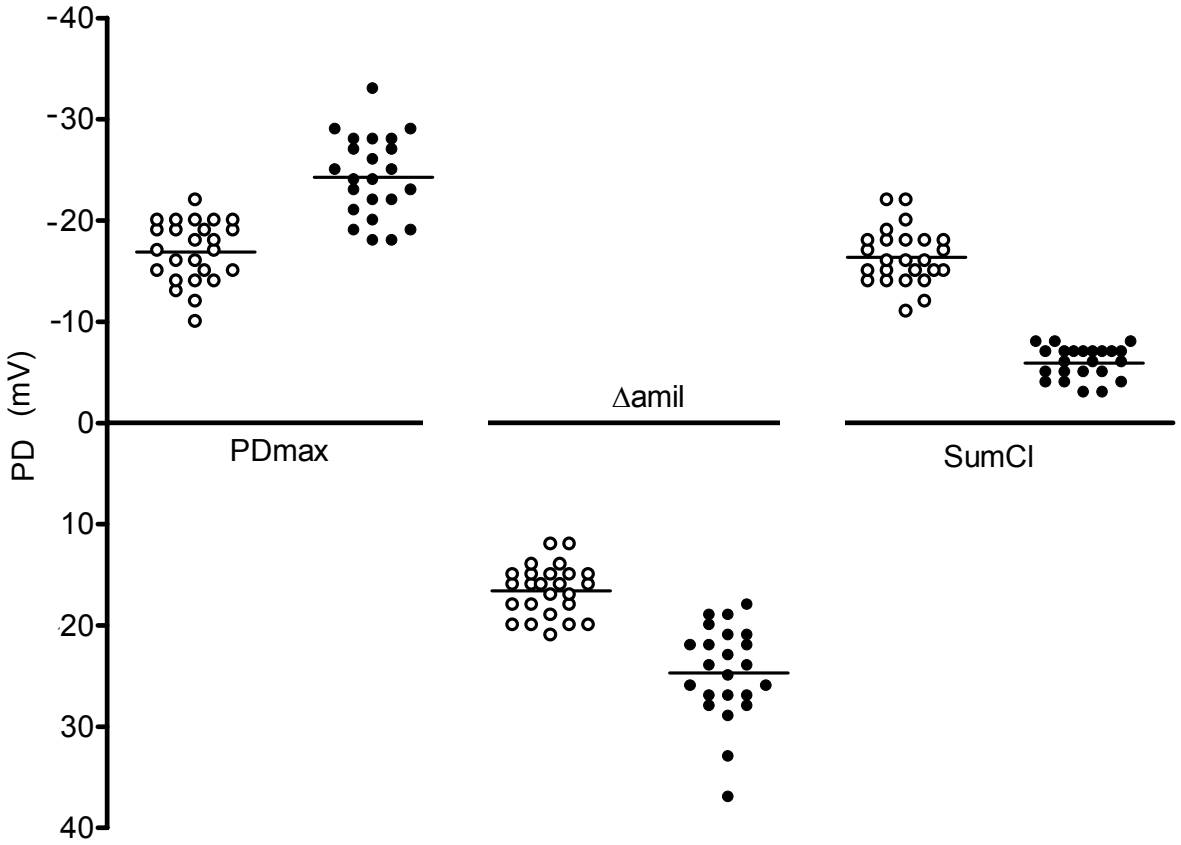

Figure 2

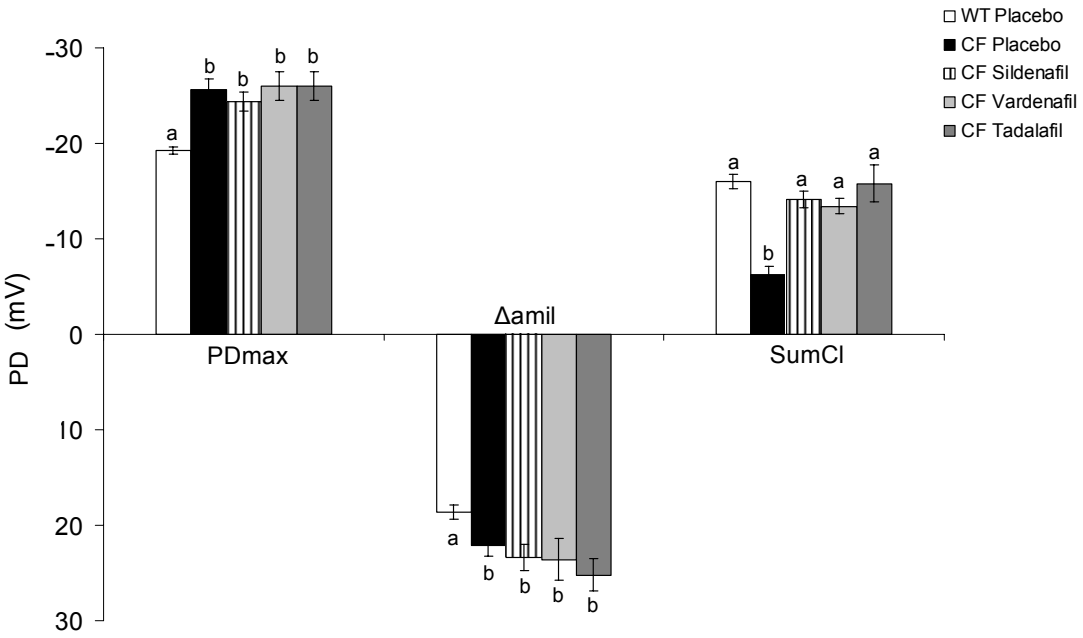
WT Placebo
CF Placebo
CF Sildenafil
CF Vardenafil
CF Tadalafil
PD (mV)
-30
-20
-10
0
10
20
30
PDmax
Δamil
SumCl
a
b
b
b
b
a
b
b
b
b
a
b
a
a
a

Figure 3

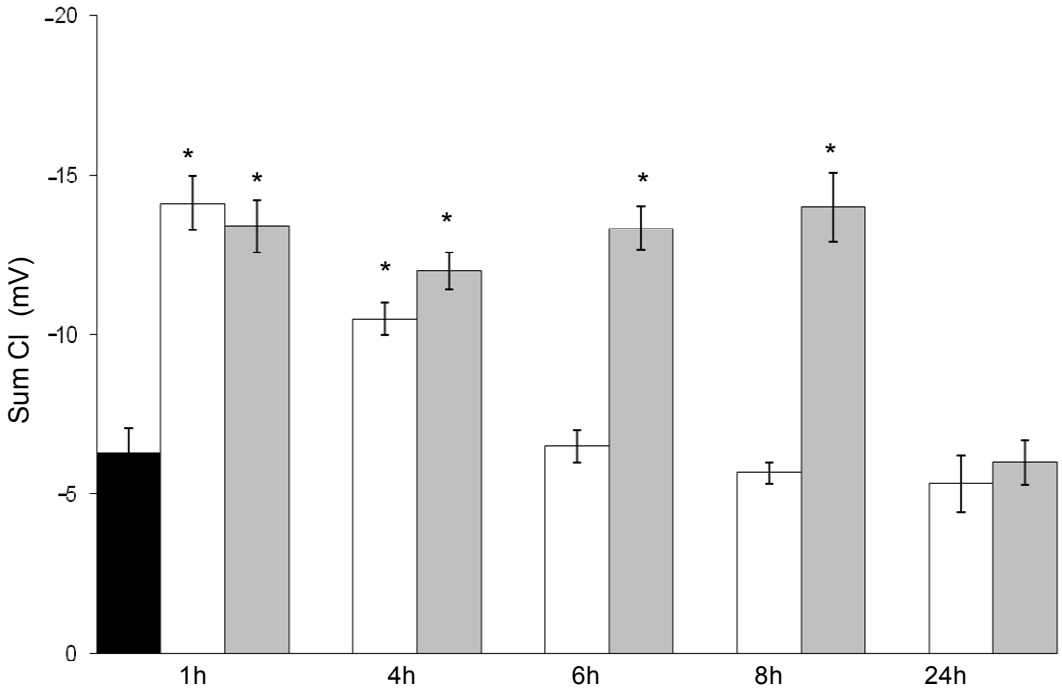

Figure 4

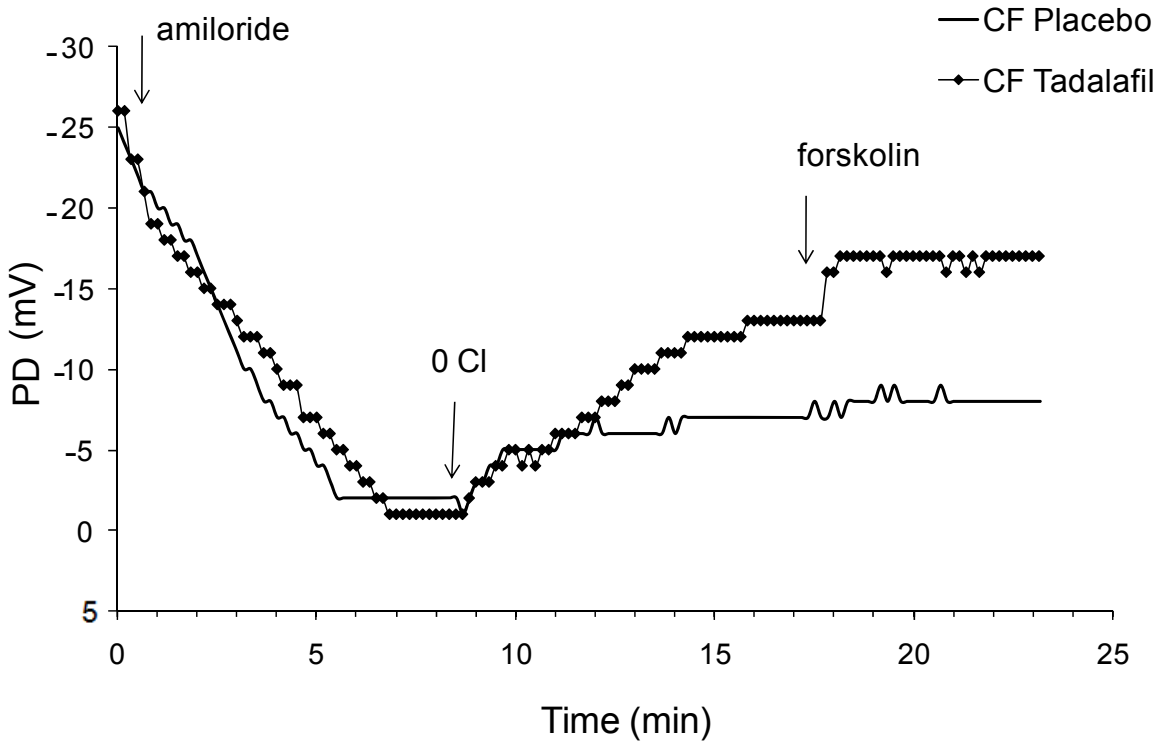
amiloride
0 Cl
forskolin
CF Placebo
CF Tadalafil
PD (mV)
-30
-25
-20
-15
-10
-5
0
5
0
5
10
15
20
25
Time (min)

Figure 5

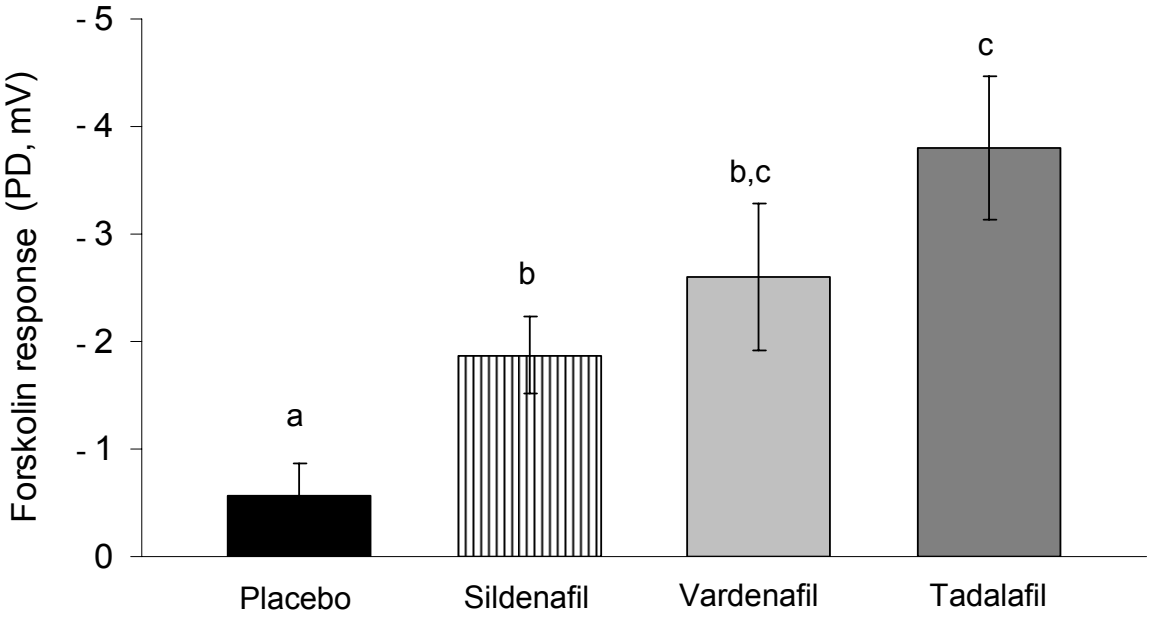
Forskolin response (PD, mV)
- 5
- 4
- 3
- 2
- 1
0
a
b
b,c
c
Placebo
Sildenafil
Vardenafil
Tadalafil

Figure 6