

Non-steroidal anti-inflammatory drugs up-regulate function of wild-type and mutant CFTR

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ABSTRACT:

Small-scale clinical trials show that treatment of cystic fibrosis (CF) patients with ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), improves the symptoms of CF and slows down the decline of lung function. Paradoxically, ibuprofen inhibits ligand-stimulated CFTR activity. The aim of this study was to investigate the effects of ibuprofen on CFTR function under different conditions.

Patch-clamp recordings were performed in two lines of human airway epithelial cells: IB3-8-3-7 cell that generates wild-type CFTR and IB3-1 cell that expresses the mutant CFTR with deletion of F580 (Δ F580CFTR).

Addition of ibuprofen to the extracellular solution caused a rapid inhibition of CFTR activity in IB3-8-3-7 cells in the presence of high intracellular concentration cAMP, whereas ibuprofen enhanced the CFTR conductance at low levels of cAMP. Introducing ibuprofen into the interior of cells occluded the enhancing effect of ibuprofen. Notably, the mutant-CFTR-mediated conductance was detected in IB3-1 cells treated with myoinositol, which was enhanced by ibuprofen at endogenous levels of cAMP.

In summary, NSAIDs increase the function of both wild-type CFTR and Δ F580CFTR in cultured human airway epithelial cells at endogenous levels of cAMP.

KEYWORDS: adenylate cyclase, chloride channel, forskolin, lung epithelium, perforated patch recording

Cystic fibrosis (CF) is one of the most common fatal genetic diseases, which is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-gated Cl^- channel. The dysfunction of mutated CFTR results in a reduction in Cl^- secretion from various epithelial cells, which in turn leads to a reduction of the volume of airway surface liquid, and, as a consequence mucus adhesion, inflammation and bacterial biofilm formation occur in the lung. Although CF is associated with altered epithelial function in multiple visceral organs including the lung, intestine and pancreas, the pulmonary complications such as chronic inflammation and mucous plugging are the major causes of morbidity and mortality in this disease [1]. In this regards, pharmacological managements is still the major approach for dealing with pulmonary complications of CF patients [1].

CF is primarily caused by the dysfunction of mutated CFTR in epithelial cells of affected organs, thus enhancing or restoring the function of mutant CFTR in CF patients should be the principle for CF treatment. Small-scale clinical studies have shown that ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), improves not only the symptoms but also preserves lung function in CF patients [2], when the gastrointestinal side effects of this medication are carefully managed. The benefit of ibuprofen treatment has been largely attributed to its anti-inflammatory action. A previous study showed that ibuprofen inhibits cAMP-activated CFTR-currents in epithelial cells by an unknown mechanism [3]. This inhibitory action on the CFTR would paradoxically be anticipated to exacerbate the symptoms of CF. Bearing this concern in mind, we reexamined the effects of NSAIDs, and specifically ibuprofen, on the function of wild-type and ΔF508 mutant CFTR ($\Delta\text{F580CFTR}$).

EXPERIMENTAL PROCEDURES

Cell culture — IB3-1 cell [4] was originally derived from airway epithelial cells of a CF subject who expressed $\Delta F580$ CFTR gene [5]. By generating a doxycycline-controlled expression system in IB3-1 cell, we created a new cell line named IB3-8-3-7, which expresses wild-type CFTR [6]. IB3-1 cells and IB3-8-3-7 cells were cultured in LHC-8 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Invitrogen), and 150 $\mu\text{g/ml}$ hygromycin B (Sigma, St. Louis, MO). Inducible CFTR expression in IB3-8-3-7 cells was triggered by addition of doxycycline ($1 \mu\text{g mL}^{-1}$), and the expression of CFTR was confirmed by RT-PCR [6] and immunocytochemistry assay. IB3-8-3-7 cells were used after 18-21 h treatment with doxycycline. Some IB3-1 cells were treated with 3 mmol L^{-1} myoinositol (Sigma) and used after 18-21 h treatment with myoinositol.

Heterologous Expression of $GABA_A$ R — IB3-8-3-7 cells were transiently transfected with cDNAs encoding human A-type, γ -aminobutyric acid receptors ($GABA_A$ R) $\alpha 1$, $\beta 2$, and $\gamma 2L$ subunit isoforms (1:1:1 ratio) by using Lipofectamine 2000 (Invitrogen). All cDNAs were subcloned into the mammalian expression vectors pCDM8 for heterologous expression. Transfected cells were grown and incubated for 24-48 h before usage.

Patch-clamp recordings — Perforated or conventional whole-cell patch-clamp recordings were performed in IB3-1 and IB3-8-37 cells using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA). The perforated patch technique was employed to avoid disturbing cell intracellular ion concentrations and metabolism. Therefore, we could detect endogenous V_M of the cells. The conventional whole-cell recordings were used to manipulate intracellular concentrations of ions and cAMP, and in order to deliver certain test compounds into the cells. Before being used for recordings, the cells were rinsed with and bathed in the standard

extracellular solution (ECS) containing (in $\text{mmol}\cdot\text{L}^{-1}$) 145 NaCl, 1.3 CaCl_2 , 5.4 KCl, 25 HEPES and 10 mM glucose (kept at 35°C). Unless specifically described otherwise in the result section, the standard intracellular solution (ICS) for whole-cell recording consisted of (in $\text{mmol}\cdot\text{L}^{-1}$) 150 KCl, 10 HEPES, 2 MgCl_2 , 1 CaCl_2 , and 0.005 K_2ATP , with or without added cAMP. ICS for perforated patch recording consisted of (in $\text{mmol}\cdot\text{L}^{-1}$) 150 KCl, 10 HEPES, 2 MgCl_2 , 1 CaCl_2 and the pore-forming agent gramicidin ($50\ \mu\text{g}\cdot\text{mL}^{-1}$, Sigma) [7]. The pH of ECS and ICS was adjusted to 7.4 and 7.3 respectively, and the osmolarity was corrected to a range of 310–315 mOsM. Patch electrodes ($3\text{--}5\ \text{M}\Omega$) were constructed from thin-walled glass (1.5 mm diameter; World Precision Instruments) using a two-stage puller (PP-830, Narishige).

Perforated patch recordings started under voltage-clamp mode. Membrane perforation was observed as a constant decrease in serial resistance after electrode seal. In most recordings, the resistance declined to a value ranging from 30 to $35\ \text{M}\Omega$ within 5–8 minutes after the seal, and then the resistance could be stable for about 40–80 minutes. To monitor a possible formation of whole-cell configuration, the serial resistance was examined approximately every 5 min during the recording. Whole-cell recordings were carried out as previously described [8,9].

Membrane potential (V_M) of the cell was measured using current-clamp technique. Under voltage-clamp mode transmembrane conductance of the cell was determined by hyperpolarizing or depolarizing voltage steps (As illustrated in Fig. 1G1 inset) or by a voltage-ramp (a steady voltage-change from -100 to 100 mV within 1.5 s, as illustrated in Fig. 2A1 inset). In accordance with the experimental purpose, testing drugs were added to the ICS or ECS. The control ECS and extracellularly-used drug(s) were applied to the cell by a computer controlled two-barrel perfusion system (SF-77B, Warner Instruments, Hamden). Testing drugs were added to the ECS at 8–12 min after whole-cell configuration, at which the amplitude of currents was steady.

Data analysis — All the electrical signals were digitized, filtered (1 kHz) and acquired online using Clampex software and analyzed offline using Clampfit software (Axon Instruments). Data analysis was performed using the Clampfit program (version 8.1). Data was expressed as mean \pm s.e.m. Means were compared with Student's unpaired or paired *t*-test where appropriate using the SigmaStat software from Jandal Scientific Co. A *P*-value <0.05 was considered as significant.

RESULTS

Electrophysiological characterization of wild-type CFTR expressed in human airway epithelial cells. The doxycycline-induced expression of wild-type CFTR in IB3-8-3-7 cells [6] was determined by immunocytochemistry (Fig. 1A). Perforated patch recordings revealed that the V_M of untreated IB3-8-3-7 cells was about -11.6 ± 3.1 mV, whereas the V_M of doxycycline-treated cells was -32.2 ± 3.0 mV (Fig. 1B), which is close to the V_M of primary human airway epithelial cells [10]. This change of V_M was likely due to the suppression of endogenous epithelial sodium channels (ENaC) that are associated with increased expression of CFTR [11,12]. CFTR activity is regulated by intracellular cAMP, and the concentration of endogenous cAMP is controlled by adenylyl cyclase (AC) [13]. Applications of the AC activator forskolin ($3 \mu\text{mol}\cdot\text{L}^{-1}$) induced a substantial and long-lasting depolarization (Figs. 1C and 1D). This depolarization is consistent with a cAMP-induced CFTR-mediated Cl^- efflux.

We then carried out whole-cell recordings with or without cAMP ($100 \mu\text{mol}\cdot\text{L}^{-1}$) in the intracellular solution (ICS) (Fig. 1E). Every cell ($n=16$, at -60 mV) responded with a slowly developing inward current in the presence of cAMP (Fig. 1E, lower trace). This current gradually increased to reach a maximal level at 4-6 minutes after whole-cell configuration and then steadily declined reaching a stable value 8-10 minutes after. In contrast, no current was recorded

in the absence of cAMP (Fig. 1E, upper trace). To explore whether this current is mediated via a Cl^- conductance we replaced Cl^- in the ICS with gluconate, a negatively-charged and membrane-impermeable anion. Under this condition, cells generated outward currents when held at 0mV (Fig. 1F). If Cl^- in the extracellular solution (ECS) was also replaced with gluconate the outward current gradually decreased to baseline (Fig. 1F). This result confirms that the cAMP-induced current is mediated by a Cl^- conductance. We also examined the transmembrane conductance by changing V_M step-by-step (from -100 mV to 100 mV) (Fig. 1G). No conductance was detected in the absence of cAMP (Fig. 1G1), whereas a voltage-independent conductance appeared when cAMP ($100 \mu\text{molL}^{-1}$) was included in the ICS (Fig. 1G2, left panel and Fig. 1G3). In addition, when V_M was held below 0mV this conductance was strongly inhibited by glybenclamide (glyben, $100 \mu\text{molL}^{-1}$), a CFTR blocker [14] (Fig. 1G2, right panel and Fig. 1G3). Taken together, these data demonstrate that the doxycycline-treated IB3-8-3-7 cells express functional CFTR channels.

Ibuprofen regulates CFTR function in a cAMP-concentration-dependent manner. We studied the effect of ibuprofen on this CFTR conductance with cAMP ($100 \mu\text{molL}^{-1}$) in the ICS. Each cell was held at 0mV and a voltage-ramp protocol was used to unveil the CFTR conductance (Fig. 2A). Once a stable CFTR conductance was observed, adding ibuprofen ($300 \mu\text{molL}^{-1}$) to the ECS produced a rapid inhibition of the conductance at V_M more hyperpolarized than -20mV (Fig. 2A1 and 1st panel in Fig. 2A2). Remarkably, ibuprofen also induced a robust and progressive enhancement of the CFTR-current (Figs. 2A1 and 2A2) including a net increase of inward current at the cell's resting V_M (-30 mV) (Figs. 2A2 and 2B).

The activity of CFTR can be up- or down-regulated by a compound depending on the concentration of intracellular cAMP levels [15], and the concentrations of endogenous cAMP in

epithelial cells are presumably at nanomolar or low micromolar ranges under physiological conditions [16]. On the other hand, ibuprofen has a high affinity for plasma proteins and free ibuprofen is less than 1% of the total plasma concentration [17]. At clinical doses reaching 50-100 $\mu\text{g}\cdot\text{mL}^{-1}$ [2], the free ibuprofen in the plasma is only about 1-2 $\mu\text{mol}\cdot\text{L}^{-1}$. We therefore tested the effects of ibuprofen at a low concentration (5 $\mu\text{mol}\cdot\text{L}^{-1}$) on CFTR activity in the presence of high (100 $\mu\text{mol}\cdot\text{L}^{-1}$) and low (5 $\mu\text{mol}\cdot\text{L}^{-1}$) concentrations of intracellular cAMP, respectively. At the high concentration of cAMP, ibuprofen caused an inhibition of the CFTR conductance (Fig. 2C). In contrast, at low cAMP ibuprofen induced an enhancement of CFTR currents (Fig. 2C) without any voltage-dependent inhibition.

Next we studied the effect of ibuprofen on CFTR activity under perforated-patch recording conditions. The voltage-ramp revealed a voltage-independent conductance in doxycycline-treated IB3-8-3-7 cells, which was enhanced by adding forskolin (5 $\mu\text{mol}\cdot\text{L}^{-1}$) to the ECS (Fig. 3A). To confirm that this forskolin-sensitive conductance in IB3-8-3-7 cells was mediated by Cl^- flux, the cells were transfected with GABA_AR subunits, which form Cl^- permeable channels [18]. Application of GABA (30 $\mu\text{mol}\cdot\text{L}^{-1}$) evoked currents in the transfected cells when held at different values of V_M (Fig. 3B, left panel). Plotting GABA-induced currents against the holding V_M gave a linear I-V relationship (Fig. 3B, right panel) and the calculated values for the reversal potential of GABA-currents (E_{GABA}) was -23 ± 2.2 mV (Fig. 3C). On the other hand, the reversal potential of the forskolin-sensitive conductance (E_{Forsk}) (Fig. 3A) was -22.4 ± 2.3 mV (Fig. 3C), which was almost identical to E_{GABA} . These data indicate that Cl^- currents underlie the forskolin-sensitive linear conductance. Remarkably, ibuprofen (5 $\mu\text{mol}\cdot\text{L}^{-1}$) substantially enhanced this Cl^- conductance (Figs. 3D and 3E) without a blockade at hyperpolarized values of V_M (Fig. 3D). This ibuprofen-enhanced Cl^- conductance was suppressed by glybenclamide (200 $\mu\text{mol}\cdot\text{L}^{-1}$)

(Fig. 3D), but persisted in the presence of $10 \mu\text{mol}\cdot\text{L}^{-1}$ amiloride (ENaC blocker) and $10 \mu\text{mol}\cdot\text{L}^{-1}$ bumetanide ($\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter inhibitor) ($n = 3$ cells, not shown), indicating that it is mediated by CFTR channels. Moreover, fast application of ibuprofen produced currents in the doxycycline-treated IB3-8-3-7 cell (Supplementary Fig. 1A) with a reversal potential at about -20 mV (Supplementary Fig. 1B). These combined data verified that ibuprofen enhances the tonic CFTR activity at endogenous concentration of cAMP.

Ibuprofen also enhanced CFTR activity in rat small intestinal epithelial cells (Figs. 3F and 3G). We conclude that ibuprofen enhances CFTR channel function under physiological intracellular conditions. Moreover, meclofenamic acid, another NSAID, similarly enhanced the CFTR conductance of doxycycline-treated IB3-8-3-7 cells at depolarized V_M but inhibited the conductance at hyperpolarized V_M (Supplementary Figure 2). The potentiation of CFTR function by NSAIDs seems not directly associated with blockade of cyclooxygenase, an enzyme that converts arachidonic acid to prostaglandins, because the former inhibits CFTR activity [19].

Ibuprofen up-regulates $\Delta F580$ -CFTR function. We made whole-cell recordings from IB3-1 CF cells, in which few $\Delta F580$ CFTR proteins are targeted to the membrane surface. Indeed, in the presence of $100 \mu\text{mol}\cdot\text{L}^{-1}$ cAMP in the ICS, sequential voltage-ramps failed to reveal a CFTR-like conductance in these cells even 10 minutes after whole-cell conformation (Fig. 4A inset).

Treating epithelial cells with myoinositol, a constituent of phospholipids, can protect the $\Delta F580$ CFTR from degradation and enhances its surface expression [20]. We treated IB3-1 cells with myoinositol for 18 hours and revealed a CFTR-like conductance in myoinositol-treated cells (Fig. 4A, $n = 9$) when $5 \mu\text{mol}\cdot\text{L}^{-1}$ cAMP was included in the ICS. This conductance was not evident when cAMP was omitted from the ICS. Also, it was blocked by glybenclamide (Fig.

4A). Given that $\Delta F508CFTR$ proteins are functional channels when targeted to the cytoplasmic membrane [21], we conclude that this cAMP-induced glybenclamide-sensitive conductance in IB3-1 cells is mediated by the $\Delta F508CFTR$; although, it exhibited a non-linear current-voltage (I-V) relationship at the hyperpolarized V_M [22]. Importantly, this $\Delta F508CFTR$ -mediated conductance was enhanced by ibuprofen (Figs. 4A and 4B).

We also made perforated patch recordings from IB3-1 cells treated with or without myoinositol. The endogenous V_M of untreated IB3-1 cells was -1.1 ± 2.3 mV. Whereas the myoinositol treatment increased V_M to -32 ± 4.8 mV (Fig. 4C). Notably, voltage-ramps revealed a conductance in the myoinositol-treated cells, which was increased by ibuprofen ($5 \mu\text{mol}\cdot\text{L}^{-1}$) (Figs. 4D and 4E). These results implied that ibuprofen enhances the function of $\Delta F508CFTR$.

Ibuprofen up-regulates CFTR activity through an intracellular mechanism. The enhancement of the CFTR conductance by ibuprofen developed slowly (Fig. 2A) suggesting that the enhancing action might require access to the intracellular compartment. To test this idea, CFTR activity in doxycycline-treated IB3-8-3-7 cells was assessed sequentially using the voltage-ramp protocol immediately after whole-cell compilation. Taking the advantage of whole-cell configuration we delivered ibuprofen ($300 \mu\text{mol}\cdot\text{L}^{-1}$, together with $100 \mu\text{mol}\cdot\text{L}^{-1}$ cAMP) into the cell. We observed that the amplitude of transmembrane conductance increased gradually, reaching the peak around 4-6 minutes after whole-cell archetype, and then declined to a steady level by about 8-10 minutes after whole-cell model (Figs. 5A and 5B). Notably, the I-V relationship of the increased currents was linear (Fig. 5A), indicating that intracellular ibuprofen does not produce voltage-dependent blockade of CFTR. Moreover, plotting the normalized amplitude (measured at the V_M of -90 mV) of successively detected currents displayed a larger conductance in the presence of intracellular ibuprofen than in the absence of ibuprofen (Fig. 5B).

This data suggests that ibuprofen up-regulates CFTR activity via intracellular mechanism(s). We also found that in the presence of intracellular ibuprofen, addition of ibuprofen to the ECS produced a rapid inhibition of the current at negative values of V_M , without enhancement at depolarized V_M (Figs. 5C and 5D). This data not only verify that ibuprofen enhances CFTR by interacting with intracellular mechanism, but also indicated that its voltage-dependent blocking effect on CFTR is likely via extracellular access to the channel.

Genistein is a known CFTR-modulating reagent [23,24], which enhances CFTR through binding to an intracellular domain [25] of the CFTR protein. We found that including genistein ($10 \mu\text{molL}^{-1}$) and cAMP ($100 \mu\text{molL}^{-1}$) in the ICS, adding ibuprofen ($200 \mu\text{molL}^{-1}$) to the ECS caused a voltage-dependent blockade without any accompanying potentiation of the CFTR current (Figs. 5E and 5F). Using perforated patch recordings we further confirmed that application of genistein to the ECS enhanced the CFTR conductance and occluded the potentiation by ibuprofen (not shown).

DISCUSSION

Results from the current study indicate that ibuprofen has at least two distinct effects on the CFTR conductance: Firstly, it at concentrations in excess of clinical doses can cause a rapid and voltage-dependent inhibition of CFTR activity, likely through a direct channel blockade. Notably, ibuprofen causes voltage-dependent blockade of CFTR only when the channel is stimulated by high concentration of cAMP. This is consistent with a previous study which showed that high concentrations of ibuprofen inhibited the CFTR conductance provided the channels were strongly activated by the forskolin or with the use of high concentrations of intracellular cAMP [3]. Secondly and most importantly, clinically relevant doses of ibuprofen substantially potentiate CFTR currents in the presence of low intracellular cAMP or in

undisturbed intracellular conditions, which better reflect the *in vivo* cell physiology. Taken together, our findings have not only revealed potent and novel actions of ibuprofen in the up-regulation of CFTR activity, but also proved that NSAIDs regulate CFTR function in a channel-activity-dependent manner through an intracellular interaction with this transmembrane protein.

The most common genetic defect in CF is the Δ F508 mutation [26]. Our data showed that ibuprofen can up-regulate the activity of Δ F508CFTR provided they are expressed on the cell surface. We postulate that CF patients who have a reduced level of Δ F508CFTR in the epithelial cell surface would benefit from the ibuprofen treatment. In addition, previous clinical studies on the determination of optimal dosages of ibuprofen for CF treatment were based on its effect on blocking neutrophil infiltration. Our results demonstrate that ibuprofen at clinical doses enhances CFTR channel activity *in vitro*. In order to assess its potential clinical benefits in CF patients, it will be necessary to confirm these findings using measurements of nasal potential difference.

Great efforts have been devoted to search for drug candidates for CF treatment. For example, genistein is considered as a CFTR-enhancing reagent [23-25] via direct interactions with CFTR molecules. The ibuprofen-induced enhancement of CFTR can be occluded by genistein, which suggests that ibuprofen regulates CFTR activity possibly by interacting with the “genistein” binding site(s) on the CFTR molecule. However, this notion needs to be demonstrated by further experiments. Genistein up-regulates CFTR function [23;24]. However, this compound is water-insoluble and strongly influences the endocrine and immune systems of newborn rats [27], thus rendering it inappropriate for clinical usage. In contrast, ibuprofen, a widely used and clinically approved NSAID, would be a much superior choice for treatment of CF patients.

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FIGURE LEGENDS

Figure 1. Inducible expression of functional CFTR Cl^- channels in IB3-8-3-7 cells. **a).** Immunocytochemical staining of CFTR in control (-doxy) IB3-8-3-7 cells (left panel) and cells treated with doxycycline (+doxy) (right panel). Note that the doxycycline-treated cells are CFTR-immunopositive. **b).** Summary of the measurement (via perforated-patch current-clamp recording) of endogenous V_M of IB3-8-3-7 cells treated with and without doxycycline (-doxycycline: -11.6 ± 3.1 mV, $n = 6$ cells; +doxycycline: -32.2 ± 3.0 mV, $n = 9$ cells; * in comparison to -doxycycline $P < 0.05$). **c).** Under perforated-patch current-clamp conditions, brief addition of $3\mu\text{M}$ forskolin to the ECS induced a long-lasting depolarization. **d).** Plotted is the summary of forskolin-induced changes of V_M (control: -30.4 ± 3.9 mV; after forskolin: -15.6 ± 4.9 mV, $n = 5$, * $P < 0.05$). **e).** Typical transmembrane currents in doxycycline-treated IB3-8-3-7 cells (clamped at -60 mV) in the absence (upper trace) and presence of $100\mu\text{M}$ intracellular cAMP (lower trace). **f).** Example trace recorded in a doxycycline-treated IB3-8-3-7 cell shows the direction-switch of trans-membrane current in response to replacing extracellular Cl^- with gluconate. Note: the cell was voltage-clamped at 0mV , and intracellular Cl^- was replaced with gluconate. **g).** In the absence of intracellular cAMP, step-changes of V_M from -100mV to $+100$ mV did not reveal transmembrane conductance in doxycycline-treated IB3-8-3-7 cell (**g1**). In the presence of intracellular cAMP ($100\mu\text{M}$), the V_M change disclosed a non-voltage-dependent conductance in a doxycycline-treated IB3-8-3-7 cell, which was inhibited by the CFTR inhibitor glybenclamide ($100\mu\text{M}$) when the cell was held at negative V_M (**g2**). The current traces in E2 were plotted against holding V_M , illustrating the current-voltage (I-V) relationship (**g3**).

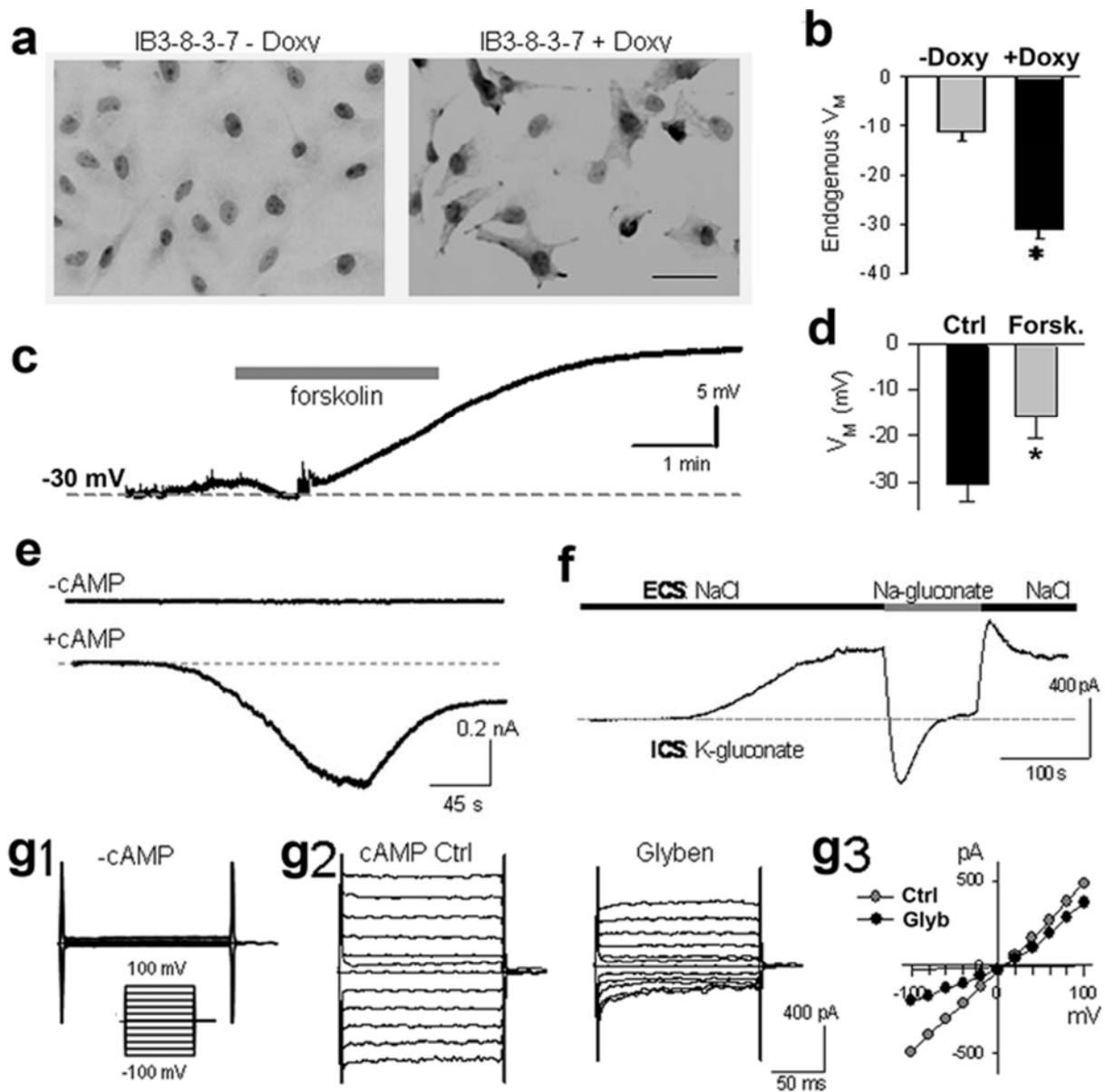


Figure 2. Ibuprofen induces dual effects on CFTR conductance. a). In the presence of $100\mu\text{M}$ intracellular cAMP, whole-cell CFTR currents in a doxycycline-treated IB3-8-3-7 cell were revealed by a voltage-ramp protocol (illustrated in the inset). Immediately after added to ECS, $300\mu\text{M}$ ibuprofen induced a blockade of CFTR current revealed at negative V_M . Besides the voltage-dependent blockade, ibuprofen gradually enhanced the CFTR current regardless of

V_M (**a1**). The dual effects of ibuprofen on the I-V relationship of CFTR conductance at different time-points were illustrated in **a2**. **b**). Plotted are CFTR currents (measured at V_M of -30mV, $n = 12$) recorded before, during and after application of ibuprofen. The amplitude of currents in the same cell at different time points is normalized to that of the current recorded just before ibuprofen application. Note the enhancement of CFTR current by ibuprofen. **c**). Summary of the effect of ibuprofen (5 μ M) on normalized whole-cell CFTR current in the presence of high and low concentrations of intracellular cAMP (100 μ M cAMP: $76.2 \pm 8.7\%$ of control, $n = 11$; 5 μ M cAMP: $148.3 \pm 15.6\%$ of control, $n = 5$). * in comparison to control, $P < 0.05$.

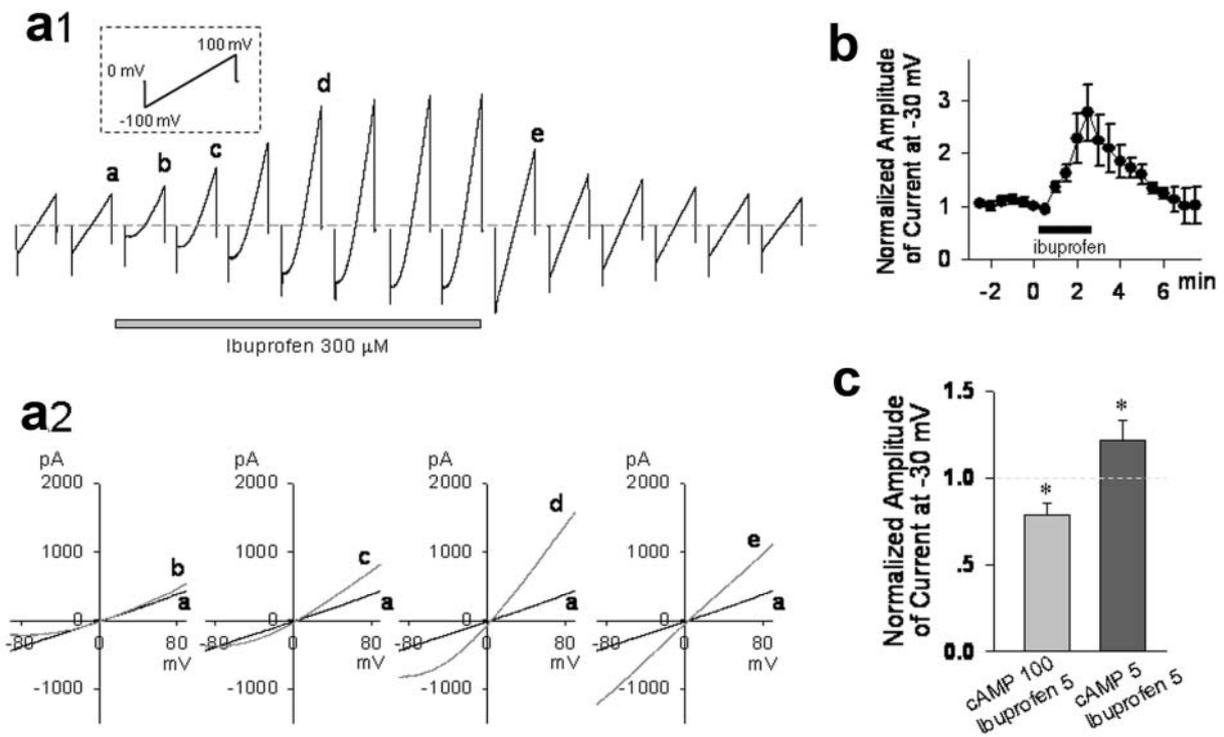


Figure 3. Ibuprofen enhances CFTR channel activity under physiological metabolic condition. All data shown in this figure were obtained by perforated patch recordings under voltage-clamp conditions with holding $V_M = 0$ mV. **a**). Voltage-ramp revealed a linear (non-voltage-gated) transmembrane conductance in a doxycycline-treated IB3-8-3-7 cell, which was

enhanced by adding 5 μ M forskolin to the ECS. **b**). The left panel shows typical GABA-evoked currents at different holding V_M (from -80 to +10 mV) in a doxycycline-treated IB3-8-3-7 cell that was transfected with cDNAs encoding $\alpha 1$, $\beta 2$ and $\gamma 2L$ subunits of GABA $_A$ R. The right panel illustrates the I-V relationship of GABA-currents. **c**). Shown are summarized data of the reverse V_M of GABA-current (E_{GABA} : -23.4 ± 2.2 mV, $n = 6$) and the reverse V_M of forskolin-sensitive currents (E_{Forsk} : -22.4 ± 2.3 mV, $n = 5$). **d**). Addition of 5 μ M ibuprofen to ECS enhanced the endogenous non-voltage-gated current at both the negative and positive V_M . Adding 100 μ M glybenclamide (glyben) to ECS inhibited the ibuprofen-enhanced current. **e**). Summarized are the normalized amplitude of voltage-ramp evoked currents (measured at -30 mV) before and during application of ibuprofen. **f**). Typical traces shows the enhancing effect of ibuprofen (5 μ M) on the voltage-ramp revealed current in rat small intestinal epithelial cells. **g**). Plotted are the amplitude of currents in the intestinal epithelial cells before (control) and 3 min after application of ibuprofen (control: 260 ± 26 pA, $n = 5$; ibuprofen: 393 ± 46 pA, $n = 5$, * in comparison to control $P < 0.05$).

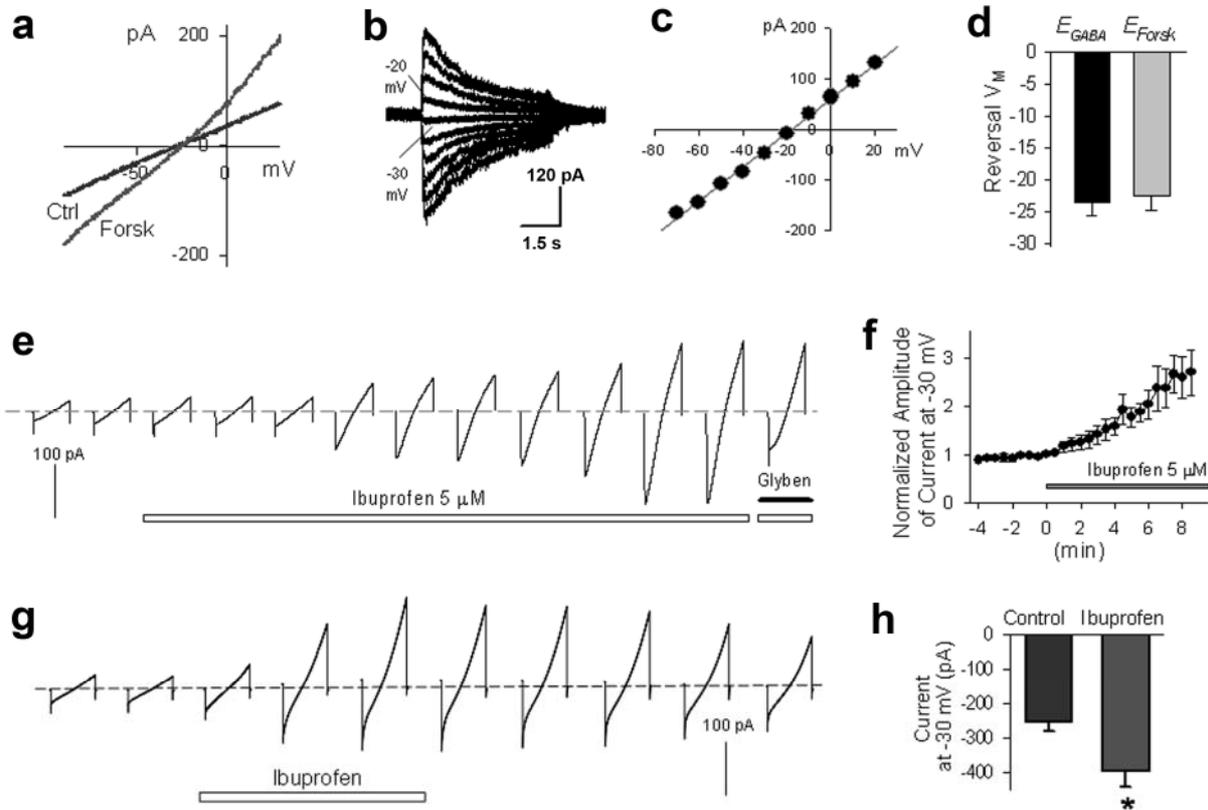


Figure 4. Ibuprofen enhances $\Delta F508$ CFTR channel function in IB3-1 cells. **a).** Under whole-cell recording condition with $5\mu\text{M}$ cAMP in the ICS, voltage-ramp revealed a conductance in myoinositol-treated IB3-1cells, but not in the untreated IB3-1cells (inset, in the presence of $100\mu\text{M}$ cAMP). This voltage-ramp-evoked conductance was enhanced by $300\mu\text{M}$ ibuprofen, and the ibuprofen-enhanced current could be suppressed by $100\mu\text{M}$ glybenclamide. **b).** Summarized data from 6 cells show the normalized amplitude of voltage-ramp evoked currents (measured at -30 mV) in myoinositol-treated IB3-1cells before, during and after application of ibuprofen. **c).** Summary of endogenous V_M measured under perforated-patch recording in control and myoinositol (MI)-treated IB3-1 cells ($-\text{myoinositol}$: $-1.1 \pm 2.3\text{ mV}$, $n = 6$; $+\text{myoinositol}$: $-32 \pm 4.8\text{ mV}$, $n = 5$; * in comparison to $-\text{myoinositol}$ $P < 0.05$). **d).** Under perforated-patch recording conditions, the voltage-ramp revealed a non-linear conductance in

myoinositol-treated IB3-1 cells, which was enhanced by addition of 5 μM ibuprofen to the ECS. e). Summarized data from perforated-patch recordings in 10 cells show the normalized amplitude of currents (measured at -30 mV) in myoinositol-treated IB3-1 cells before and during application of 5 μM ibuprofen.

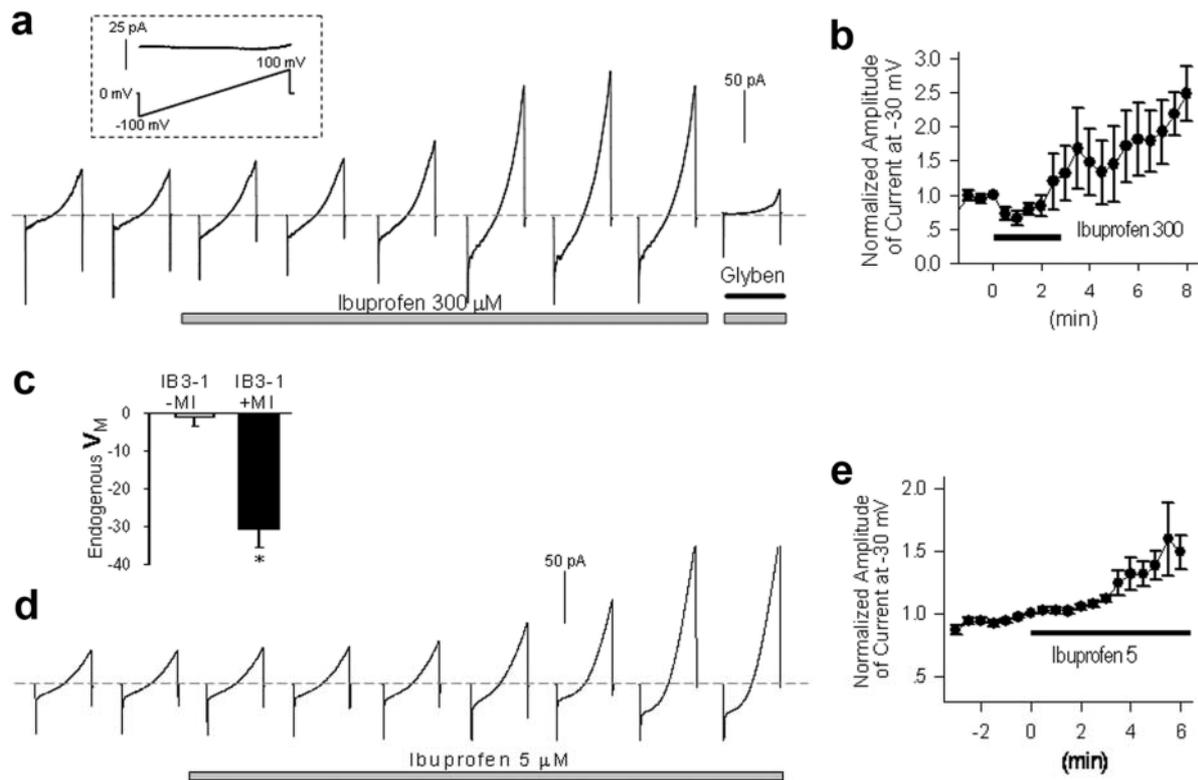


Figure 5. Ibuprofen exerts enhancing and inhibitory effects on CFTR-conductance via intracellular and extracellular mechanism, respectively. All data shown in this figure were obtained by whole-cells recordings in doxycycline-treated IB3-8-3-7 cells. **a**). Typical traces of voltage-ramp-evoked currents recorded at different time points of recording in the presence of 300 μM ibuprofen in the ICS. 0 min = immediately after whole-cell configuration. **b**). Plotted are normalized amplitudes of voltage-ramp-evoked currents at V_M of -90 mV of cells in the absence (n = 6 cells) and presence (n = 6 cells) 300 μM ibuprofen in the ICS. Note: Currents were

normalized to the one recorded immediately after whole-cell configuration. **c**). In the presence of 300 μ M ibuprofen in the ICS, 300 μ M ibuprofen was added to the ECS (indicated by the open bar) at 11 min after whole-cell configuration, and sequential traces illustrate the effect of extracellular ibuprofen on the evoked CFTR-currents. **d**). Amplitudes of the currents recorded during perfusion of ibuprofen were normalized accordingly to the current evoked 30s before addition of ibuprofen to the ECS. The current amplitudes during perfusion of ibuprofen were $55 \pm 6.5 \%$, $84 \pm 3.0 \%$ and $97 \pm 2.6 \%$ of control size at -90, -60 and -30 mV of holding V_M , respectively (n = 4 cells; ***, $P < 0.0001$; **, $P < 0.001$). **e**). Successive traces show the effect of addition of 300 μ M ibuprofen to the ECS on evoked CFTR currents in the company of 10 μ M genistein in the ICS. **f**). Plotted are the normalized amplitude of currents recorded during perfusion of ibuprofen. The current amplitudes during perfusion of ibuprofen were $50 \pm 2.6 \%$, $81 \pm 3.4 \%$ and $98 \pm 2.5 \%$ of control size at -90, -60 and -30 mV of holding V_M , respectively (n = 4 cells).

