

Metformin Treatment of Diabetes Mellitus Increases the Risk for Pancreatitis in Patients Bearing the CFTR-mutation S573C

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Key Words

Cystic fibrosis transmembrane conductance regulator • CFTR AMP • PKA AMP-activated protein kinase • S573C • Pancreatitis • Metformin • Pancreas • Chloride secretion

Abstract

Metformin use in diabetes can cause acidosis and might be linked to pancreatitis. Here, we mechanistically focus on this relationship via a point mutation in the cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7). CFTR is an ATP-hydrolyzing, cAMP/PKA-activated anion channel regulating pancreatic bicarbonate/chloride secretion across duct-facing apical membranes in epithelia. CFTR has two nucleotide binding domains (NBD1/2) which clamp two ATP molecules across their opposed, inverted interfacial surfaces which generates anion-conductance after ATP hydrolysis. Notably, CFTR mutations not causal for classical cystic fibrosis segregate with unexplained pancreatitis and one of these lies in NBD1 near its ATP-clamp (S573C; close to the Walker B aspartate D572). We recently showed that after raising [cAMP], wt-CFTR chloride-conductance, when expressed in *Xenopus* oocytes,

remains elevated despite the presence of metformin. Yet here, we find that S573C-CFTR manifests a metformin-inhibitable whole cell chloride-conductance after cAMP elevation. In the absence of metformin, cAMP-activated S573C-CFTR also displays a reduced anion-conductance relative to wt-CFTR. Furthermore, intra-oocyte acidification inhibited wt-CFTR and abolished S573C-CFTR conductance. We conclude that defective S573C-CFTR remains both poorly conducting and inhibited by metformin and intracellular acidosis. This might explain the propensity to pancreatitis with this rare CF mutation.

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Introduction

Pancreatitis arises from a complex amalgam of genetic and environmental factors. In one instance, pancreatitis has an onset during fetal life in the common inherited disease, cystic fibrosis (CF) that affects about 1 in 4000 babies in the developed world. CF is caused by mutations in a phosphorylation and ATP-regulated chloride channel, the cystic fibrosis transmembrane-conductance regulator (CFTR). Even before CFTR was cloned two

decades ago, the classical form of CF was recognized as a disease associated with low volumes of pancreatic fluid secretion containing less bicarbonate compared to non-CF pancreatic juice. Although the role of CFTR in bicarbonate secretion by CFTR in the pancreatic duct is not fully elucidated [1], it is now almost axiomatic that CFTR can conduct anions other than chloride and HCO_3^- is foremost amongst such candidates (another is glutathione). Bicarbonate transport via CFTR recycles with luminal Cl^- across the apical membrane of pancreatic duct cells and is therefore functionally coupled to luminal anion exchangers in charge of net HCO_3^- flux.

In fact CFTR may physically interact with two bicarbonate exchangers SLC26A6 and DRA (down regulated in adenoma) [2-4]. Activation of CFTR by DRA is facilitated by the C terminus of CFTR where PDZ ligands can interact through a conserved motif whereas the binding of the SLC26T STAS domain occurs through the CFTR regulatory (R) domain. The R domain is unique to CFTR when compared to other members of the ABC family and is the site of multiple regulation by protein kinases such as PKA (cAMP-dependent), PKC (lipid and calcium regulated) and AMP-activated kinase (a metabolic regulator). Binding of the STAS and R domains was shown to be regulated by PKA-mediated phosphorylation of the R domain [5, 6] which we and others find to dramatically alter its structure (as judged by NMR and gel shift analysis) after phosphorylation by PKA. Thus R domain plasticity, associated changes in CFTR function and regulated HCO_3^- secretion appear to be tightly coupled. These ideas underpin classical Cystic fibrosis (CF) which is also characterized by a chronic lung disease that follows airway obstruction and chronic infection by many bacteria but particularly *Pseudomonas aeruginosa*. Other features include elevated sweat electrolyte concentrations, male infertility and almost always in such cases, there exists a severe degree of pancreatic insufficiency that follows the precipitation of pancreatic enzymes within the pancreas itself. Precipitation and premature activation lead to pancreatic autodigestion due to a failure of duct fluid secretion [7]. Moreover, the variability in repeat numbers of certain intronic elements at exon-intron boundaries CFTR gene characterized by the 5T poly-pyrimidine tract genotype have also been found to be associated with chronic pancreatitis [8-12]. This complexity is exacerbated by a wider spectrum of non-classical CF that is 'organ-confined' affecting one or more organs but sparing the lung. Thus an emerging view is that it is possible for a patient to have CF of the organs and not CF as a whole.

Here we expand this theme by focusing on one mutation in CFTR that lies close to one of the Walker B motifs involved in ATP binding to the first nucleotide binding domains of CFTR.

CFTR is regulated by many protein kinases and the adenosine monophosphate activated protein kinase (AMPK) is known to inhibit CFTR function [13, 14]. AMPK is a ubiquitous Ser/Thr kinase with a substantial basal constitutive activity independent of AMP (hence the designation as AMP-activated rather than the AMP-regulated) and is the downstream effector of a cascade that is sensitive towards cellular energy. Upon ATP depletion, the substantial basal activity of AMPK is further activated by a rise in AMP which then stimulates catabolic pathways that ultimately lead to restoration of ATP production. In AMP-activated mode, AMPK also downregulates anabolic pathways that consume ATP, by direct phosphorylation of metabolic enzymes or by regulating gene expression [15]. Biguanidine compounds such as metformin (or the more potent but clinically toxic phenformin) activate AMPK in part by inhibiting complex I of the respiratory chain but additional mechanisms may also be operant [16, 17]. Metformin is widely used in the clinical treatment of type II diabetes. Phenformin and metformin both promote insulin-stimulated glucose uptake in muscle tissues and lower hepatic glucose output. Metformin slightly increases the risks of lactic acidosis (much more so with phenformin) and patients using this compound can develop pancreatitis secondary to metformin poisoning, or at therapeutic metformin doses when a patient presents with renal failure [18, 19]. Phenformin has been withdrawn from clinical use due to the high incidence of lactic acidosis and it was replaced by metformin, which still has some risk of lactic acidosis and pancreatitis [17, 18].

CFTR binds two ATP molecules using a sandwich structure consisting of nucleotide binding domains (NBDs) 1 and 2. We investigated the S573C point mutation that lies within the first nucleotide binding domain of CFTR adjacent to the Walker B aspartate at D572. S573C appears not to induce classical CF but instead segregates with pancreatitis of unknown etiology [20, 21]. We tested the hypothesis that this was merely a chance finding by investigating S573C-CFTR function. Should we find no obvious change in CFTR function compared to wild type then we would have to conclude that the mutation was just a polymorphic variant of no particular consequence with just a random association with pancreatic failure. We find that this is not the case and present a testable hypothesis about the means by which this mutant CFTR

might induce pancreatitis. We speculated that the presence of the *CFTR* gene variant S573C in non-CF diabetes patients renders them more susceptible towards the development of pancreatitis and tested the potential role of metformin. We therefore examined the function of S573C and other point mutants of CFTR at this site by overexpression in *Xenopus* oocytes. The data indicate a lower channel activity of S573C-CFTR with a higher sensitivity towards metformin-induced closure. Impaired Cl⁻ channel function and regulatory function of S573C-CFTR may explain the higher incidence of this mutant in patients with pancreatitis.

Materials and Methods

cRNAs for CFTR and double electrode voltage clamp

Oocytes were injected with cRNA (10 ng, 47 nl double-distilled water) encoding wtCFTR, S573C-CFTR, and S573A-CFTR. All mutants were generated by PCR and correct sequences were confirmed by restriction digest and by sequencing. Water injected oocytes served as controls. 2–4 days after injection, oocytes were impaled with two electrodes (Clark Instruments Ltd, Salisbury, UK), which had a resistances of < 1 MΩ when filled with 2.7 mol/l KCl. Using two bath electrodes and a virtual-ground head stage, the voltage drop across the serial resistance was effectively zero. Membrane currents were measured by voltage clamping (oocyte clamp amplifier, Warner Instruments LLC, Hamden CT) in intervals from -60 to +40 mV, in steps of 10 mV, each 1 s. The bath was continuously perfused at a rate of 5 ml/min. All experiments were conducted at room temperature (22 °C).

In vitro phosphorylation

In vitro phosphorylation experiments were conducted as described recently [14].

Materials and statistical analysis

All compounds used were of highest available grade of purity and were purchased from SIGMA or Calbiochem. Student's t-test was used for statistical analysis. A p value of <0.05 was regarded as significant.

Results

S573C attenuates CFTR whole cell Cl⁻ conductance and is inhibited by metformin

Because S573C-CFTR has been found in patients with pancreatitis, we examined the ability of both wtCFTR and S573C-CFTR to generate Cl⁻ currents by maximal stimulation of *Xenopus* oocytes with IBMX (1 mM) and forskolin (2 μM). Under these conditions which elevate cAMP and activate PKA, whole cell currents and

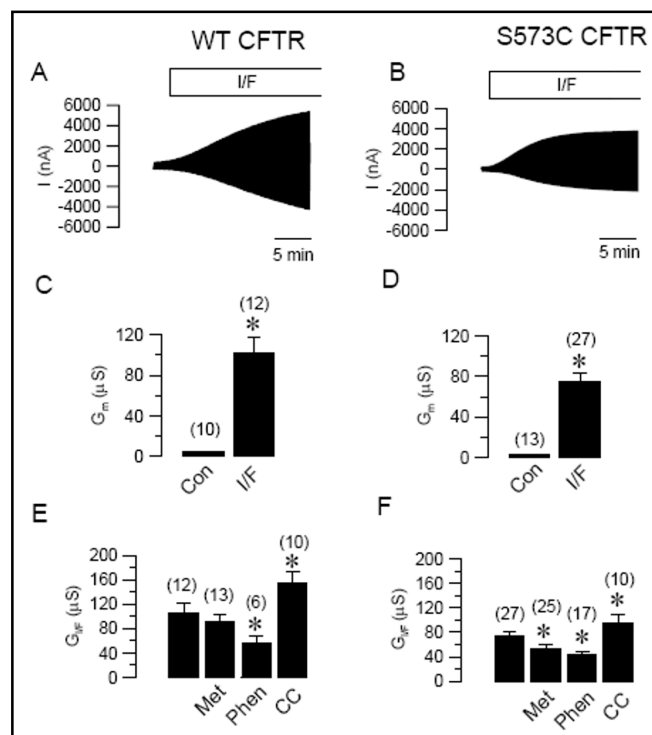


Fig. 1. S573C attenuates CFTR whole cell Cl⁻ conductance. Current recordings obtained in *Xenopus* oocytes expressing wt-CFTR (A) or S573C-CFTR (B) showing activation of whole cell Cl⁻ currents by stimulation with IBMX (1 mM) and forskolin (2 μM). Summaries of whole cell conductances under control conditions and after stimulation with I/F (G_m) (C,D). Summaries of whole cell conductances activated by IBMX and forskolin (G_{I/F}) in oocytes expressing wt-CFTR (E) and S573C-CFTR (F), and effects of the activators of AMPK metformin (Met; 2 mM) and phenformin (Phen; 5 mM) or the AMPK-inhibitor compound C (CC; 80 μM). Mean ± SEM, (n) = number of cells measured. *significant increase in whole cell conductance (paired t-test).

conductances produced by S573C-CFTR were reduced (~20%) when compared to those produced by wt-CFTR (Fig. 1A-D). AMP-activated protein kinase (AMPK) has been shown previously to inhibit CFTR and to be relevant *in vivo* [13, 14, 22, 23]. The biguanide metformin that is used for treatment of type 2 diabetes mellitus, is also known to activate AMPK and therefore may inhibit CFTR, similarly to the more potent phenformin [14, 17]. We examined the effect of 500 μM metformin on CFTR-conductance and compared the effect with that of phenformin (1 mM). Metformin only inhibited S573C-CFTR but not wt-CFTR (compare second bars in Fig. 1 E,F), in contrast to the more potent activator of AMPK, phenformin that inhibited both S573C-CFTR and wt-CFTR (Fig. 1E,F). Thus mutation of serine 573 to a close analogue merely bearing an S replacing an oxygen in an

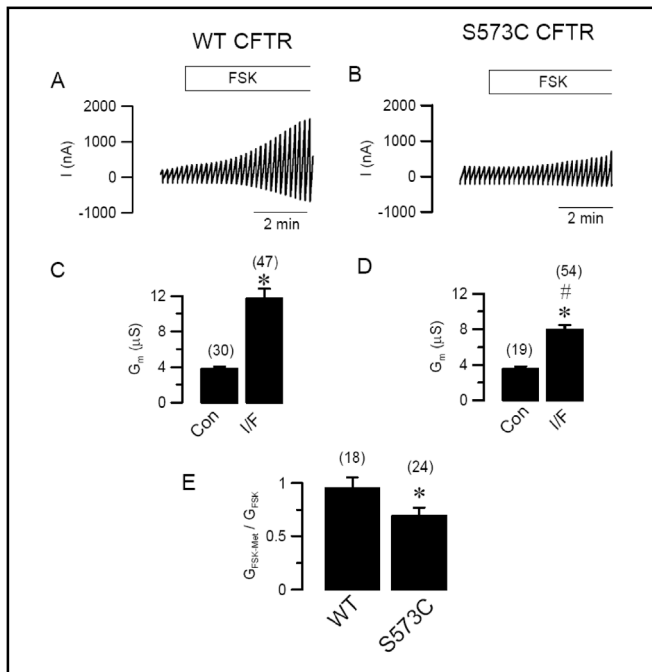


Fig. 2. Metformin inhibits S573C-CFTR but not wt-CFTR. Current recordings obtained in a *Xenopus* oocyte expressing wt-CFTR (A) or S573C-CFTR (B) showing activation of whole cell Cl^- currents by stimulation with only forskolin (20 μM). Summaries of the whole cell conductances under control conditions and after stimulation with I/F (G_m) (C,D). Summaries of the ratio of whole cell conductances activated in the presence of metformin or under control conditions in oocytes expressing wt-CFTR and S573C-CFTR (E). Mean \pm SEM, (n) = number of cells measured. *significant increase in whole cell conductance (paired t-test). # significant difference when compared to wt-CFTR.

alcoholic side chain reduces CFTR-conductance and appears to enhance both sensitivity towards AMPK-induced closure and conversely, this mutant fails to almost double CFTR conductance after pharmacological inhibition of AMPK using compound C (Fig. 1E,F).

We demonstrated recently that CFTR is more susceptible towards inhibition by AMPK at lower cAMP-induced activation levels [14]. Therefore we stimulated oocytes with forskolin alone (20 μM), which caused about 10 % of maximal activation of CFTR (Fig. 2A-D). Under these conditions it became quite obvious that S573C-CFTR has a reduced Cl^- conductance compared to wtCFTR (Fig. 2C,D) and that 500 μM metformin did not inhibit wtCFTR and yet inhibited S573C-CFTR by about 25% (Fig. 2E), thus confirming the enhanced sensitivity of S573C-CFTR for inhibition by metformin.

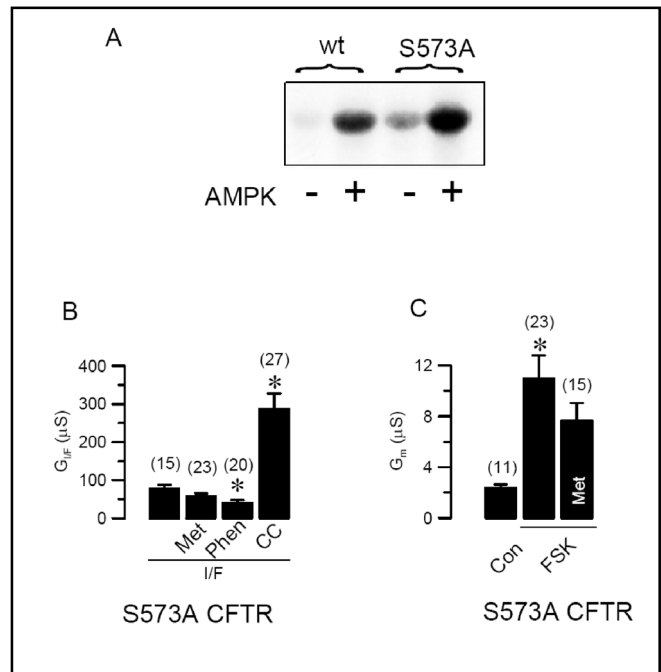


Fig. 3. Phosphorylation and activation of S573A-CFTR. Phosphorylation by AMPK of the first nucleotide binding domains (NBD1) of wtCFTR and S573A-CFTR (A). Summary of the whole cell conductances activated by IBMX (1 mM) and forskolin (20 μM) ($G_{I/F}$) obtained in *Xenopus* oocyte expressing S573A-CFTR (B) or S573A-CFTR (C) and effects of activators (metformin, phenformin) or an inhibitor (compound C) of AMPK. Mean \pm SEM, (n) = number of cells measured. *significant effects on whole cell conductance (paired t-test).

Phosphorylation of NBD1 is enhanced by the mutation S573A

Previously we found that two serines in the regulatory (R) domain of CFTR, S737 and S768, formerly identified as inhibitory PKA sites, are phosphorylated by AMPK and are the residues essential for inhibition by AMPK [14]. Since S573 is located in the first nucleotide binding domain (NBD1) of CFTR and because inhibition by the AMPK-activator metformin is enhanced for S573C-CFTR, the present results suggest that NBD1 may contribute to regulation of CFTR by AMPK. We therefore examined whether eliminating the serine at position 573, or a specific exchange of serine by cysteine is reducing CFTR activity. We investigated a S573A-CFTR mutant and found that it behaved like wild type and critically, has lost its inhibitory metformin sensitivity when stimulated

by a rise in cAMP (Fig. 3B). This suggested that CFTR is able to discriminate between a methylene group on the side chain at position S573 bearing either an OH (wild type) or the bulkier SH group. We interpreted this data to suggest that the S573C defect might lie downstream of AMPK due to perturbation of the milieu around either the adjacent Walker B aspartate (for example at D572) or near another local cysteine, as suggested by Chen et al during their pH studies [24]. We were particularly interested in the role of pH in this process because Sheppard and colleagues had found that this very same region might be involved in pH sensing (Chen et al). To rule out a direct effect of AMPK on this serine, we examined phosphorylation of isolated NBD1 *in vitro* and found that AMPK indeed phosphorylated this domain of CFTR as expected (Fig. 3A). We mutated serine 573 to an alanine and found that elimination of this potential phosphorylation site did not abolish phosphorylation of NBD1 by AMPK (Fig. 3A). These results were consistent with existence of other phosphorylation sites for AMPK in NBD1, which are different from serine 573. Although *in vitro* phosphorylation data do not allow for quantitative assessment, it appears that AMPK-phosphorylation of the S573A mutant was somewhat augmented but this may equally be an artifact caused by slight differences relative amounts of protein. This was not taken further here because mutation at S573 did not have a dramatic effect on phosphorylation by AMPK compared to other CFTR sites (S768 for example) we had previously encountered leading us to concentrate on pH effects.

Acidification equally inhibits wtCFTR, S573A-CFTR and S573C-CFTR.

Lactic acidosis with the consequence of pancreatitis may be caused by the use of antidiabetic biguanide drugs such as phenformin or metformin [18, 19, 25, 26]. We wondered whether change in external pH and in particular acidosis may be an additional factor that inhibits the function of wild type CFTR. Also, the inhibitory effect of metformin may be potentiated in the presence of extracellular acidosis which could explain the pancreatitis observed upon treatment with biguanide drugs and lactic acidosis [18]. Non-injected control oocytes remained completely unaffected by extracellular acidification (Fig. 4A,B). Moreover extracellular pH does not *per se* affect ion currents of *Xenopus* oocytes expressing CFTR (data not shown). Surprisingly, metformin (500 μ M), when applied in the presence of acidic pH (pH 5.5), did not inhibit forskolin (20 μ M) activated Cl^- currents, generated

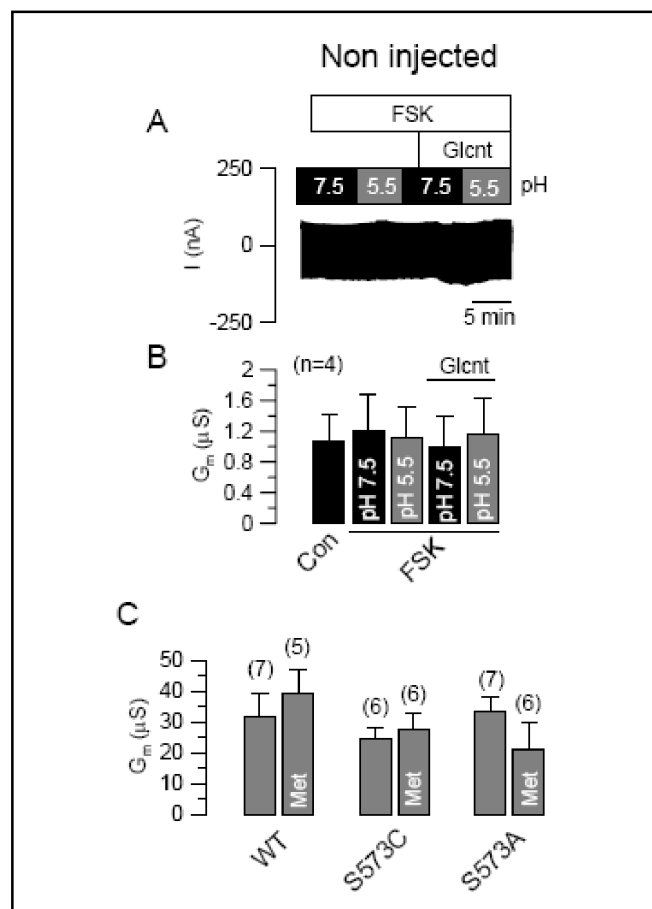
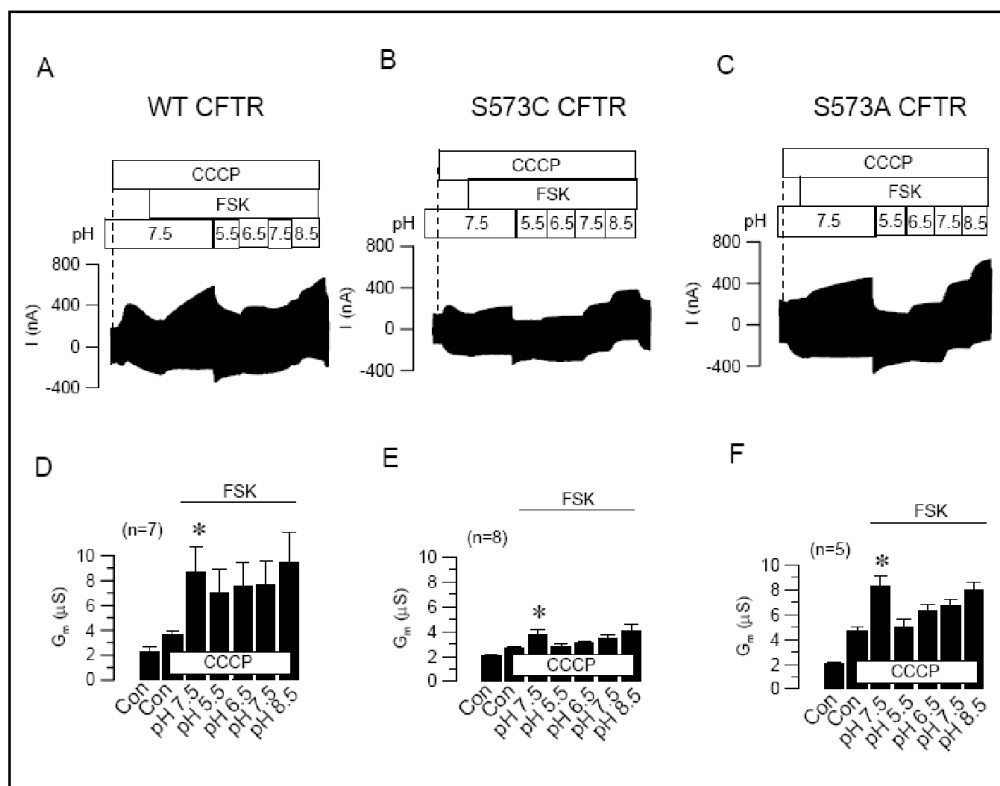


Fig. 4. Extracellular acidification does not affect control oocytes. Current recording obtained in a non-injected *Xenopus* oocyte indicating lack of effects of forskolin or extracellular pH 5.5 (A). Summary of whole cell conductances obtained in control oocytes in the absence or presence of extracellular acidosis or stimulation with forskolin (B). Summary of the whole cell conductances generated by wt-CFTR, S573C-CFTR and S573A-CFTR when activated by forskolin (20 μ M) in the presence of pH 5.5, and effects of metformin (500 μ M) (C). Mean \pm SEM, (n) = number of cells measured.

by wt-CFTR, S573C-CFTR, or S573A-CFTR (Fig. 4C). Thus the S573C CFTR which had previously retained metformin-induced inhibition despite the presence of forskolin and IBMX, had now 'become wild type' just like S573A.

We further tested whether intracellular acidification differentially affects whole cell currents produced by wt-CFTR, S573C-CFTR, or S573A-CFTR. To that end oocytes were exposed to the protonophore carbonyl cyanide m-chloro phenylhydrazone (CCCP; 10 μ M) at different extracellular pH ranging from pH 5.5 to pH 8.5. We observed regular activation of wt-CFTR and S573A-

Fig. 5. Inhibition of wtCFTR, S573C-CFTR, and S573 A-CFTR by intracellular acidosis. Current recordings obtained in a *Xenopus* oocyte expressing wt-CFTR (A), S573C-CFTR (B), or S573 A-CFTR (C). Summary of the whole cell conductances under control conditions and after stimulation with forskolin (20 μ M), in the presence of CCCP at different extracellular pH. Acidification (pH 5.5, pH 6.5) but not alkalization (pH 8.5) inhibits Cl^- currents generated by wt-CFTR, S573C-CFTR and S573 A-CFTR. Acidosis completely inhibited currents produced by S573C-CFTR and S573A-CFTR. (D-F). Mean \pm SEM, (n) = number of cells measured. *significant increase in whole cell conductance (paired t-test).



CFTR by forskolin (20 μ M) in the presence of CCCP and at physiological pH (pH 7.5), while activation of S573C-CFTR was largely reduced (Fig. 5A-C). Subsequent, acidification inhibited Cl^- currents produced by wt-CFTR, S573C-CFTR, or S573A-CFTR, while subsequent alkalization did not affect whole cell Cl^- currents (Fig. 5A-C). In fact acidosis completely inhibited S573C-CFTR currents. Taken together the present results are consistent with a model whereby the S573C-CFTR mutation predisposes to pancreatitis because it i) may sensitize CFTR towards inappropriate regulation by AMPK, ii) is inhibited by the therapeutic biguanide metformin and iii) is inhibited potently by acidification.

Discussion

Pancreatitis is not a common complication during metformin therapy; however, there are clinical cases of pancreatitis associated with metformin treatment [18]. CFTR has a clear role in alkalinizing the pH of the pancreatic juice, thereby solubilizing secreted enzymes, neutralizing acid chyme that is entering the duodenum and preventing premature activation of enzymes within the pancreatic duct [4, 27]. Notably, fluid and HCO_3^- secretion occurs in a coordinated manner and it has been

demonstrated recently that basolateral HCO_3^- entry via the $\text{Na}^+/\text{HCO}_3^-$ cotransporter, pNBC1, and exit at the luminal side by the CFTR/SLC26 transporter-complex is coordinated by the protein IRBIT (inositol-1,4,5-trisphosphate receptors binding protein released with IP3) [6]. This is discussed further and in depth elsewhere [24] but our data suggest that near the site of ATP binding in NBD1, subtle alterations induced by and O-S group substitution next to the Walker B aspartate motif in NBD1 not only reduce the conductance of CFTR (as might be expected by a potential effect on ATP binding) but, unexpectedly, alter the ability of CFTR to respond to a step change in pH or metformin application. Moreover, functional CFTR is also essential for bicarbonate secretion in the small intestine [28] and CFTR-dependent duodenal HCO_3^- secretion in the mouse colon is strictly dependent on colocalization of β_2 -adrenergic receptors and CFTR and the function of the Na^+/H^+ exchanger regulatory factor, NHERF1 [29].

The team of Argent and Gray found inhibition of CFTR Cl^- currents in guinea pig pancreatic duct cells by extracellular HCO_3^- that may have clear implications for the current models of pancreatic ductal HCO_3^- secretion [30]. This may also be of clinical relevance, since a cystic fibrosis phenotype has been observed in a previously healthy adolescent presenting with metabolic alkalosis

[31]. In contrast, CFTR does not seem to be regulated by extracellular pH, according to previous studies on isolated sweat ducts [32]. In contrast Reddy and coworkers found inhibition of phosphorylation by intracellular acidification, which might explain inhibition of CFTR currents by intracellular acidosis observed in the present study through interactions with other protein kinases [32]. The result of the present study pose challenging new questions with respect to the molecular mechanisms for predisposal of S573C towards AMPK-phosphorylation, the residues within NBD1 that are phosphorylated by AMPK, and how S573C within NBD1 affects pH sensitivity of CFTR. Despite these open

questions, the current results suggest caution when biguanide drugs are used in a cystic fibrosis patient or patients carrying one of the CFTR variants predisposing to pancreatitis.

Acknowledgements

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