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Sildenafil Acts as Potentiator and Corrector of CFTR but Might be not Suitable for the Treatment of CF Lung Disease

Geraldine Leier, Nadine Bangel-Ruland, Katja Sobczak, Yvonne Knieper and Wolf-Michael Weber

Institute of Animal Physiology, Westphalian Wilhelms-University, Muenster

Key Words

CFTR • cGMP • Corrector • Cystic fibrosis • Potentiator • *Xenopus laevis* oocyte

Abstract

The phosphodiesterase-5 inhibitor sildenafil is an established and approved drug to treat symptoms of a variety of human diseases. In the context of cystic fibrosis (CF), a genetic disease caused by a defective CFTR gene (e.g. ΔF508-CFTR), it was assumed that sildenafil could be a promising substance to correct impaired protein expression. This study focuses on the molecular mechanisms of sildenafil on CFTR recovery. We used ΔF508-CFTR/wt-CFTR expressing *Xenopus laevis* oocytes and human bronchial epithelial cell lines (CFBE41o$^-$/16HBE14o$^-$) to investigate the pathways of sildenafil action. Cells were treated with sildenafil and cAMP-mediated current (I_m), conductance (G_m), and capacitance (C_m) were determined. Sildenafil increased I_m, G_m, and C_m of wt-CFTR and functionally restored ΔF508-CFTR in oocytes. These effects were also seen in CFBE41o$^-$ and 16HBE14o$^-$ cells. Transepithelial measurements revealed that sildenafil mediated increase (wt-CFTR) and restoration (ΔF508-CFTR) of channel activity. cGMP pathway blocker inhibited the activity increase but not CFTR/ΔF508-CFTR exocytosis. From these data we conclude that sildenafil mediates potentiation of CFTR activity by a cGMP-dependent and initiates cGMP-independent functional insertion of CFTR/ΔF508-CFTR molecules into the apical membranes. Thus, sildenafil is a corrector and potentiator of CFTR/ΔF508-CFTR. Yet, the necessary high doses of the drug for CFTR recovery demonstrate that sildenafil might not be suited as a therapeutic drug for CF lung disease.

Introduction

Besides its function in the therapy of erectile dysfunction and pulmonary arterial hypertension, the phosphodiesterase-5 (PDE-5) inhibitor sildenafil seems to have putative effects in cystic fibrosis (CF) [1, 2]. This inherited autosomal recessive disease is caused by a mutation in the *cystic fibrosis transmembrane conductance regulator* (CFTR) gene [3]. The ABC transporter CFTR

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Prof. Dr. Wolf-Michael Weber
Institute of Animal Physiology, Westphalian Wilhelms-University
Hindenburgplatz 55, 48143 Muenster (Germany)
Tel. +49 251 83 21782, Fax +49 251 83 21785
E-Mail wmw@uni-muenster.de

is located at the apical membrane of chloride secreting epithelia and tissues including sweat glands, pancreas, colon, and lung, where it acts as a cAMP regulated chloride channel [4, 5]. As an important second messenger cAMP activates the proteinkinase A (PKA), which phosphorylates the CFTR protein at its regulatory domain (R-domain) [6]. Moreover, the protein kinases C and G II (PKC; PKGII) also have the ability to phosphorylate the R-domain, which represents a necessary condition for CFTR activation [7, 8]. In this process the PKG depends on the second messenger cGMP [9].

In case of the most common CFTR mutation, the deletion of a phenylalanine at the position 508 (ΔF508-CFTR) of the CFTR sequence causes misfolding of the protein inducing its retention in the endoplasmatic reticulum (ER) and proteosomal degradation by ER associated protein degradation (ERAD) [10]. Subsequently, the majority of CFTR molecules fail to reach the plasma membrane leading to a strongly reduced chloride conductance. Interestingly, 75% of the wildtype (wt)-CFTR are degraded in the proteasome, too [11]. If the ΔF508-CFTR protein is inserted into the plasma membrane the misfolded protein is able to conduct chloride [12]. The insertion of only 10-15% of the ΔF508-CFTR into the plasma membrane seems to be sufficient to restore the chloride conductance and thus to alleviate CF symptoms [13, 14]. Restoration of CFTR function is an extremely essential goal since CFTR is, beside its chloride channel function, one of the most important regulatory proteins of epithelial cells and controls several central tasks in secretory epithelia [15, 16]. For this reason numerous therapeutic approaches focus on the restoration of the chloride conductance by repairing the trafficking and insertion of the ΔF508-CFTR protein by the use of so called correctors. In addition, the search for compounds that enhance the activation of the inserted CFTR molecules (potentiators) is also currently in progress [17]. Using a combinational therapy of a corrector and a potentiator seems to be a promising perspective to achieve the maximum therapeutic benefit [18]. Until now, several potentiators like VRT-532 [19], genistein and apigenin were identified [20]. A few potentiators are already in clinical trials as therapeutic substances for CF and very recently the drug VX-770 has been approved by the food and drug administration for the therapy of cystic fibrosis [21]. Application of correctors including the chaperones corr-4a [22, 23] and corr-2b [22] as well as RDR1 [24], VRT-532, and VRT-325 [19] evoke a partial restoration of ΔF508-CFTR plasma membrane expression. The best characterized corrector corr-4a induces a 8% restoration of the ΔF508-CFTR plasma membrane expression in primary human bronchial epithelial cells and enhances the stability of the rescued ΔF508-CFTR protein [22, 23]. The underlying mechanisms, how these correctors and potentiators influence CFTR is not fully understood. In the last years some of the mechanisms of correctors could be identified. Wang et al. showed a direct interaction of VRT-325 as well as corr-4a with the CFTR protein [25], assuming that these correctors induced the conformational rearrangement of the CFTR protein, thereby leading to partial CFTR activity. However, the postulated binding site is still unknown [26]. Moreover, RDR1 also influences CFTR directly by binding to the first nucleotide binding domain of CFTR, which contains the ΔF508-CFTR mutation [24].

In the ongoing progress of identifying different CFTR correcting molecules, the class of phosphodiesterase (PDE) inhibitors as active substances are under closer scrutiny. Besides the PDE inhibitor milrinone, other classes of PDE inhibitors were screened as prospective CFTR correctors or potentiators [27]. PDEs cleave the phosphodiester bond within cyclic nucleotides including cAMP and cGMP. Due to their specification to hydrolyze either cAMP or cGMP, PDEs were divided into 10 classes. PDE-4s are highly specific for cAMP, whereas PDE-5s and PDEs-6 are cGMP-specific, thus PDE-1s show a mixed specificity for cAMP and cGMP [28].

Based on several high throughput screenings the PDE-5 inhibitor sildenafil, which is the active agent of Viagra®, was identified as a CFTR corrector [1, 2, 29, 30]. Dormer et al. reported for the first time an enhanced insertion of ΔF508-CFTR in isolated epithelial cells from CF patients due to incubation with 150 µmol l^{-1} sildenafil for 2 hours [1]. Additionally, another group showed that application of the clinical dose of 0.5 µmol l^{-1} sildenafil to ΔF508-CFTR mice corrected the reduced chloride conductance [2, 31, 32]. Since 2008, sildenafil is in a phase I / II clinical trial to investigate the *in vivo* effect of sildenafil on CFTR in CF lung disease (www.ClinicalTrials.gov: NCT00659529).

Originally, sildenafil was developed as a smooth muscle relaxant for pulmonary arterial hypertension, because dilatation of arterial muscles leads to a slight blood pressure decrease. Based on this effect sildenafil and its analogues were previously approved and are commercially available as Viagra® and similar compounds for the therapy of erectile dysfunction [33]. Until now, the mechanism that allows sildenafil to act as a CFTR corrector is still matter of intensive discussions. One possibility is that sildenafil modulates the cGMP concentration via altera-

tions in the NO-dependent cGMP pathway as described for the sildenafil therapy in erectile dysfunction and pulmonary arterial hypertension [28, 34, 35]. Furthermore, it is possible that sildenafil interacts directly with the CFTR protein as already shown for the correctors corr-4a, VRT-325 as well as RDR 1 [24, 25].

With respect to the effect of sildenafil as a corrector of CFTR, we aimed at analysing the mechanisms of sildenafil action on CFTR in more detail with regard to a potential use of this drug as a therapeutic substance in cystic fibrosis. Therefore, we blocked the NO-dependent cGMP pathway via the guanylate cyclase inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) as well as the PKG by its inhibitor KT 5823 [36, 37]. We were able to show a restoration of the chloride conductance via electrophysiological measurements in ΔF508-CFTR expressing oocytes as well as via transepithelial experiments with the human bronchial epithelial cell line CFBE41o$^-$ (ΔF508-CFTR$^{+/+}$) by 60 - 400 µmol l^{-1} sildenafil. Furthermore, using wt-CFTR expressing oocytes and human bronchial epithelial wt-CFTR cells (16HBE14o$^-$) an enhancement of CFTR activity and exocytosis of CFTR molecules could be detected. The enhanced CFTR activity by sildenafil could be inhibited by ODQ and KT 5823, whereas the sildenafil stimulated exocytotic insertion of CFTR was not altered using the respective cGMP pathway blocker. Moreover, Western blot analysis as well as immunofluorescence approaches confirmed these results. In conclusion, we present data illustrating that sildenafil seems to be a CFTR corrector as well as a potentiator acting via two different mechanisms: one being cGMP-dependent and one being cGMP-independent. However, the observed positive effects of sildenafil could be only achieved with quite high doses that could cause severe adverse effects when used for the treatment of lung disease in cystic fibrosis patients.

Materials and Methods

Oocyte preparation and injection of cRNA

Oocytes were isolated from female *Xenopus laevis* (purchased from the African Xenopus Facility, Knysna, Africa; approved by the ethic committee of the university of Muenster) as described previously [38]. Briefly, the *Xenopus laevis* (*X. laevis*) female was anaesthetized with tricaine (MS 222, 1.5 g/l pH 7.0) before the ovarian lobes were removed by laparotomy. The connective tissue of the ovarian lobes was removed by incubation for 2 hours with collagenase (1 mg/ml; Serva, Heidelberg, Germany) in oocyte Ringer solution (ORi; 90 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} HEPES; pH 7.6). The oocytes were washed in ORi 3 times. For defolliculation, oocytes were incubated for 10 min in Ca^{2+}-free ORi (90 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 1.5 mmol l^{-1} EGTA, 5 mmol l^{-1} HEPES; pH 7.6) and subsequently washed 3 times in ORi. Healthy looking stage V-VI oocytes were selected and stored in ORi at 16°C. The oocytes were injected with human wt-CFTR cRNA (23 ng, 50.6 nl) or ΔF508-CFTR cRNA (40 ng; 50.6 nl) into the cytosplasm, respectively as described elsewhere [38]. 3 - 6 days after cRNA injection the oocytes were injected with 60 µmol l^{-1} sildenafil additionally (27.6 nl; Molekular, Taufkirchen, Germany) 2 hours before voltage clamp measurements.

cRNA preparation

For *in vitro* transcription (IVT) pSTI-wt-CFTR (a kind gift from the laboratory of J. Rosenecker) and pAlter ΔF508-CFTR (a kind gift from K. Kunzelmann) were used. The linearized plasmids were extracted with phenol/chloroform and precipitated with ethanol. The IVT reaction was carried out using the mMessage mMachine Kit (Ambion, Foster City, USA), followed by a purification using the RNeasy plus Mini Kit (Qiagen, Hilden, Germany) and subsequent ethanol precipitation to achieve good quality mRNA.

Voltage clamp (VC) measurements

All measurements were performed under voltage-clamp conditions using the standard two-microelectrode technique as described in detail elsewhere [38]. Briefly, the oocytes were placed into a 1 ml Plexiglas chamber and were perfused continuously with ORi. Oocytes were impaled with two microelectrodes, which had a resistance between 0.5 - 3 MΩ and were filled with 1 mol l^{-1} KCl. Additionally, two Ag/AgCl pellets were placed in the bath as reference and to supply a virtual ground circuitry. The electrodes were associated with a voltage clamp amplifier (OC 725C, Warner Instruments Inc., Hamden, USA), which is further connected via two digital processing boards (DSP-boards, Model 310B, Dalanco Spry, Rochester, NY, USA) to a computer. This equipment (designed by Prof. Willy Van Driessche, KU Leuven, Belgium) was described in detail elsewhere [38], allowed us to record simultaneously membrane current (I_m), conductance (G_m) and capacitance (C_m), the latter representing the surface area of the oocytes [38].

During the measurements the oocyte membrane potential was clamped to -60 mV and subsequently CFTR was activated by an application of the cell permeable cAMP analog 8-(4-chlorophenylthio) cAMP (cpt-cAMP, 100 µmol l^{-1}, in ORi). The cAMP cocktail also contains the phosphodiesterase inhibitor isobutylmethylxantine (IBMX, 1 mmol l^{-1}). In one set of experiments the cAMP analog was substituted by the cGMP analog 8-(4-chlorophenylthio)-cGMP (cpt-cGMP, 100 µmol l^{-1}). For data analysis, cAMP- or cGMP-mediated electrical responses were determined in relation to the initial values after clamping the potential to -60 mV.

Before and after sildenafil injection, wt-CFTR expressing oocytes were incubated 30 min with the PKG inhibitor KT5823 (3 µmol l^{-1}; Enzo Lifescience, Lörrach, Germany) or the guanylate cyclase blocker ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 50 µmol l^{-1}; Enzo Lifescience) to verify that the action of

sildenafil was cGMP dependent. Moreover, oocytes were incubated for 24 hours in N-[2-(methylamino)ethyl-5) isoquinolinesulfonamidedihydrochlorid (H8; 30 µmol l^{-1}) to inhibit the PKA-dependent exocytosis.

Cell culture

16HBE14o$^-$ and CFBE41o$^-$ cells were obtained from Dr. Dieter Gruenert (California Pacific Medical Center Research Institute, San Francisco, USA). The 16HBE14o$^-$ (endogenous wt-CFTR) and CFBE41o$^-$ (ΔF508-CFTR) epithelial lung cells were cultivated on coated flasks (0.13 % bovine serum albumin (7.5%), 3% bovine collagen type I (1 mg/ml), 1% human fibronectin (1 mg/ml), 9.87% sterile water and LHC basal medium) in Minimal Essential Medium Eagle (MEM) supplemented with 10 % fetal bovine serum (FBS), 1% L-glutamin (200 mmol l^{-1}) and 1% Penicillin/Streptomycin (10000 U/ml). The cells were incubated at 37°C, 95% air and 5% CO_2.

For transepithelial measurements 1.1 x 10^5 cells were seeded on permeable filters with a pore size of 0.4 µm (0.33 µm²; Costar Transwell, Corning Inc., NY, USA), whereas for immunofluorescence analyses 3.5 x 10^5 cells were seeded on coated glass cover slips (12 mm diameter; Roth, Karlsruhe, Germany), which were placed in petri dishes.

Transepithelial measurements

After approximately 7 days the cells on the filter reached confluence and were mounted into the Ussing chamber, which was heated to 37°C. Within the measurement the apical and basolateral compartment was continuously perfused with cell culture Ringer (130 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} glucose and 10 mmol l^{-1} HEPES, 37°C). Voltage and current electrodes were electrically coupled to the chamber by KCl-agar bridges. The transepithelial potential (V_t) was clamped to zero. The applied compensational current (short circuit current, I_{sc}) reflects the transported net charges over the epithelium and depends on the conductance of the epithelium. I_{sc}, conductance (G_t) and capacitance (C_t) were continuously monitored using the computer program ImpDsp1.4 (Prof. Willy Van Driessche). All parameters were normalized to 1 cm².

After stabilization of all electrical parameters a cAMP cocktail (cpt-cAMP (100 µmol l^{-1})/ IBMX (1 mmol l^{-1}) in cell culture Ringer) was applied on the basolateral side to activate CFTR. Subsequently, CFTR was inhibited using the CFTR inhibitor $CFTR_{inh}$-172 apically (Tocris Bioscience, Bristol, UK). Sildenafil was added to the cells in various concentrations (30 µmol l^{-1}, 45 µmol l^{-1}, 60 µmol l^{-1}, 200 µmol l^{-1}, 400 µmol l^{-1}) as well as in combination with KT 5823 (3 µmol l^{-1}) and ODQ (10 µmol l^{-1}) to the apical site. The sildenafil containing solutions also contained a final concentration of 9% DMSO to increase the solubility of sildenafil. Because of the presence of DMSO in the sildenafil solutions, additional monolayers were incubated with 9% DMSO as a control and the changes in the parameters were subtracted from the sildenafil effect. In case of KT 5823 or ODQ application, the cells were incubated 15 min before and during the measurements with these inhibitors.

Protein biochemistry

Cells were incubated with sildenafil (0 µmol l^{-1}, 60 µmol l^{-1}, 400 µmol l^{-1}) and 60 µmol l^{-1} sildenafil in combination with 10 µmol l^{-1} ODQ. After 2 hours of incubation the membrane and cytosolic proteins were isolated separately by using the ProteoExtract® Transmembrane Protein Extraction Kit (Novagen, Merk, Darmstadt, Germany) with the supplied extraction buffer A. The protein concentrations were measured colorimetrically using the BCA test (Pierce, Rockford, IL). 40 µg membrane proteins and 10 µg cytosolic proteins were separated via SDS-PAGE (7.5% acrylamide) and subsequently transferred to a polyvinylidenfluorid (PVDF) membrane. Non-specific binding sites were blocked by incubation with 5% milk powder (Roth), dissolved in Tris-buffered saline/Tween (TBST; 10 mmol l^{-1} Tris HCl (pH 7.4), 1.5 mmol l^{-1} NaCl, 0.05% Tween 20), for 2 hours. CFTR protein was detected by using a mouse anti-CFTR antibody (MA1-935; Dianova, Hamburg, Germany) in a 1: 500 dilution. This antibody binds to the first extracellular loop of CFTR (amino acid residues 103-117) and detects only the mature, complexly glycosylated CFTR protein, which exhibits a molecular mass around 170 kDa. As loading control α-tubulin was detected using a mouse anti-α-tubulin antibody (Dianova) at a concentration of 1:250. In both samples the antibodies were diluted in 5% milk powder in TBST and incubated overnight at 4°C. Afterwards, the membrane was washed 3 times with TBST before the secondary antibody coupled to a horseradish peroxidase (anti-mouse, 1:10000; Dianova) was applied for 1 hour at room temperature. To identify and compare the protein amount of the detected bands, the PVDF membranes were digitized and analyzed semi quantitatively by densitometry, using the ImageJ analysis software 1.3.6 (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). Furthermore, the overall protein amount in the cells was normalized using the loading control α-tubulin.

Immunofluorescence staining

Cells grown on glass cover slips were incubated with sildenafil (0 µmol l^{-1}, 45 µmol l^{-1}, 60 µmol l^{-1}, 400 µmol l^{-1}) and 60 µmol l^{-1} sildenafil in combination with 3 µmol l^{-1} KT5823 for 2 hours. Afterwards cells were fixed with 0.05% glutaraldehyde in HEPES buffer (140 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} glucose, 10 mmol l^{-1} HEPES) for 10 minutes at 37°C. The antibody staining was performed as described previously [39]. Briefly, after fixation cells were washed 5 times for 5 min with phosphate buffered saline (PBS; 140 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 4 mmol l^{-1} Na_2HPO_4, 1 mmol l^{-1} KH_2PO_4, pH 7.4) at room temperature. Subsequently, an incubation with 100 mmol l^{-1} glycine/PBS for 60 min followed. After washing 5 times for 5 min with PBS, the cells were blocked with 10% normal goat serum (NGS) at room temperature for 30 min. Afterwards, the mouse anti-CFTR antibody (MA1-935; Dianova) was applied in a concentration of 1:500 in NGS for 1 hour at room temperature in a humidity chamber. Cells were washed 5 times for 5 min before the Quantum Dots (QD; QD655 $F(ab')_2$ anti-mouse IgG conjugates; Invitrogen, Karlsruhe, Germany) were added at a concentration of 1:200 in NGS for 1 hour at room temperature in the dark. Cells were washed again

5 times for 5 min with PBS, two times with HEPES buffer for 5 min and fixed with 0.05% glutaraldehyde in HEPES buffer for 10 min. Cells were then washed 3 times for 5 min in PBS, 5 times briefly in deionised water and were mounted on a microscope slide using Dako Fluorescent mounting medium (Dako, Glostrup, Denmark). As a negative control 16HBE14o$^-$ and CFBE41o$^-$ cells were incubated with the secondary antibody without application of the primary antibody.

Image analysis

The immunofluorescence images were acquired with an inverted fluorescence microscope (Axiovert 200; Zeiss, Jena, Germany) equipped with a 100 x 1.45 oil immersion objective and a fluorescence filter with 488 nm excitation and an emission of 655 nm (F31-118Q; AHF Analysetechnik AG, Tübingen, Deutschland) as well as a FITC filter. For image acquisition it was focused on the upper membrane of the cells. Afterwards the amount of fluorescence spots, representing quantum dot labeled CFTR molecules, were counted manually using the ImageJ analysis software 1.3.6 and the means were corrected by the negative controls.

Statistics

Data are presented as arithmetic means (± SEM). Variances were checked using the F-test and subsequently the appropriate statistical method was used. Most of the statistical evaluations were made by Student´s t-test, whereas a significance level of $p \leq 0.05$ (*) or $p \leq 0.01$ (**) was assumed. In oocyte experiments N reflects the number of donor frogs, whereas n represents the number of measured oocytes. In the immunofluorescence and protein biochemistry experiments n is the number of evaluated cells/experiments and N the number of immunofluorescence staining procedures/protein isolations. In transepithelial measurements n gives the number of preparations.

Results

Part 1: *Effects of sildenafil on wt-CFTR/ΔF508-CFTR expressed in Xenopus laevis oocytes*

Determination of endogenous PDE-5 expression in Xenopus laevis oocytes. In order to verify the endogenous PDE-5 expression in oocytes total RNA was isolated from *X. laevis* oocytes and a fragment of the PDE-5 was amplified, cloned, and sequenced. The nucleotide sequence was compared with the published PDE-5 sequence from *Xenopus laevis* spleen (GenBank: NM_001094802). Thereby, a portion of the PDE-5 cDNA sequence is identical, which confirmed the expression of PDE-5 in the *X. laevis* oocytes.

Effects in non-injected oocytes. Previous experiments revealed that H_2O-injected oocytes and non-injected oocytes showed no differences in I_m, G_m as well as C_m [40]. Therefore, non-injected oocytes were used as controls. Application of the cAMP cocktail caused only small changes in I_m, G_m and C_m. For instance, I_m increased about 39 ± 21 nA in control oocytes, whereas cAMP stimulated I_m by 1692 ± 126 nA in wt-CFTR expressing oocytes. An additional incubation with H8, KT 5823 and ODQ did not alter the response of control oocytes to the cAMP cocktail. Moreover, sildenafil decreased the cAMP response in non-injected control oocytes. Taken together, cAMP-mediated changes in I_m, G_m and C_m after incubation with the test substances were negligible as compared to CFTR-expressing oocytes (Fig. 1), demonstrating that H8, KT5823 and ODQ had no effect on endogenous transport systems of the oocytes. Therefore, these substances can be used to characterize the effect of sildenafil in CFTR expressing oocytes.

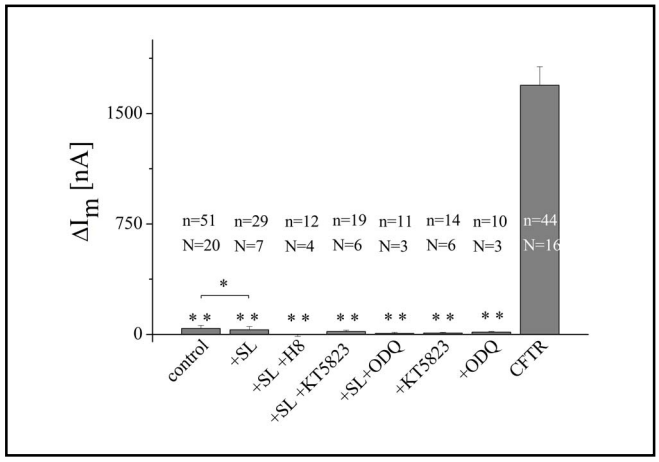

Fig. 1. cAMP mediated increases in I_m of non-injected *Xenopus laevis* oocytes (control). Injection of sildenafil (SL) decreased the cAMP response significantly, whereas incubation with H8, KT 5823 and ODQ did not affect cAMP responses, demonstrating that these substances have no effect on the endogenous transport mechanisms of the oocytes. Compared with the cAMP induced increase in I_m in CFTR expressing oocytes, cAMP response of control oocytes is negligible.

Effect of sildenafil on ΔF508-CFTR expressing oocytes. To investigate the effect of the PDE-5 inhibitor sildenafil on ΔF508-CFTR expressing oocytes, we injected 27.6 nl of a 60 µmol l^{-1} sildenafil containing solution into the oocyte cytoplasm 2 hours prior to measurements. The final sildenafil concentration in the oocyte cytoplasm was calculated to be ~1.5 µmol l^{-1} assuming an average oocyte volume of 0.95 µl [41]. Oocytes were then clamped to -60 mV and the cAMP/IBMX cocktail was applied. As a control we used ΔF508-CFTR expressing oocytes, which

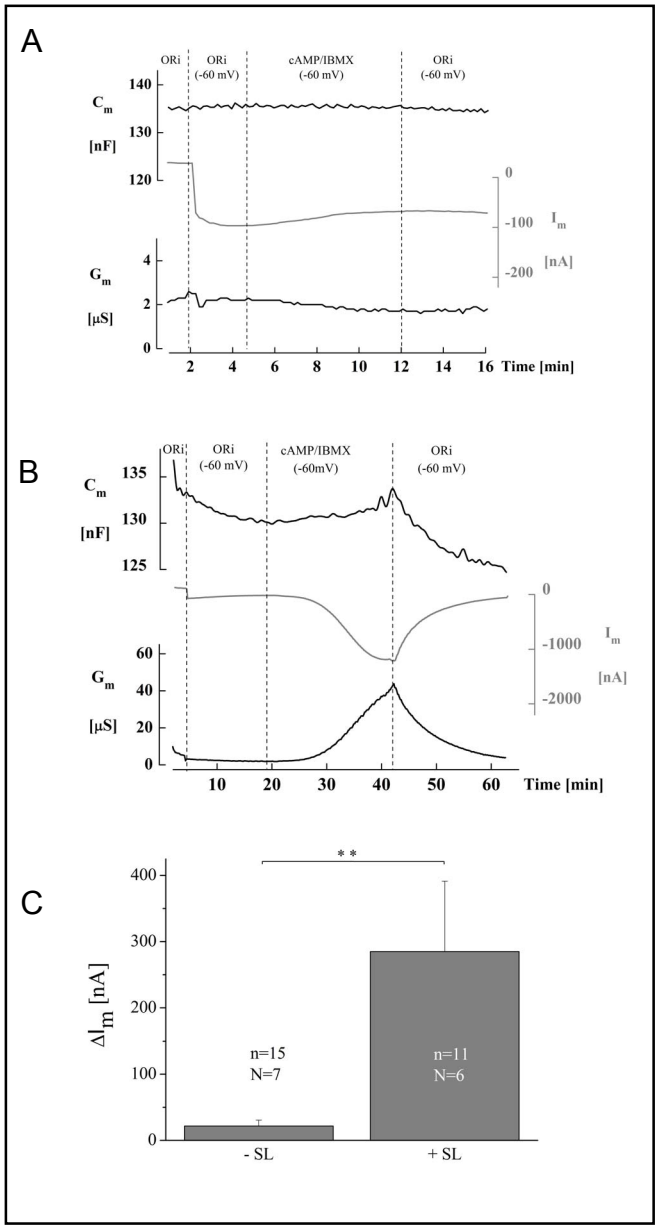

Fig. 2. SL effect on ΔF508-CFTR expressing oocytes. A + B: Representative measurements of ΔF508-CFTR expressing oocytes with (B) and without (A) preceding sildenafil (SL) injection. After stabilization of I_m, G_m, and C_m the membrane potential was clamped to -60 mV before the cAMP/IBMX cocktail was applied. A: Without sildenafil injection no significant changes by cAMP/IBMX application could be observed. B: After sildenafil injection cAMP/IBMX stimulates I_m and G_m. This stimulation could be reversed by cAMP cocktail washout. C: Statistical evaluation showed a significant 13-fold higher cAMP mediated current increase after sildenafil injection.

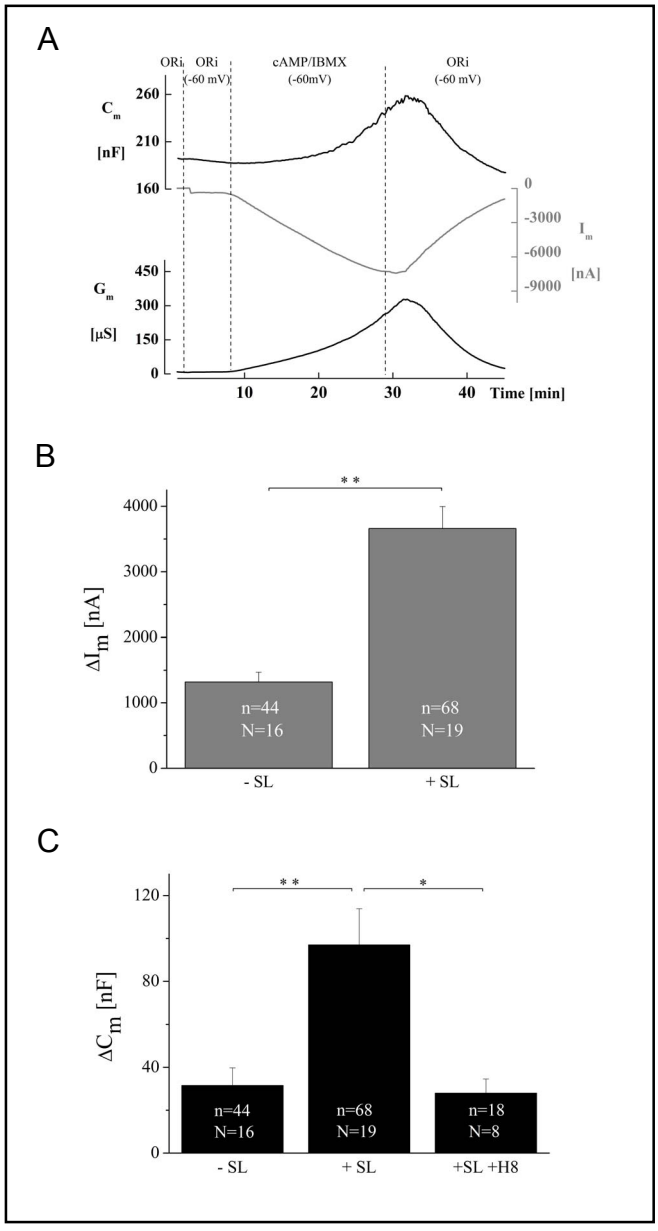

Fig. 3. SL effect on wt-CFTR expressing oocytes. A: Typical measurement of a wt-CFTR expressing oocyte after sildenafil (SL) injection. Membrane potential was clamped to -60 mV before a cAMP/IBMX cocktail was applied. I_m, G_m, and C_m rose significantly in a reversible way. B + C: Comparison of cAMP mediated increases of sildenafil treated and sildenafil untreated wt-CFTR expressing oocytes. Sildenafil increased the cAMP induced current (B) and membrane capacitance (C). Additional incubation with the PKA inhibitor H8 blocked the sildenafil mediated increase in C_m completely (C). These data show that the sildenafil induced capacitance increase is evoked by PKA dependent exocytosis of CFTR carrying vesicles.

were not injected with sildenafil. These controls showed no bioelectrical alterations after cAMP/IBMX application (Fig. 2A), demonstrating that ΔF508-CFTR could not be activated by cAMP. In contrast to this, in sildenafil-injected oocytes cAMP stimulated I_m (Fig. 2B; 285 ± 106 nA; n =11, N = 6) and G_m (7.51 ± 3.8 µS; n=11, N = 6).

Statistical evaluation revealed a 13-fold increase of the ΔF508-CFTR current after sildenafil injection (Fig. 2C), whereas there were only tiny or no changes in C_m.

Effect of sildenafil on wt-CFTR expressing oocytes. To find a general mechanism independently from the ΔF508 mutation, we analyzed the impact of sildenafil on wt-CFTR. Therefore, we used wt-CFTR expressing oocytes and the same experimental setup as for ΔF508-CFTR reported above. wt-CFTR expressing oocytes were injected with 60 µmol l^{-1} sildenafil and cAMP-mediated changes were registered. We observed an increase in I_m, G_m and C_m in the sildenafil injected (Fig. 3A) and non-sildenafil injected wt-CFTR expressing oocytes, which could be reversed by washout of the cAMP cocktail. Interestingly, cAMP-mediated I_m and G_m increased after sildenafil application (Fig. 3B). In average, sildenafil injected oocytes showed a cAMP induced increase of I_m of 3662 ± 334 nA (n = 68, N = 19), whereas non-sildenafil injected oocytes revealed only an increase of 1318 ± 151 nA (n = 44, N = 16). These data demonstrate that ~1.5 µmol l^{-1} sildenafil leads to a significant activation of the wt-CFTR in the *Xenopus laevis* oocytes.

Furthermore, these experiments clearly show that sildenafil treatment of the wt-CFTR expressing oocytes enhanced also the cAMP-stimulated increase in C_m significantly (sildenafil injected oocytes: 97 ± 17 nF (n = 68, N = 19); sildenafil untreated oocytes: 32 ± 8 nF (n = 44, N = 16; Fig. 3C). As C_m reflects the membrane surface area of the oocyte, the measured C_m increases are probably evoked by cAMP-induced insertion of CFTR carrying vesicles into the oocyte membrane as already observed in previous studies [38].

To verify the assumption that this cAMP-mediated rise in C_m after sildenafil injection is caused by exocytotic insertion of CFTR carrying vesicles into the plasma membrane of the oocyte, we inhibited the PKA dependent exocytosis pathway with the specific PKA inhibitor H8. For these experiments, wt-CFTR expressing oocytes were incubated with 30 µmol l^{-1} H8 for at least 24 hours prior to the VC measurements. 2 hours before the oocytes were voltage-clamped sildenafil was injected into the oocytes. These experiments demonstrated that the PKA inhibitor H8 blocked the sildenafil stimulated C_m increase completely to the level of the non-sildenafil-njected oocytes (Fig. 3C), indicating that sildenafil had a strong positive effect on exocytotic events.

Mechanism of sildenafil action on wt-CFTR expressing oocytes. Until now studies on the PDE-5 inhibitor sildenafil have been mainly focusing on the direct

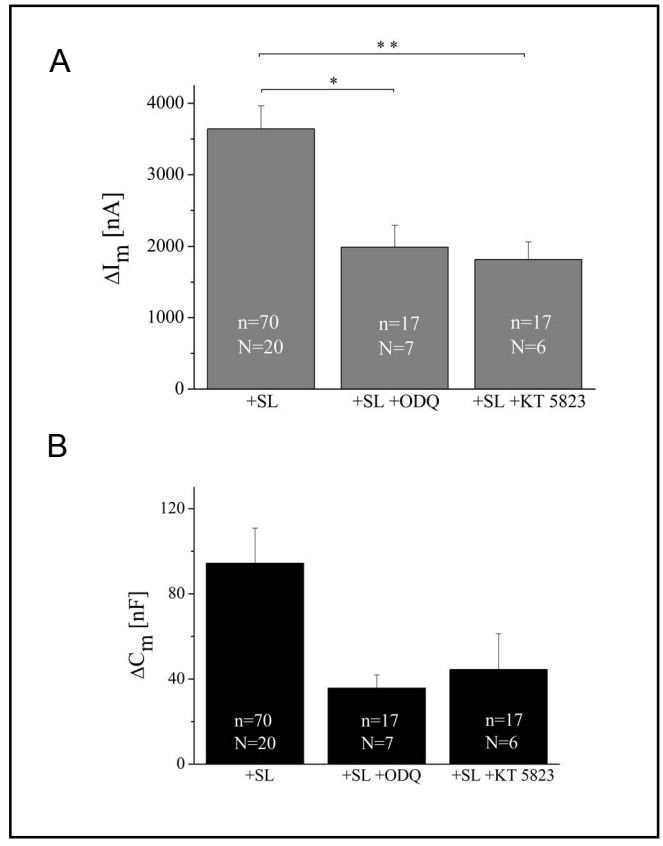

Fig. 4. Effect of ODQ and KT5823 on SL-injected wt-CFTR expressing oocytes. cAMP-mediated I_m (A) and C_m (B) in absence and presence of ODQ, KT 5823 on wt-CFTR-expressing and SL-treated oocytes. ODQ and KT 5823 inhibited cAMP induced I_m significantly (A). In contrast to that the cAMP-mediated increases in C_m were not significantly influenced by ODQ or KT 5823 (B). This demonstrates that the inhibition of the cGMP pathway by ODQ or KT 5823 inhibited the sildenafil mediated CFTR current but not the sildenafil mediated wt-CFTR exocytosis.

effects of sildenafil on the CFTR protein. Here we investigated the overall underlying molecular mechanisms of sildenafil effects on CFTR. First, we analyzed the pathway, where sildenafil activates CFTR by inhibiting PDE-5-mediated cGMP degradation and subsequent elevation of cellular cGMP levels. Therefore, we applied a cGMP cocktail to wt-CFTR expressing oocytes clamped to -60 mV. cGMP increased I_m (1402 ± 378 nA; n = 12, N = 3) and G_m (41 ± 7 µS; n = 12, N = 3). Interestingly, C_m remained unaffected by cGMP, indicating that cGMP has no potency to induce exocytosis as we showed for cAMP. cGMP did not influence those parameters in non-injected control oocytes. These results support the hypothesis of

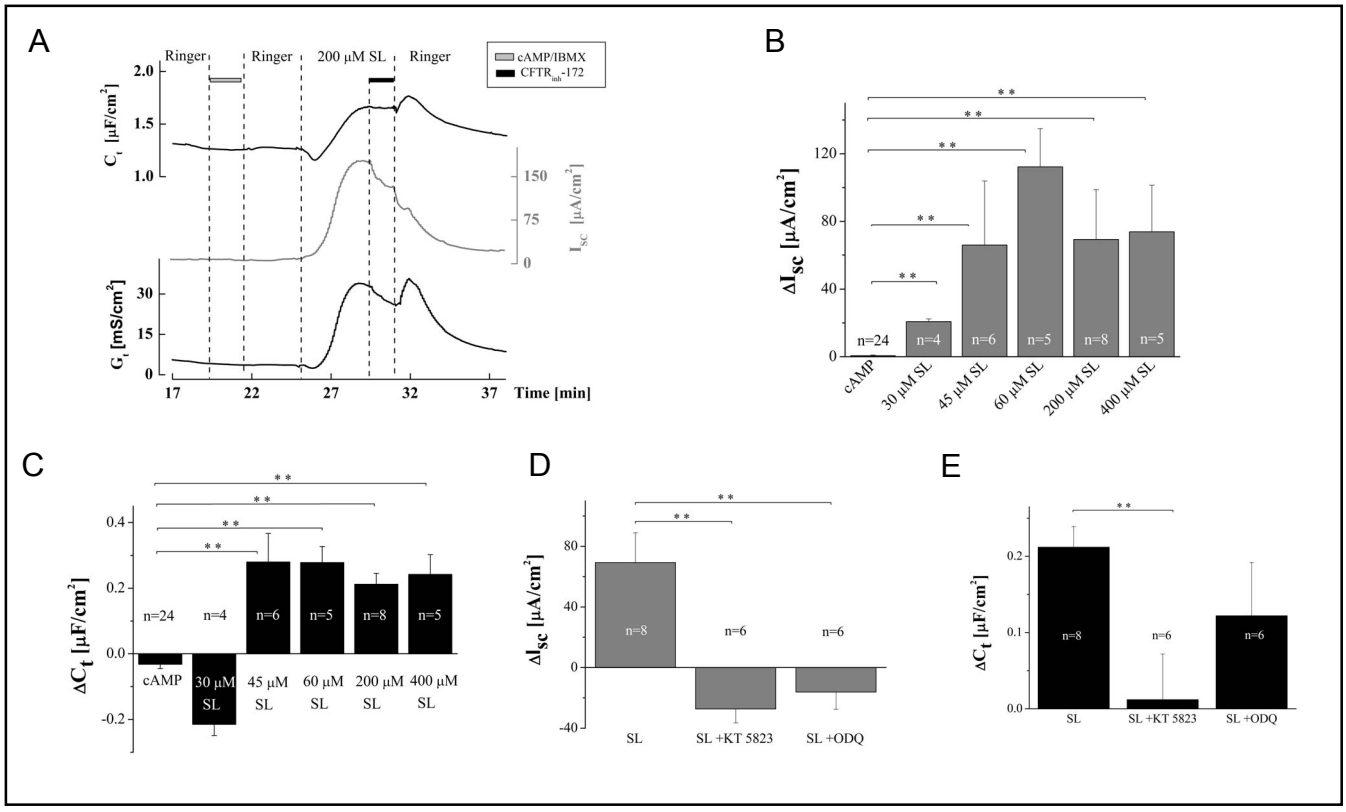

Fig. 5. Effects of sildenafil (SL), ODQ, and KT 5823 on transepithelial transport in CFBE41o$^-$ cells. A: Time course of a typical Ussing chamber measurement. Shown are I_{SC}, G_t and C_t. Application of cAMP had no effect, whereas sildenafil (200 µmol l^{-1}) led to significant increases in I_{SC}, G_t and C_t, which could be inhibited partially by $CFTR_{inh}$-172 (10 µmol l^{-1}). B + C: Averaged changes in I_{SC} and C_t after application of cAMP or different concentrations of sildenafil. In these figures mol l^{-1} is abbreviated by M. D + E: Influences of ODQ or KT 5823. Both inhibitors blocked the sildenafil-stimulated I_{SC} completely (D), while they had different effects on C_t (E).

Table 1. Initial electrophysiological parameters of CFBE41o$^-$ and 16HBE 14o$^-$ cells in presence and absence of ODQ or KT 5823.

Parameter	CFBE 41o$^-$	CFBE 41o$^-$ +ODQ	CFBE 41o$^-$ +KT 5823
I_{sc} [µA/cm²]	5.2 ± 1.1	3.1 ± 2.3	10.4 ± 1.7
R_t [Ω*cm²]	332.6 ± 17.4	407.3 ± 66.4	311.5 ± 27.8
C_t [µF/cm²]	1.22 ± 0.02	2.87 ± 0.55	3.34 ± 0.3
N	27	6	6

Parameter	16HBE14o$^-$	16HBE14o$^-$ +ODQ	16HBE14o$^-$ +KT 5823
I_{sc} [µA/cm²]	9.7 ± 3.1	4.2 ± 1.2	3.9 ± 3.1
R_t [Ω*cm²]	389.3 ± 43.3	351.3 ± 50.8	427.4 ± 135.9
C_t [µF/cm²]	1.8 ± 0.06	1.5 ± 0.1	1.36 ± 0.05
N	20	5	6

a cGMP-dependent activation of wt-CFTR without any influence of the second messenger on exocytotic events.

In a second step we inhibited some components of the cGMP pathway selectively to further enlighten the molecular mechanisms of sildenafil effects. The wt-CFTR expressing oocytes were incubated with the respective blocker 30 min before sildenafil injection. VC experiments were started 2 hours after sildenafil injection. During the application of the cAMP cocktail the respective blocker was also added. cAMP-mediated changes in I_m, G_m and

C_m were determined in presence and absence of ODQ or KT 5823, respectively.

The cGMP-producing guanylate cyclase was inhibited by the blocker ODQ (50 µmol l^{-1}), while the cGMP-activated PKG was blocked by KT 5823 (3 µmol l^{-1}). ODQ led to a significant decrease of the cAMP-mediated I_m after sildenafil injection (45 ± 8 %). Moreover, incubation with KT5823 reduced the SL-mediated increase in I_m by 50 ± 7% (Fig. 4A). Similarly, sildenafil-mediated increases in G_m were inhibited by ODQ (63 ± 6%) and KT5823 (57 ± 7%; data not shown). The increase in C_m after sildenafil injection was not affected by ODQ. KT 5823 produced similar effects (Fig. 4B).

Part 2: *Effects of sildenafil on wt-CFTR/ΔF508-CFTR in human bronchial epithelial cells*

Sildenafil effects on ΔF508-CFTR. After having studied the basic effects of sildenafil on wt-CFTR/ΔF508-CFTR in the oocyte expression system we turned towards human cells and used confluent monolayers of the human bronchial epithelial cell lines 16HBE14o$^-$ (wt-CFTR) and CFBE41o$^-$ (ΔF508-CFTR$^{+/+}$) to measure transepithelial short circuit current (I_{SC}), conductance (G_t) and capacitance (C_t) in modified Ussing chambers. The initial values of the 16HBE14o$^-$ and CFBE41o$^-$ monolayers are summarized in Table 1.

Due to the ΔF508 mutation of CFTR in CFBE41o$^-$ cells, application of cAMP did not significantly change the bioelectrical parameters of the monolayers (Figs. 5A, B). Subsequent application of sildenafil produced a considerable dose-dependent increase in I_{SC}, G_t, and C_t. This activating effect starts at a concentration of 30 µmol l^{-1} sildenafil with maximal responses at a concentration of 60 µmol l^{-1} sildenafil (Figs. 5 A-C). I_{SC} at 60 µmol l^{-1} sildenafil increased by 112.2 ± 22.7 µA/cm^2 (n = 5) and cell surface area rose remarkably by 22.7 ± 4 % measured as increases in C_t. Higher sildenafil concentrations did not produce further ΔF508-CFTR activation. Sildenafil-mediated I_{SC} and G_t increases could be partially inhibited by the CFTR-specific inhibitor $CFTR_{inh}$-172 (10 µmol l^{-1}; Fig. 5A). A comparable $CFTR_{inh}$-172 inhibition (~50 %) of ΔF508-CFTR mediated current after corr-4a treatment was reported by Jurkuvenaite et al. with far higher inhibitor concentrations (100 µmol l^{-1}) [18]. From these data we conclude that sildenafil was able to stimulate ΔF508-CFTR activation in CFBE41o$^-$ cells.

In another set of experiments the cells were incubated for 15 min before and during the measurements

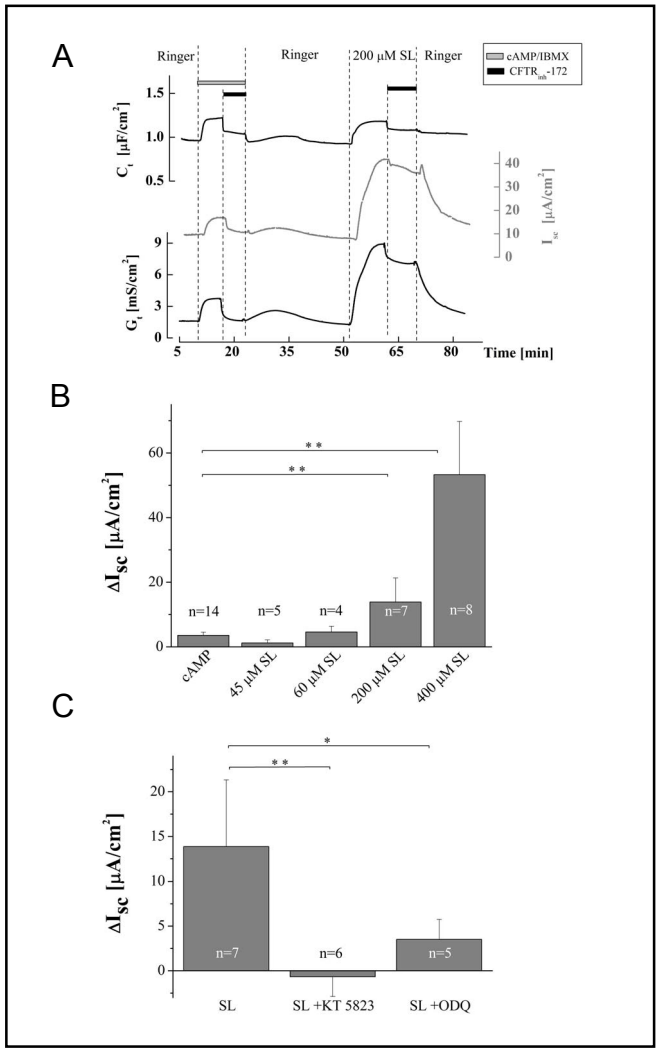

Fig. 6. Effects of sildenafil (SL) on wt-CFTR in 16HBE14o$^-$ cells. A: Typical measurement of a 16HBE14o$^-$ monolayer. cAMP increased I_{SC}, G_t and C_t, which could be inhibited by $CFTR_{inh}$-172 (10 µmol l^{-1}). Addition of sildenafil (200 µmol l^{-1}) led to a significantly higher I_{SC}, G_t and C_t increase than cAMP. B: Dose response relationship of sildenafil. In these figures mol l^{-1} is abbreviated by M. C: ODQ and KT 5823 inhibited the sildenafil induced I_{SC} increase.

with the cGMP pathway inhibitors ODQ or KT5823 in presence of sildenafil. Inhibition of different steps in the cGMP pathway with ODQ or KT 5823 prevented the sildenafil induced activation of ΔF508-CFTR mediated changes in I_{SC} completely, indicating a cGMP dependence of the sildenafil effect in CFBE41o$^-$ cells (Fig. 5D). Interestingly, KT5823 inhibited exocytotic delivery of ΔF508-CFTR carrying vesicles to the apical membranes of the cells, while ODQ had nearly no influence on exocytosis (Fig. 5E).

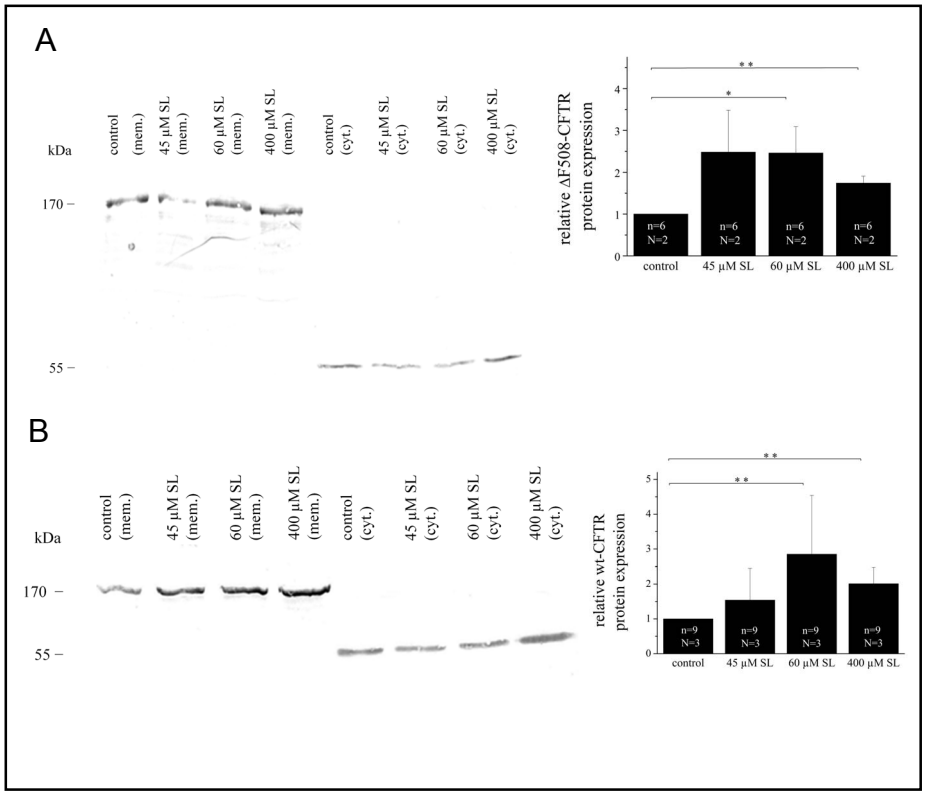

Fig. 7. Western blot analyses of CFBE41o$^-$ and 16HBE14o$^-$ cells with and without sildenafil (SL). Isolated membrane (mem.; 40 µg) and cytosolic proteins (cyt.; 10 µg) were separated in a 7.5% SDS-PAGE. ΔF508-CFTR and wt-CFTR were identified by a specific CFTR antibody, which detected one specific band in the range of 170 kDa in the membrane protein fraction. As a loading control a α-tubulin-specific band was determined in the cytoplasmatic protein samples. In the labeling of the blots mol l^{-1} is abbreviated by M. Densitometric quantification of the protein amount of the non-sildenafil-incubated cells (control) was set to a value of 1.0, whereas the protein amount of the other samples was normalized to the control. The representative images as well as the statistic evaluation showed an enhanced protein amount in CFBE41o$^-$ (A; n = 6, N = 2) and in 16HBE14o$^-$ cells (B, n = 9, N = 3) after incubation with various sildenafil concentrations.

Sildenafil effects on wt-CFTR. Prompted by the effects of sildenafil on wt-CFTR in the *Xenopus laevis* oocytes we were interested in possible effects of sildenafil on wt-CFTR and performed the same procedures with 16HBE14o$^-$ cells. The initial values of I_{SC}, G_t and C_t of these cells are given in Table 1. Since these cells express wt-CFTR, cAMP application increased all parameters significantly, which could be partly inhibited by $CFTR_{inh}$-172 (Fig. 6A). Addition of sildenafil (200 µmol l^{-1}) led to a higher activation of wt-CFTR currents and conductances than cAMP (Fig. 6A). Interestingly, 45 µmol l^{-1} sildenafil had no effect on wt-CFTR and maximal activation was only achieved with 400 µmol l^{-1} sildenafil arguing for different modes of sildenafil activation of ΔF508-CFTR and wt-CFTR. Figure 6B shows the dose response relationship of sildenafil-induced increases in I_{SC}. These sildenafil-induced increases in I_{SC} could be inhibited by ODQ and KT 5823 (Fig. 6C).

Western blot analyses. To analyze the effect and mechanism of sildenafil on ΔF508-CFTR and wt-CFTR on the protein level, we performed semi quantitative Western blot analyses with a specific CFTR antibody. CFBE41o$^-$ and 16HBE14o$^-$ cells, respectively, were incubated with different sildenafil concentrations (45 µmol l^{-1}, 60 µmol l^{-1}, 400 µmol l^{-1}) prior to isolation of membrane and cytosolic proteins. This allowed to access whether sildenafil stimulates exocytosis of ΔF508-CFTR and wt-CFTR proteins. In the cytosolic protein samples a α-tubulin specific band was determined to normalize the variations in the membrane protein fraction. The densitometrically detected protein amount of the non-sildenafil-incubated sample (control) was set to a value of 1.0. Incubation of CFBE41o$^-$ cells with 60 µmol l^{-1} sildenafil increased the ΔF508-CFTR protein amount 2.5-fold while 400 µmol l^{-1} evoked a 1.7-fold increase compared to the controls (Fig. 7A).

The samples of the 16HBE14o$^-$ cells showed nearly the same results: Incubation with 60 µmol l^{-1} sildenafil increased the wt-CFTR protein amount 2.9-fold and incubation with 400 µmol l^{-1} sildenafil 2-fold (Fig. 7B). The sildenafil (60 µmol l^{-1}) induced increase in ΔF508-CFTR protein amount in the CFBE41o$^-$ cells was 264% (n = 3) larger than the wt-CFTR protein amount of the non-sildenafil-incubated 16HBE14o$^-$ cells. Incubation of CFBE41o$^-$ cells with 400 µM sildenafil brought the ΔF508-CFTR protein amount to a value of 88% of the wt-CFTR protein amount in the 16HBE14o$^-$ control cells (n=3; data not shown).

We also analyzed the impact of ODQ on the CFTR correcting effect mediated by sildenafil. CFBE41o$^-$ and

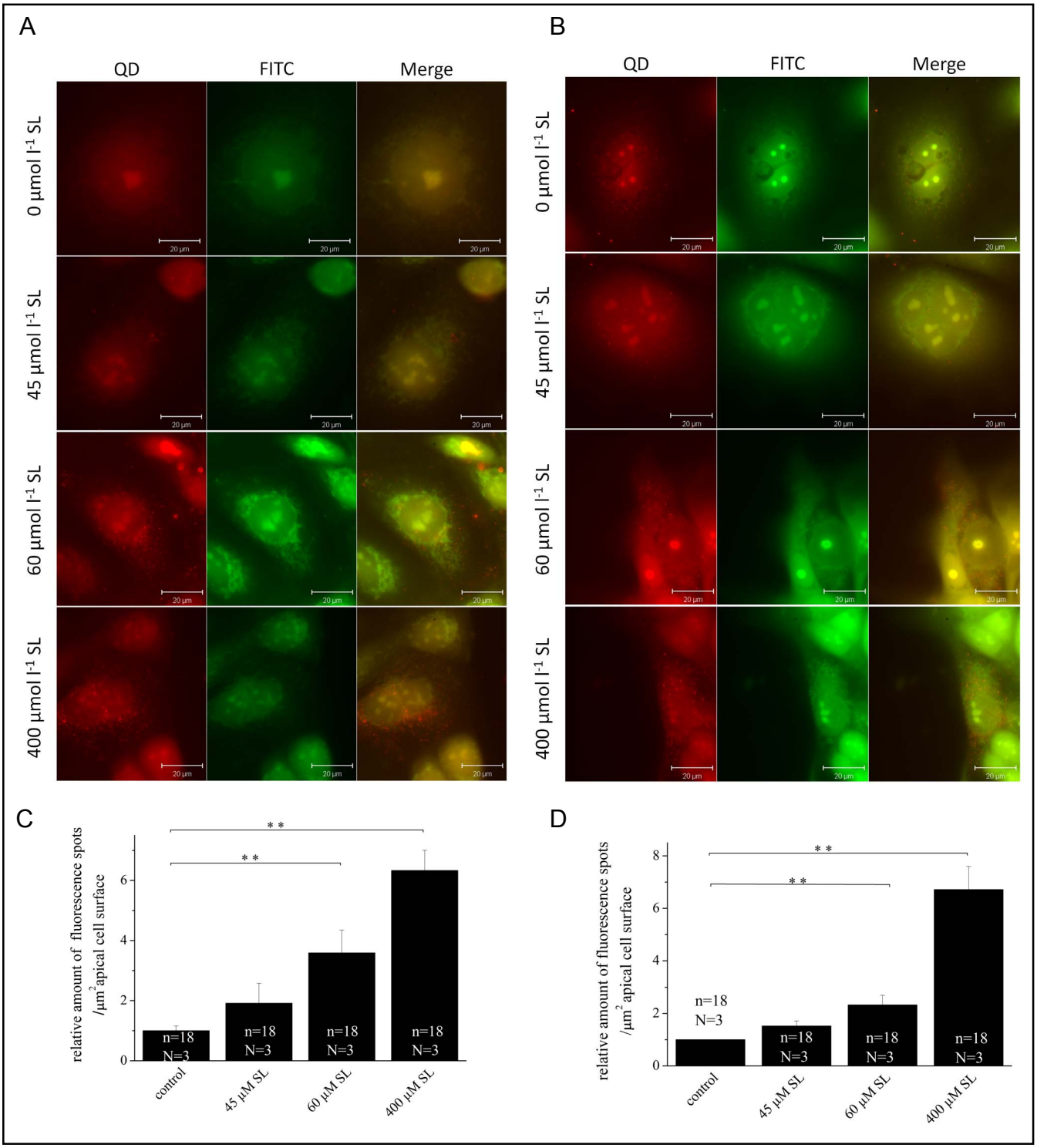

Fig. 8. Quantification of fluorescence spots at the apical surface of CFBE41o- and 16HBE14o- cells. CFBE41o- (A) and 16HBE14o- cells (B) were seeded on glass cover slips. ΔF508-CFTR/wt-CFTR molecules were detected using a CFTR-specific antibody and a QD-labeled anti-mouse F(ab´)2 conjugated secondary antibody, represented in the QD images. After incubation with 45 µmol l^{-1}, 60 µmol l^{-1} or 400 µmol l^{-1}sildenafil (SL) the cells were fixed with 0.05 % glutaraldehyde, which caused the background staining, demonstrated in the FITC channel. Scale bar corresponds to 20 µm.The merge panels represent the overlay of the QD and FITC channels. The average amount of fluorescence spots in the merge panel, representing ΔF508-CFTR and wt-CFTR molecules in the non-sildenafil-incubated cells, was normalized to 1.0, 200 µmol l^{-1} or 400 µmol l^{-1}. Sildenafil led to a significantly increased amount of fluorescence spots in the CFBE41o- cells (ΔF508-CFTR; C) and in the 16HBE14o- cells (wt-CFTR; D), respectively. In the figures C and D mol l^{-1} is abbreviated by M.

16HBE14o$^-$ cells were incubated with 60 µmol l^{-1} sildenafil and ODQ (10 µmol l^{-1}). We found no significant suppression of the sildenafil effect by ODQ in CFBE41o$^-$ or in 16HBE14o$^-$ cells on the protein level: ODQ did not change the CFTR protein amount neither in CFBE41o$^-$ cells nor in 16HBE14o$^-$ cells (data not shown). This is a hint of a cGMP-independent exocytosis mediated by sildenafil that could also be observed in C_m measurements.

Immunofluorescence experiments. We used immunofluorescence experiments for the detection and quantification of the sildenafil effect on ΔF508-CFTR and wt-CFTR molecules at the apical membrane. Therefore, CFBE41o$^-$ and 16HBE14o$^-$ cells were seeded on glass cover slips and afterwards incubated for 2 hours with different sildenafil concentrations or with sildenafil (60 µmol l^{-1}) and KT 5823 (3 µmol l^{-1}). To localize the CFTR molecules QD-labeled anti-mouse F(ab´)2 conjugates were used as secondary antibodies. Due to the glutaraldehyde fixation (FITC channel), which does not permeabilize the cell membrane [39], and the binding of the used antibody at the first extracellular domain of CFTR, the fluorescence spots represent quantum dot (QD) labeled wt-CFTR (16HBE14o$^-$) or ΔF508-CFTR molecules (CFBE41o$^-$) at the cell surface (Fig. 8 A,B; QD channel). The specific amount of CFTR molecules per µm^2 at the apical cell surface could not be determined because of the resolution limitations of the fluorescence microscopy, where two quantum dots labeled CFTR molecules in a distance closer than 200 nm would be detected as one fluorescence spot. For this reason Figure 8 (C;D) demonstrates relative amounts of fluorescence spots per µm^2 cell surface indicating relative ratios of cell surface located wt-CFTR/ΔF508-CFTR between untreated and sildenafil-treated cells (16HBE14o$^-$/CFBE41o$^-$). For comparison the fluorescence spots amount of the non-sildenafil-treated samples were set to 1.0 (control) and the amount of fluorescence spots of the sildenafil-treated samples are expressed in relation to the control. We detected an elevated amount of fluorescence spots at the apical cell surface of the CFBE41o$^-$ cells (ΔF508-CFTR) after incubation with sildenafil, indicating an exocytotic stimulation of ΔF508-CFTR insertion into the plasma membrane. Incubation with 60 µmol l^{-1} sildenafil led to a 3.6-fold higher amount of fluorescence spots, whereas 400 µmol l^{-1} sildenafil increased the fluorescence spots amount by 6.3-fold (Fig. 8C). Appropriate experiments with the wt-CFTR cell line 16HBE14o$^-$ also demonstrated a significant 2.3-fold higher amount of fluorescence spots after incubation with 60 µmol l^{-1} sildenafil and a 6.7-fold higher amount after application of 400 µmol l^{-1} sildenafil (Fig. 8D).

Interestingly, 60 µmol l^{-1} sildenafil led to ~30% more fluorescence spots at the apical membranes of CFBE41o$^-$ cells in comparison to non-treated control cells (16HBE14o$^-$). 400 µmol l^{-1} sildenafil enhanced the amount of fluorescence spots even by 300%. In this set of experiments application of ODQ or KT 5823 had no significant effects on the amount of sildenafil-mediated fluorescence spots in the CFBE41o$^-$ or 16HBE14o$^-$ cells.

Discussion

In the present study we investigated the effects and mechanism of action of the PDE-5 inhibitor sildenafil on ΔF508-CFTR and wt-CFTR using two model systems: *X. laevis* oocytes and human bronchial epithelial cells. Oocytes heterologously expressing human ΔF508-CFTR and wt-CFTR yield quick results, while human cell lines produce more clinical relevant data. We used both model systems for continuous and simultaneous measurements of ΔF508-CFTR or wt-CFTR current, conductance and membrane capacitance, the latter being a direct measurement of cell surface area. This technique allows us to follow exo- and endocytotic processes in real time.

Until now the modes of sildenafil action on wt-CFTR/ΔF508-CFTR are still matter of ongoing discussions. While Dormer et al. [1] classified sildenafil as a CFTR corrector using isolated epithelial cells from CF patients, other groups regarded the compound as a potentiator of CFTR in ΔF508-CFTR $^{(+/+)}$ mice [2]. A corrector is thought to restore the impaired trafficking to the apical membranes, while a potentiator should boost the reduced Cl^- conductance by the mutated CFTR. Direct effects of sildenafil on the CFTR molecule are also discussed [1, 2]. We made use of our model systems to investigate the molecular pathways of sildenafil.

In the first part of the project we used *X. laevis* oocytes for the expression of ΔF508-CFTR or wt-CFTR. An important prerequisite to the use of the *X. laevis* oocytes for the evaluation of the PDE-5 inhibitor sildenafil effects was the presence of an endogenous PDE-5. Since we found no information on PDE-5 of the oocytes in the literature we set out to verify the endogenous expression of the PDE-5 in these cells. By isolation of oocyte total RNA, cloning and sequencing we were indeed able to prove the endogenous expression of the PDE-5 in

X. laevis oocytes. Therefore, the oocytes are well suited to study the effects of sildenafil via inhibition of the endogenous PDE-5 and the effects on wt-CFTR or ΔF508-CFTR.

We expressed human ΔF508-CFTR in *X. laevis* oocytes and observed strongly enhanced cAMP mediated ΔF508-CFTR current and conductance after sildenafil treatment. Sildenafil led in average to a 13-fold increase in the cAMP-induced ΔF508-CFTR current, indicating the restoring potency of the compound. Without sildenafil treatment no cAMP response could be measured. Since the capacitance of the oocyte membrane showed only tiny changes, sildenafil obviously restored the defective ΔF508-CFTR Cl^- function without additional exocytotic delivery of ΔF508-CFTR containing vesicles in contrast to the observations for wt-CFTR earlier [38].

To now only a few groups described the effects of sildenafil on wt-CFTR and the data is contradictory: Dormer et al. [1] observed no influence of sildenafil on the wt-CFTR, while Cobb et al. [42] found an activation of wt-CFTR by the PDE-5 blocker. Therefore, in a next set of experiments we expressed human wt-CFTR in the oocytes and treated them with sildenafil. We saw an approximate triplication of cAMP-induced CFTR current. To our surprise and in contrast to ΔF508-CFTR, the cAMP-mediated membrane capacitance increase also tripled. From these data we conclude that sildenafil activated wt-CFTR already present in the oocyte membrane and additionally initiated exocytotic delivery to and insertion of CFTR carrying vesicles into the oocyte membrane. This exocytotic insertion of CFTR molecules into the plasma membrane is dependent on protein kinase A (PKA) [38] and the PKA-specific inhibitor H8 removed completely the sildenafil-induced enhancement of cAMP-stimulated capacitance increase, demonstrating that sildenafil has an enormous influence on the exocytosis of wt-CFTR, but unfortunately not of ΔF508-CFTR. Therefore, in the oocytes sildenafil acts on wt-CFTR via two different pathways, i.e. direct activation and exocytosis, whereas ΔF508-CFTR function is solely restored by activation without exocytotic delivery from intracellular vesicle pools to the oocyte membrane.

To further evaluate the basic mechanisms of sildenafil action we substituted cAMP with cGMP in our activating cocktail. cGMP increased wt-CFTR mediated current and conductance, yet had no effect on the membrane capacitance. Obviously, cGMP has no influence on exocytotic events in the oocytes, an observation that could also explain why sildenafil does not stimulate exocytosis of ΔF508-CFTR.

In the second part of the project we used the human bronchial epithelial cell lines CFBE41o$^-$ and 16HBE14o$^-$ to investigate the modes of action of sildenafil. The CFBE41o$^-$ cell line was generated from bronchi of an one year old CF patient who was homozygous for the ΔF508 mutation (ΔF508-CFTR$^{(+/+)}$) [43, 44]. The 16HBE14o$^-$ cells are widely used as controls and were generated from human bronchial non-CF tissue [43, 44]. Both cell lines serve since 2004 as model systems for the investigation of the molecular mechanisms that cause CF and are of special value in the search for therapeutically active compounds [45, 46]. Furthermore, these cells are particularly well suited for transepithelial electrophysiological measurements since they form tight confluent monolayers with a considerable transepithelial resistance that was in this study around 350 Ω*cm^2; a value that is in good accordance with those reported in the literature [44].

For the analysis of the effects of sildenafil on the ΔF508-CFTR function we performed Ussing chamber experiments with CFBE41o$^-$ cells. Application of the cAMP cocktail had no measurable effect on I_{SC}, G_t and C_t of the CFBE41o$^-$ monolayers. Yet, the application of sildenafil in various concentrations led to intense increases in I_{SC}, G_t and C_t, resulting in a more or less bell shaped curve, with the strongest effect at a sildenafil concentration of 60 µmol l^{-1}. Lower and higher concentrations had lesser effects on I_{SC} and G_t, while all concentrations of sildenafil from 45 µmol l^{-1} to 400 µmol l^{-1} produced similar increases in C_t. Parts of the increases in I_{SC} and G_t could be blocked by the CFTR specific inhibitor $CFTR_{inh}$-172. From these results and from those of the ΔF508-CFTR expressing oocytes we conclude that sildenafil enhanced the remaining ΔF508-CFTR chloride conductance, which confirms the classification of sildenafil as a potentiator of CFTR [2].

Moreover, in the bronchial cells sildenafil stimulates additional delivery of ΔF508-CFTR molecules to the apical membranes of the CFBE41o$^-$ cells, indicating a potentially auxiliary impact of sildenafil in the treatment of CF symptoms. To confirm and extend the observation that sildenafil induced exocytotic delivery of ΔF508-CFTR molecules we used Western blot and immunofluorescence approaches. In the Western blots we observed a 2.5-fold increase in the amount of ΔF508-CFTR protein in the membranes of CFBE41o$^-$ cells after sildenafil application (60 µmol l^{-1}). This was further confirmed in the immunofluorescence experiments that showed a 3.6-fold higher amount of fluorescence spots representing ΔF508-

CFTR molecules at the apical membranes following incubation with 60 µmol l^{-1} sildenafil and a 6.3-fold higher amount of fluorescence spots with 400 µmol l^{-1} sildenafil. These results revealed that sildenafil corrects the trafficking defect of the ΔF508-CFTR protein, leading to an exocytotic insertion of the protein into the apical plasma membrane. This mechanism represents an effect of sildenafil as a CFTR corrector as also demonstrated earlier in nasal epithelial cells isolated from CF patients [1]. The enormous exocytotic potency of sildenafil is further demonstrated by our observation that the amount of ΔF508-CFTR protein in the membranes of CFBE41o$^-$ cells following sildenafil exposure is nearly three times higher than the numbers of wt-CFTR copies in the 16HBE14o$^-$control cells. This is a highly encouraging aspect of the sildenafil action since elevating the amount of functional CFTR in CF epithelia by only 10 - 15% is sufficient to restore proper epithelial function [13, 14]. From this point of view sildenafil could be a very promising candidate for the treatment of CF patients.

Using two blocker that interfere with the cGMP pathway on different levels, i.e. ODQ and KT 5823, we identified two modes of action of sildenafil: one pathway that is independent of cGMP and one that depends on cGMP. In the first mode of action sildenafil inhibits the PDE-5 and prevents the degradation of cGMP. Increasing cellular cGMP levels activate PKG followed by ΔF508-CFTR phosphorylation, thereby correcting the defective protein function. In the case of the cGMP-independent mode of action, sildenafil obviously activates the exocytotic delivery of CFTR (wt-CFTR or ΔF508-CFTR) molecules to and insertion into the plasma membrane, thereby drastically increasing the number of CFTR molecules in the membrane.

Taken together, our results from both model systems show that the PDE-5 inhibitor sildenafil restores the function of ΔF508-CFTR and simultaneously induces the insertion of the ΔF508-CFTR molecules into the apical plasma membrane. Thus, sildenafil acts as a CFTR corrector as well as a potentiator of CFTR. This combination of a corrector and a potentiator should achieve the maximum therapeutic benefits in CF as stated recently [18, 47]. However, and that's the real sad message of our study, the concentrations of sildenafil that are necessary to induce the beneficial effects exceed by far those used for the treatment of erectile dysfunction. 60 µmol l^{-1} sildenafil yielded the best effect in our studies, yet this concentration is roughly 120 times larger than the commonly used clinical concentration of 0.5 µmol l^{-1} for the treatment of erectile dysfunction [32]. It is to be feared that this high concentration of sildenafil could have unwanted severe adverse effects in patients, such as headache, flushing, nasal congestion, dyspepsia, priapism and sudden hearing loss [48]. Rare but serious adverse effects could be severe hypotension, myocardial infarction and ventricular arrhythmias [49]. However, on the other hand some other positive effects of sildenafil have been described. It could be shown that sildenafil mediates a reduced bacterial adhesion of *P. aeruginosa* in the mouse respiratory tract as well as an anti-inflammatory effect in human bronchial epithelial cells and in guinea pigs [29, 50]. Because the respiratory CF symptoms are highly influenced by the colonisation with *P. aeruginosa* and *S. aureus*, this additional effect of sildenafil is another hint that the PDE-5 inhibitor might have a high importance in the therapy of CF. Moreover, sildenafil is reported to inhibit Na^+ absorption through epithelial Na^+ channels (ENaC) [29]. This could be of therapeutic benefit since CF patients suffer from Na^+ hyperabsorption in the respiratory tract leading to massive loss of water in the airway surface liquid and increased viscosity of the mucus [51]. Furthermore, there are attempts underway to investigate chemical modifications of sildenafil to enhance the biochemical potency and selectivity of the compound, thereby offering the possibility to apply the newly developed drug in lower concentrations with fewer putative adverse effects [52]. Since the most severe manifestations in CF are found in the lower airways sildenafil could be administered by aerosol inhalation, which could lead to effective drug concentration in the bronchial epithelia as has been shown in mice [31].

Conclusions

Our results strongly provide evidence that sildenafil recovers the Cl^- channel defect in CF acting as a ΔF508-CFTR potentiator and corrector via two mechanisms: one being cGMP-dependent (potentiator) and one being cGMP-independent (corrector). From these data we conclude that the development of PDE-5 inhibitors more selective and more sensitive than sildenafil could be of therapeutic importance for the treatment of CF symptoms until we are eventually able to cure the disease by repairing the underlying basic molecular defects.

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References

1 Dormer RL, Harris CM, Clark Z, Pereira MM, Doull IJ, Norez C, Becq F, McPherson MA: Sildenafil (Viagra) corrects DeltaF508-CFTR location in nasal epithelial cells from patients with cystic fibrosis. Thorax 2005;60:55-59.

2 Lubamba B, Lecourt H, Lebacq J, Lebecque P, de Jonge HR, Wallemacq P, Leal T: Preclinical evidence that sildenafil and vardenafil activate chloride transport in cystic fibrosis. Am J Respir Crit Care Med 2008;177:506-515.

3 Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL: Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989;245:1066-1073.

4 Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MJ: Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. Science 1991;253:202-205.

5 Pilewski JM, Frizzell RA: Role of CFTR in airway disease. Physiol Rev 1999;79:S215-S255.

6 Ostedgaard LS, Baldursson O, Welsh MJ: Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- channel by its R domain. J Biol Chem 2001;276:7689-7692.

7 Berger HA, Travis SM, Welsh MJ: Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- channel by specific protein kinases and protein phosphatases. J Biol Chem 1993;268:2037-2047.

8 French PJ, Bijman J, Edixhoven M, Vaandrager AB, Scholte BJ, Lohmann SM, Nairn AC, de Jonge HR: Isotype-specific activation of cystic fibrosis transmembrane conductance regulator-chloride channels by cGMP-dependent protein kinase II. J Biol Chem 1995;270:26626-26631.

9 Vaandrager AB, Tilly BC, Smolenski A, Schneider-Rasp S, Bot AG, Edixhoven M, Scholte BJ, Jarchau T, Walter U, Lohmann SM, Poller WC, de Jonge HR: cGMP stimulation of cystic fibrosis transmembrane conductance regulator Cl^- channels co-expressed with cGMP-dependent protein kinase type II but not type Ibeta. J Biol Chem 1997;272:4195-4200.

10 Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE: Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 1990;63:827-834.

11 Ward CL, Kopito RR: Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. J Biol Chem 1994;269:25710-25718.

12 Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE, Welsh MJ: Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature 1992;358:761-764.

13 Farmen SL, Karp PH, Ng P, Palmer DJ, Koehler DR, Hu J, Beaudet AL, Zabner J, Welsh MJ: Gene transfer of CFTR to airway epithelia: low levels of expression are sufficient to correct Cl^- transport and overexpression can generate basolateral CFTR. Am J Physiol Lung Cell Mol Physiol 2005;289:L1123-L1130.

14 Johnson LG, Olsen JC, Sarkadi B, Moore KL, Swanstrom R, Boucher RC: Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis. Nat Genet 1992;2:21-25.

15 Schwiebert EM, Benos DJ, Egan ME, Stutts MJ, Guggino WB: CFTR is a conductance regulator as well as a chloride channel. Physiol Rev 1999;79:S145-S166.

16 Kunzelmann K, Schreiber R: CFTR, a regulator of channels. J Membr Biol 1999;168:1-8.

17 Riordan JR: CFTR function and prospects for therapy. Annu Rev Biochem 2008;77:701-726.

18 Jurkuvenaite A, Chen L, Bartoszewski R, Goldstein R, Bebok Z, Matalon S, Collawn JF: Functional stability of rescued delta F508 cystic fibrosis transmembrane conductance regulator in airway epithelial cells. Am J Respir Cell Mol Biol 2010;42:363-372.

19 Van Goor F, Straley KS, Cao D, Gonzalez J, Hadida S, Hazlewood A, Joubran J, Knapp T, Makings LR, Miller M, Neuberger T, Olson E, Panchenko V, Rader J, Singh A, Stack JH, Tung R, Grootenhuis PD, Negulescu P: Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. Am J Physiol Lung Cell Mol Physiol 2006;290:L1117-L1130.

20 Illek B, Fischer H: Flavonoids stimulate Cl conductance of human airway epithelium *in vitro* and *in vivo*. Am J Physiol 1998;275:L902-L910.

21 Antoniu SA: Cystic fibrosis transmembrane regulator potentiators as promising cystic fibrosis therapies. Expert Opin Investig Drugs 2011;20:423-425.

22 Pedemonte N, Lukacs GL, Du K, Caci E, Zegarra-Moran O, Galietta LJ, Verkman AS: Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. J Clin Invest 2005;115:2564-2571.

23 Varga K, Goldstein RF, Jurkuvenaite A, Chen L, Matalon S, Sorscher EJ, Bebok Z, Collawn JF: Enhanced cell-surface stability of rescued DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) by pharmacological chaperones. Biochem J 2008;410:555-564.

24 Sampson HM, Robert R, Liao J, Matthes E, Carlile GW, Hanrahan JW, Thomas DY: Identification of a NBD1-binding pharmacological chaperone that corrects the trafficking defect of F508del-CFTR. Chem Biol 2011;18:231-242.

25 Wang Y, Loo TW, Bartlett MC, Clarke DM: Correctors promote maturation of cystic fibrosis transmembrane conductance regulator (CFTR)-processing mutants by binding to the protein. J Biol Chem 2007;282:33247-33251.

26 Loo TW, Bartlett MC, Clarke DM: Correctors promote folding of the CFTR in the endoplasmic reticulum. Biochem J 2008;413:29-36.

27 O'Grady SM, Jiang X, Maniak PJ, Birmachu W, Scribner LR, Bulbulian B, Gullikson GW: Cyclic AMP-dependent Cl^- secretion is regulated by multiple phosphodiesterase subtypes in human colonic epithelial cells. J Membr Biol 2002;185:137-144.

28 Corbin JD, Francis SH: Cyclic GMP phosphodiesterase-5: target of sildenafil. J Biol Chem 1999;274:13729-13732.

29 Poschet JF, Timmins GS, Taylor-Cousar JL, Ornatowski W, Fazio J, Perkett E, Wilson KR, Yu HD, de Jonge HR, Deretic V: Pharmacological modulation of cGMP levels by phosphodiesterase 5 inhibitors as a therapeutic strategy for treatment of respiratory pathology in cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 2007;293:L712-L719.

30 Carlile GW, Robert R, Zhang D, Teske KA, Luo Y, Hanrahan JW, Thomas DY: Correctors of protein trafficking defects identified by a novel high-throughput screening assay. Chembiochem 2007;8:1012-1020.

31 Lubamba B, Lebacq J, Reychler G, Marbaix E, Wallemacq P, Lebecque P, Leal T: Inhaled phosphodiesterase type 5 inhibitors restore chloride transport in cystic fibrosis mice. Eur Respir J 2011;37:72-78.

32 Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA: Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. N Engl J Med 1998;338:1397-1404.

33 Goldenberg MM: Safety and efficacy of sildenafil citrate in the treatment of male erectile dysfunction. Clin Ther 1998;20:1033-1048.

34 Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM: Effects of sildenafil on the relaxation of human corpus cavernosum tissue *in vitro* and on the activities of cyclic nucleotide phosphodiesterase isozymes. J Urol 1998;159:2164-2171.

35 Koesling D, Mullershausen F, Lange A, Friebe A, Mergia E, Wagner C, Russwurm M: Negative feedback in NO/cGMP signalling. Biochem Soc Trans 2005;33:1119-1122.

36 Jiang LH, Gawler DJ, Hodson N, Milligan CJ, Pearson HA, Porter V, Wray D: Regulation of cloned cardiac L-type calcium channels by cGMP-dependent protein kinase. J Biol Chem 2000;275:6135-6143.

37 Taglialatela M, Pannaccione A, Iossa S, Castaldo P, Annunziato L: Modulation of the K^+ channels encoded by the human ether-a-gogo-related gene-1 (hERG1) by nitric oxide. Mol Pharmacol 1999;56:1298-1308.

38 Weber WM, Cuppens H, Cassiman JJ, Clauss W, Van Driessche W: Capacitance measurements reveal different pathways for the activation of CFTR. Pflugers Arch 1999;438:561-569.

39 Kusche-Vihrog K, Sobczak K, Bangel N, Wilhelmi M, Nechyporuk-Zloy V, Schwab A, Schillers H, Oberleithner H: Aldosterone and amiloride alter ENaC abundance in vascular endothelium. Pflugers Arch 2008;455:849-857.

40 Kusche-Vihrog K, Segal A, Grygorczyk R, Bangel-Ruland N, Van Driessche W, Weber WM: Expression of ENaC and other transport proteins in *Xenopus* oocytes is modulated by intracellular Na^+. Cell Physiol Biochem 2009;23:9-24.

41 Kelly SM, Butler JP, Macklem PT: Control of cell volume in oocytes and eggs from *Xenopus laevis*. Comp Biochem Physiol A Physiol 1995;111:681-691.

42 Cobb BR, Fan L, Kovacs TE, Sorscher EJ, Clancy JP: Adenosine receptors and phosphodiesterase inhibitors stimulate Cl^- secretion in Calu-3 cells. Am J Respir Cell Mol Biol 2003;29:410-418.

43 Meng QH, Springall DR, Bishop AE, Morgan K, Evans TJ, Habib S, Gruenert DC, Gyi KM, Hodson ME, Yacoub MH, Polak JM: Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. J Pathol 1998;184:323-331.

44 Gruenert DC, Willems M, Cassiman JJ, Frizzell RA: Established cell lines used in cystic fibrosis research. J Cyst Fibros 2004;3 Suppl 2:191-196.

45 Dragomir A, Bjorstad J, Hjelte L, Roomans GM: Curcumin does not stimulate cAMP-mediated chloride transport in cystic fibrosis airway epithelial cells. Biochem Biophys Res Commun 2004;322:447-451.

46 Ehrhardt C, Collnot EM, Baldes C, Becker U, Laue M, Kim KJ, Lehr CM: Towards an *in vitro* model of cystic fibrosis small airway epithelium: characterisation of the human bronchial epithelial cell line CFBE41o-. Cell Tissue Res 2006;323:405-415.

47 Schmidt A, Hughes LK, Cai Z, Mendes F, Li H, Sheppard DN, Amaral MD: Prolonged treatment of cells with genistein modulates the expression and function of the cystic fibrosis transmembrane conductance regulator. Br J Pharmacol 2008;153:1311-1323.

48 Gresser U, Gleiter CH: Erectile dysfunction: comparison of efficacy and side effects of the PDE-5 inhibitors sildenafil, vardenafil and tadalafil-review of the literature. Eur J Med Res 2002;7:435-446.

49 McCullough AR: Four-year review of sildenafil citrate. Rev Urol 2002;4 Suppl 3:S26-S38.

50 Toward TJ, Smith N, Broadley KJ: Effect of phosphodiesterase-5 inhibitor, sildenafil (Viagra), in animal models of airways disease. Am J Respir Crit Care Med 2004;169:227-234.

51 Rückes-Nilges C, Weber U, Lindemann H, Munker G, Clauss W, Weber WM: Minor role of Cl^- secretion in non-cystic fibrosis and cystic fibrosis human nasal epithelium. Cell Physiol Biochem 1999;9:1-10.

52 Robert R, Carlile GW, Pavel C, Liu N, Anjos SM, Liao J, Luo Y, Zhang D, Thomas DY, Hanrahan JW: Structural analog of sildenafil identified as a novel corrector of the F508del-CFTR trafficking defect. Mol Pharmacol 2008;73:478-489.