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Chemical rescue of ΔF508-CFTR in C127 epithelial cells reverses aberrant extracellular pH acidification to wild-type alkalization as monitored by microphysiometry

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ABSTRACT

Cystic fibrosis (CF) is caused by mutations in the gene for CFTR, a cAMP-activated anion channel expressed in apical membranes of wet epithelia. Since CFTR is permeable to HCO_3^-, and may regulate bicarbonate exchangers, it is not surprising evidence of changes in extracellular pH (pHo) have been found in CF. Previously we have shown that tracking pHo can be used to differentiate cells expressing wild-type CFTR from controls in mouse mammary epithelial (C127) and fibroblast (NIH/3T3) cell lines. In this study we characterized forskolin-stimulated extracellular acidification rates in epithelia where chemical correction of mutant ΔF508-CFTR converted an aberrant response in acidification (10%+ increase) to wild-type (25%+ decrease). Thus treatment with corrector (10% glycerol) and the resulting increased expression of ΔF508-CFTR at the surface was detected by microphysiometry as a significant reversal from acidification to alkalization of pHo. These results suggest that CFTR activation as well as correction can be detected by carefully monitoring pHo and support findings in the field that extracellular pH acidification may impact the function of airway surface liquid in CF.

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is an apical membrane anion channel involved in salt transport and epithelial fluid secretion [1]. CFTR is a member of the ABC transporter family, requiring ATP to regulate the gating of ions such as chloride and bicarbonate [2]. Cystic fibrosis (CF) is a result of genetic mutation within the CFTR gene [3]. The most prevalent mutation of the CFTR gene is ΔF508, where the resulting protein is targeted and degraded by the ubiquitin–proteasome pathway before reaching the apical membrane [1]. The disease pathology is attributed primarily to impaired Cl^- conductance [4], however, manifestations of CF have been difficult to attribute solely to a defect in Cl^- conductance [5].

Because CFTR has been found to transport Cl^- and HCO_3^- [6] it may contribute to the regulation of pH within and without the cell [7]. As a result there have been a number of studies examining potential pH regulation associated with CFTR [7,8]. CFTR expression has been shown to impact intracellular pH in a pancreatic cell line [9] and transfected fibroblasts [10], yet perhaps the most applicable experiments examined extracellular pH changes [11] as the most important CF pathophysiology appears to arise from alterations in external fluids [12]. For example, increased acidity of secretions containing enzymes or mucins can lead to premature activation of enzymes, increased thickness of mucus, and consequently ductal blockage and irritation [5]. Smith et al. (1996) also reported that the primary cause of CF lung disease may reside in the extracellular milieu, the airway surface fluid (ASF). Nonfunctional CFTR is thought to be the regulating factor of acidification of ASL pH in the CF pig and neonates [13,14].

To examine CFTR's effect on extracellular pH (pHo) more closely, we used a highly sensitive pH biosensor that allows real-time measurement of changes in pHo and can assay (10^6 cells per channel) and detect subtle changes (~0.001 pH unit) in the rates at which cells acidify their environment [15]. Changes in extracellular acidification rates result from alterations in production of acidic products of metabolism (principally lactic acid, H^+ and CO_2) and the mechanisms regulating cellular pH (principally Na/H exchange, and HCO_3^- flux) [7].

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In an effort to address the underlying problems associated with CFTR mutations, chemical rescue using correctors and potentiators has been investigated [16,17]. Correctors are designed to amend folding and cellular processing of the CFTR protein and potentiators are designed to induce proper ion channel gating [18]. This report presents an attempt to finely tune the application of microphysiometry to determine effects of CFTR correction on pHo.

2. Materials and methods

2.1. Materials

Unless otherwise indicated, chemicals were obtained from Sigma (St. Louis, MO) and culture media from GIBCO BRL (Grand Island, NY). Supplies for the Cytosensor™ Microphysiometer were obtained from the manufacturer, Molecular Devices Corporation (Sunnyvale, CA).

2.2. Cell culture

NIH mouse fibroblast cell lines (NIH/3T3, 3T3/WT) were obtained from M.J. Welsh. They are untransfected parental cells (NIH/3T3) and a stable cell line cotransfected with a retroviral vector containing human CFTR cDNA and a neomycin resistance vector, pSV2neo (3T3/WT). C127 mouse mammary epithelial control cells, and cells stably expressing wild-type CFTR or mutant ΔF508-CFTR (BPV, 2WT2, 508 cells respectively) were obtained from Genzyme Corporation (Dr. Seng Cheng). They are stable cell lines transfected with the BPV vector (bovine papilloma-based vector with a neomycin resistance gene) alone (BPV cells), or the BPV vector with the cDNA for wild-type CFTR (2WT2 cells) or ΔF508 CFTR (508 cells). C127 cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.3. Isotopic flux studies

I-125 efflux (for Cl^-) was measured using complete sample replacement with 30-sec intervals. Efflux buffer was (in mM): 10 N-2-hydroxy ethylpiperazine-N′-2-ethane (HEPES; pH 7.4), 5.4 KCl, 1.8 $CaC1_2$, 1.0 NaH_2PO_4, 0.8 $MgSO_4$, and 1 mg/ml glucose at 22 °C. Protocol and efflux rate constants were followed as according to Venglarik et al. [19].

2.4. Immunoblot

Cells grown to confluency in a T-75 flask were scraped free in PBS, centrifuged at 1200 rpm for 10 min, resuspended in lysis buffer (5 mM Tris–HCL at pH 7.4 plus 5 mM EDTA with 1% Triton X-100), and held on ice with frequent vortexing for 30 min. Gels were blotted onto Hybond-ECL (nitrocellulose) membrane using a Genie Blotter (24 V for 2 h); the membrane was then removed and blocked with 5% nonfat dry milk in TBST (20 mM Tris-base, 137 mM NaCl, 0.05% Tween-20) for at least 1 h at 22 °C before incubating overnight at 4 °C with anti-CFTR monoclonal antibodies "a-1468" (1:10,000 dilution). "a-1468" antibody recognizes a cytoplasmic C-terminus region of CFTR. After rinsing, the membrane was incubated @ 22 °C for 1 h in HRP rabbit anti-mouse IgG diluted at 1:1000 in TBST-1% BSA, and then washed for at least 1 h in fresh TBST-1% BSA.

2.5. Glycerol experiments

BPV, 2WT2 and 508 cells were pre-incubated with 10% glycerol in DMEM media 24 h prior to the experiment as described by Zsembery et al. [20]. Three hours prior to the experiment media was diluted to 0% glycerol over the course of several dilutions. Forskolin stimulations were repeated in series during an experiment with one cell capsule to better evaluate the response. The data was normalized, averaged, and presented with statistical analysis.

2.6. Measurement of extracellular acidification

NIH/3T3 and C127 cell lines were seeded into 12-mm diameter disposable 3.0 μm porous polycarbonate cell capsules at 3×10^5 cells/well in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum as described previously [21]. To enhance the detection of subtle pH changes sensor chambers were perfused with nominally bicarbonate-free DMEM with a low buffering capacity (1.0 mM sodium phosphate and ~100 μM HCO_3^- from atmospheric CO_2). Media was also degassed/debubbled and warmed to 37 °C before perfusing the cells where pHo was continually monitored. Reservoirs of medium were directed to the sensor chambers by software driven valves and flow was set to 100 μl/min. Each cycle lasted 1–2 min and consisted of a perfusion phase and an interruption phase. During the interruption phase the pumps were inactivated and the rate of acidification within the chamber was calculated, recorded and plotted by the software. Flow during this phase was interrupted for an interval of 40–80 s. The flow was then resumed and the next cycle begun, with rates of acidification returning again to baseline.

2.7. Statistical analysis

Except where noted, data are reported as means ± standard error of the mean (S.E.M.). Statistical significance was assessed with two-tailed Student's *t*-test for samples with unequal variances as implemented in Excel (Microsoft Corporation) as appropriate. To clarify the origin of all data, in microphysiometry the notation "$n = x(y)$" is used where "x" is the traditional trial number. A single trial, "$n = 1$", represents data acquisition from a single capsule of cells in one experiment. The notation "y" is the total number of multiple replications performed on capsules of cells during experiments. Standard procedure is that multiple curves would be averaged to represent the response from a single capsule of cells. For example, if a single capsule is tested twice, this is represented as $n = 1(2)$. For all figures and statistical calculations one capsule was represented by one averaged measurement and statistical tests were done using the number of capsules as trial number.

3. Results

3.1. Confirmation of CFTR expression and anion current

Cell lines were first studied for expression of CFTR protein through Western blot analysis. Immunoblots densely stained a 170kD band in 3T3/WT cell lines transfected with wild-type CFTR but no band was detected in untransfected NIH/3T3 mouse fibroblasts (Fig. 1A inset). In addition, epithelial cell lines transfected with ΔF508-CFTR and wild-type CFTR (508 cells and 2WT2, respectively) expressed detectable levels of CFTR protein staining at appropriate molecular weights, while BPV cells transfected with vector alone expressed no CFTR protein (Fig. 1B inset).

The presence of functional CFTR was confirmed by cAMP-dependent activation of Cl^- channels in Iodide-125 efflux experiments. Fibroblast cells expressing wtCFTR (3T3/WT) showed a substantial increase in I-125 efflux compared to controls when treated with 10 μM forskolin (Fig. 1A). Similarly, epithelial cells expressing wild type CFTR (2WT2) showed robust increase in efflux while cells expressing mutant ΔF508-CFTR (508 cells) and no CFTR (BPV) show little change in response to a 1 μM forskolin treatment (Fig. 1B).

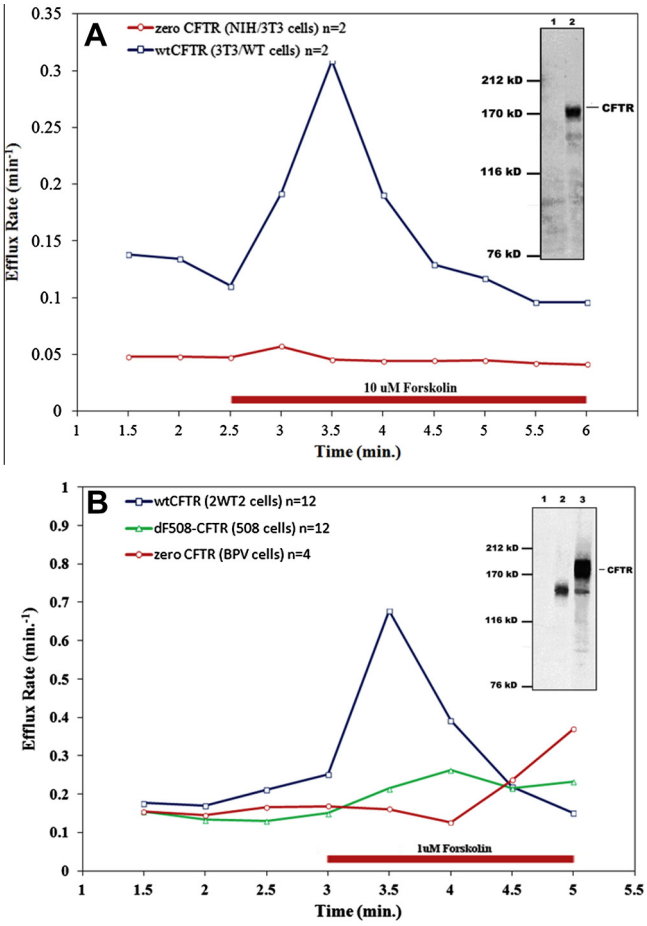

Fig. 1. I-125 efflux increased in cells expressing wild type CFTR in response to forskolin. (A) Mouse fibroblasts expressing wild type CFTR (3T3/WT, gel lane 2) show substantial increase in anion efflux when stimulated with 10 μM forskolin. Non-expressing CFTR cells (NIH/3T3, lane 1) show minimal changes in ion efflux rate. (B) Epithelia expressing wild type CFTR (2WT2, lane 3) also show substantial increase in iodide efflux in response to treatment with 1 μM forskolin. Cells expressing mutant (ΔF508-CFTR, lane 2) and those lacking CFTR (BPV, lane 1) show little change.

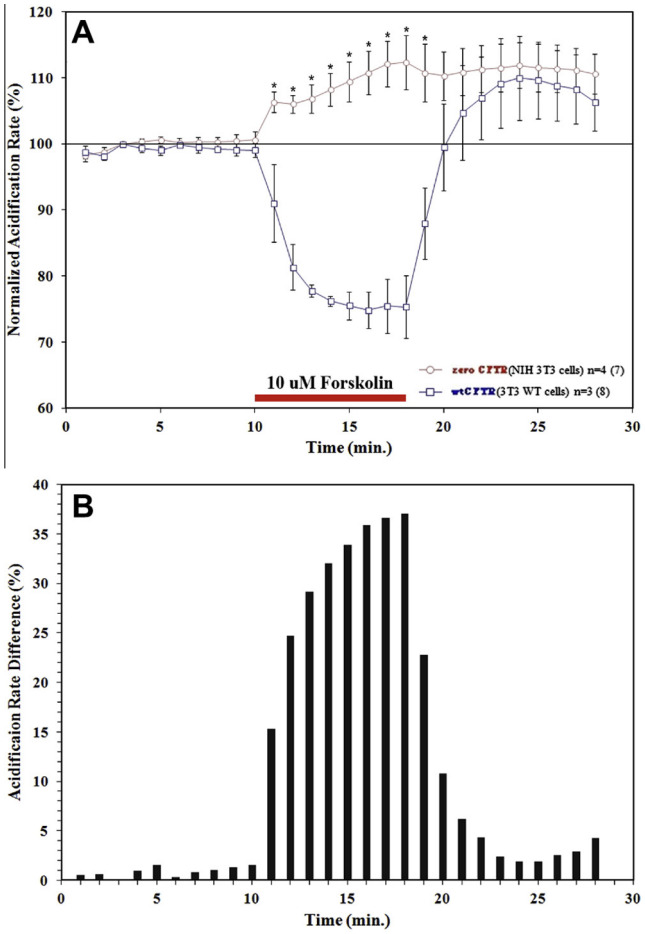

Fig. 2. Effect of 10 μM forskolin treatment on normalized acidification responses of CFTR-expressing (3T3/WT) and CFTR-deficient (NIH/3T3) NIH fibroblasts. (A) Fibroblasts expressing CFTR showed greatly reduced acidification rates in response to forskolin treatment compared to fibroblasts not expressing CFTR (*$p < 0.05$). (B) 10 μM forskolin-stimulated pH response profile generated as the absolute value of the difference between the two responses.

3.2. CFTR-dependent reduction in extracellular acidification rates

Fibroblasts expressing wild-type CFTR (3T3/WT) show greatly reduced acidification rates in response to 10 μM forskolin treatment when compared to controls (NIH/3T3) (Fig. 2A). Response curves from CFTR-expressing cells were subtracted from CFTR-deficient cells to find the absolute value of the difference between the two responses. The difference between 3T3/WT and NIH/3T3 cell lines when activated with 10 μM forskolin peaked at a 38% reduction in acidification rate (Fig. 2B).

Initial acidification studies were performed on mouse fibroblast cells using a high dose of forskolin. To be sure the effects seen were not cell-specific, and due solely to an increase in $[cAMP]_i$, tests using a lower dose of stimulant were repeated with the most relevant cell type, epithelia, expressing wild-type, mutant CFTR protein and controls.

Epithelial cells expressing CFTR (2WT2) also show reduced acidification rates in response to 1uM forskolin treatment when compared to controls (BPV) (Fig. 3A). As seen in fibroblasts, there was sustained suppression of acidification in C127 CFTR-expressing epithelial cells following forskolin washout. The peak difference in 2WT2 and BPV cells when activated with 1 μM forskolin was a reduction in acidification rate of 18% (Fig. 3B).

Whether in mouse fibroblasts or human epithelial cells, the activation of wild-type CFTR resulted in reduced acidification in response to treatment with forskolin compared to controls.

3.3. Chemical rescue reverses CF aberrant pH response profile

Epithelial cells expressing mutant ΔF508-CFTR (508 cells) also show increased acidification rates in response to 1 μM forskolin treatment in a profile that is similar but separate to CFTR-deficient controls (BPV cells) (Fig. 4A). To restore mutant ΔF508-CFTR to wild type functionality, 508 cells were incubated in 10% glycerol 24 h prior to experimentation. Following glycerol chemical rescue, 508 cells showed a reduction in forskolin-stimulated acidification rate compared to controls (BPV) (Fig. 4). Not only did mutant 508 cells assume a functionality like that of wild type (2WT2) cells, they surpassed their acidification rates. Prior to glycerol pre-treatment, 508 cells exposed to 1 μM forskolin had a peak increase in acidification of 10%, however, when 508 cells were pre-treated with 10% glycerol, the response to forskolin reversed to a reduction in acidification by roughly 50%. Glycerol chemical rescue of mutant ΔF508-CFTR appeared at first to result in profile that exceeded that of wild type.

Wild type (2WT2) cells pre-treated with 10% glycerol also showed a enhanced reduction in forskolin-stimulated acidification

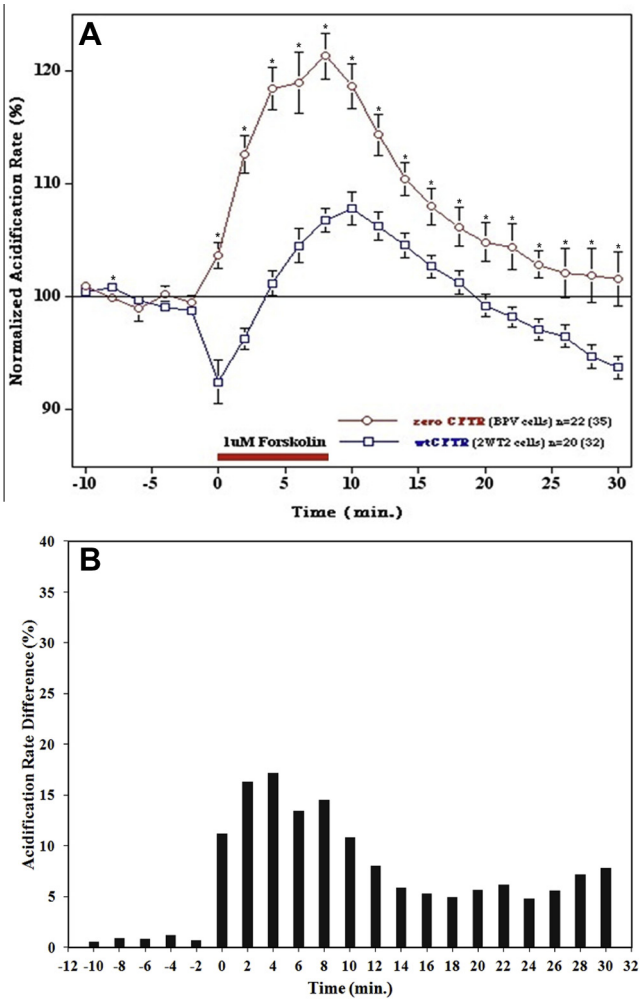

Fig. 3. Effect of 1 μM forskolin treatment on normalized acidification responses of CFTR-expressing (2WT2) and non-expressing (BPV) epithelia. (A) Epithelial cells expressing CFTR showed reduced acidification rates in response to forskolin treatment when compared to controls (*$p < 0.05$). (B) 1 μM forskolin-stimulated pH response profile difference between the two responses.

rates when compared to controls (BPV) (Fig. 4). The peak difference in response between 2WT2 and BPV cells was 18% prior to chemical rescue. Following glycerol pre-treatment, the difference in response widened to approximately 56% because a great number of wild type CFTR proteins were also rescued from removal by quality control systems of the cell. Response of BPV cells with or without glycerol pre-treatment showed no significant differences (Fig. 4).

4. Discussion

Since CFTR is permeable to HCO_3^- [22] and may regulate a family of bicarbonate exchangers [23] its absence in CF has been hypothesized to alter extracellular fluid composition which may extend to other organs beyond the accepted pH effects observed in the pancreas [13,14]. Previously we have shown that cell lines expressing wild-type CFTR can be detected and differentiated from controls using microphysiometry [21]. The microphysiometer, a semiconducter-based instrument, can detect subtle changes in extracellular pH (pHo) in real time as a result of transient acid/base fluxes such as those caused by short-lived transporter activity [15].

After confirming expression and ion conductance of CFTR in cell lines studied, stimulation of CFTR-deficient NIH/3T3 fibroblasts with 10 μM forskolin, which elevates [cAMP], was found to increase the acidification rate of pHo as might be expected for any stimulus that impacts metabolism. The magnitude of the increase, 10%, represents an increased flux of $\sim 10^7$ protons·s^{-1}-$cell^{-1}$ and is within typical ranges observed [24]. In marked contrast, the same forskolin stimulus produced a roughly 25% decrease in the acidification rate of wild-type CFTR expressing fibroblasts. Forskolin activates CFTR via cAMP-dependent phosphorylation [25], with a time course that is consistent with the alkaline signal that was observed. After forskolin washout, the reduced acidification rates of CFTR-expressing cells appear to be sustained, much like that of CFTR channel activity.

To avoid the possibility that the alkalization signal might be cell-specific and not linked to CFTR, we then measured pHo responses in C127 mouse mammary epithelial cell lines, using a 10-fold lower concentration of forskolin and a shorter exposure time to increase the possibility that any response observed would result from elevation of $[cAMP]_i$. Epithelial cells showed CFTR-dependent changes much like those seen in NIH fibroblasts. Consistent results across these conditions argues for a specific effect. To examine the pH response profile, the control cell acidification rates were subtracted from CFTR-expressing cell rates revealing biphasic extracellular alkalization responses in both cell lines studied.

In this study we also find forskolin-stimulated extracellular acidification rates of epithelia (508 cells) expressing mutant ΔF508-CFTR are distinct from wild-type and controls. In addition we find that pre-treatment with glycerol (10% glycerol for 24 h prior to the experiment), acting by chemical rescue, can correct the trafficking of ΔF508-CFTR and lead to a significant decrease in acidification of pHo (essentially a conversion of pHo phenotype from defect to wild-type). Even a 10-fold lower concentration of forskolin and a shorter exposure time (in the more disease-relevant cell type, epithelia) increased the acidification rates of 508 cells not treated with glycerol by 13% while the same forskolin stimulus decreased the acidification rates of glycerol-treated 508 cells by 27%. Thus treatment with glycerol and increased expression of CFTR at the surface was detected by microphysiometry as roughly a net 40% increase in alkalization of pHo.

While one might have expected the acidification curve for 508 cells to move to match that of 2WT2 cells after glycerol treatment, the dramatic change and conversion beyond that point is predictable when glycerol treatment of 2WT2 cells reveals wild-type CFTR also suffers a trafficking deficit during biosynthesis. As is the case with essentially all mutant ΔF508-CFTR proteins, a majority of wild-type CFTR molecules are also lost during biosynthesis and undergo ER-associated degradation through the ubiquitin–proteasome pathway [26]. Thus it is appropriate that the glycerol rescue also greatly impacts 2WT2 cell lines expressing wild type CFTR.

The CFTR-dependent decrease in acidification rates could represent either a decrease of metabolic activity or the export of base that transiently masks the acidification signal. In fact, the biphasic nature of the response is consistent with two competing processes, export of acid (based on increased metabolism) and export of base (based on the opening of the CFTR conductance pathway for HCO_3^-). We believe all evidence supports that the CFTR-dependent decrease in acidification rates represents, at least in part, the export of base. In some cell types CFTR may also activate anion exchangers [23] and that could contribute to the responses seen.

These results indicate that the expression of functional CFTR (or lack thereof in CF) may significantly alter extracellular pH in organs beyond just the pancreas. Our work and that of others [6,11–14,27–32] suggest that CFTR activity may alter pHo via HCO_3^- efflux.

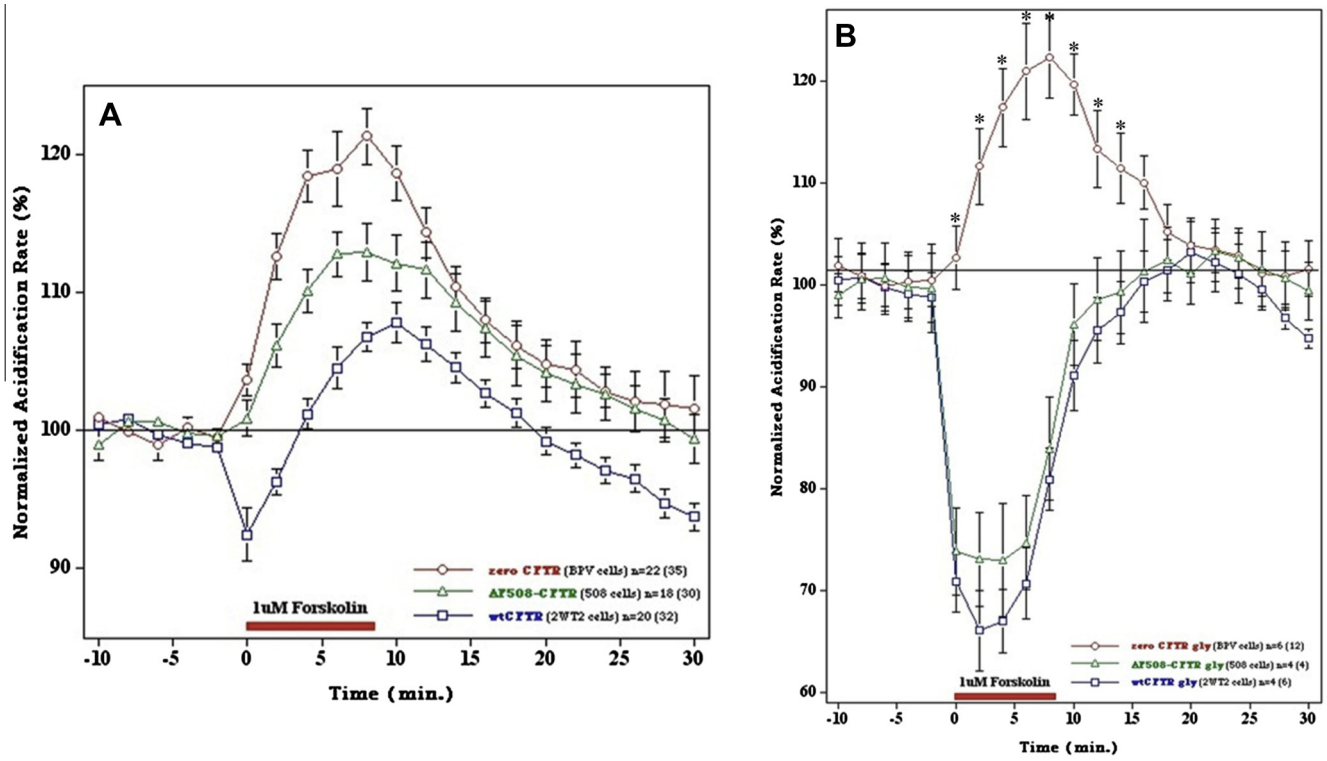

Fig. 4. Effect of 1 μM forskolin treatment on normalized acidification responses in C127 epithelial cells before and after chemical rescue by glycerol incubation. (A) Responses of epithelial cells expressing wild type CFTR (2WT2 cells), mutant ΔF508-CFTR (508 cells), and no CFTR (BPV cells) in response to treatment with 1 μM forskolin. (B) Glycerol pre-treatment greatly reduced the acidification response in wild type and mutant CFTR expressing cell lines (2WT2 and 508) in response to forskolin ($^{*}p < 0.05$).

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