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Arsenite Regulates Cystic Fibrosis Transmembrane Conductance Regulator and P-glycoprotein: Evidence of Pathway Independence

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Key Words
CFTR • P-glycoprotein • Arsenite • MAPK • Transcription

Abstract
In the past, people have argued for and against the theory of reciprocal regulation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and P-glycoprotein (Pgp). Data have indicated that this may occur *in vitro* during drug-induced selection of cells, and *in vivo* during development. Much of this debate has been caused by a severe lack of mechanistic details involved in such regulation. Our past data indicate that certain Pgp modulators can affect CFTR expression and function. The goal of this study was to investigate the effects of trivalent arsenic (arsenite), a known transcriptional activator of Pgp, on CFTR expression. *In vitro* analyses in T-84 cells that express basal levels of Pgp and CFTR were conducted using a variety of molecular techniques. Expressions of both genes were altered following treatment with arsenite in a dose- and time-dependent fashion. CFTR expression was suppressed almost three-fold by arsenite, along with a concomitant increase in P-glycoprotein expression. We also report that a member of the MAPK-family, the ERK-mediated signaling cascade is implicated in suppression of CFTR expression following treatment with arsenite. However, this particular pathway is not involved in regulation of P-glycoprotein expression in T-84 cells following treatment with arsenite. Thus, the regulatory pathways that control functional expression of CFTR and P-glycoprotein following arsenite treatment in T-84 cells are distinct and independent.

Introduction

P-glycoprotein (Pgp), product of the human MDR-1 gene and the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) are members of the ATP-binding cassette family of proteins (ABC proteins) [1, 2]. CFTR forms a chloride channel that plays an important role in regulating ion and water transport in epithelial cells. Mutations to the CFTR gene cause abnormal physiological conditions in several organs [3]. CF is the most common lethal genetic disease in Caucasians, accounting for 1 in 3000 live births. Multidrug

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resistance involving Pgp over-expression occurs in about 60% of all cancers [4]. Expression of this protein poses a serious hurdle to cancer chemotherapy and is associated with poor survival. Additionally, Pgp is hypothesized to be involved in transporting toxins out of cells and provide an overall protective role in the human body. Bacterial homologues of this protein act as heavy metal transporters [5]. Pgp activation by stress stimuli, such as heat shock, differentiation agents, certain metals, like arsenic and cadmium, and ultraviolet light have provided evidence for this role [6, 7].

Past studies have argued for and against reciprocal regulation of Pgp and CFTR [8-11]. However, the exact mechanism by which this effect takes place is not well understood. For example, prolonged selection of HT-29 cells in media containing Colchicine, a substrate of Pgp, was shown to induce Pgp expression and inhibit CFTR expression. In mice, a developmental pattern of reciprocal expression was reported among different tissues at the mRNA level. Additionally, some reports suggest that these proteins may have overlapping functions [12, 13].

The environmental toxicant arsenic has found use over the years both as a poison and a therapeutic agent in cancer chemotherapy. Arsenic manifests itself in organic compounds such as monomethylarsenic and dimethylarsenic, or in inorganic compounds such as arsenic trioxide and sodium arsenite [14]. Inorganic arsenic is found in both trivalent (III) and pentavalent (V) forms. Arsenite(III) is more toxic to most biological systems, which may be related to greater cellular uptake of this form compared to arsenite(V) [15]. Recent use of arsenic as a therapeutic compound has taken advantage of the potent capability of this agent to induce apoptosis in leukemias [16].

Several studies indicate that arsenite induces Pgp expression. This has been postulated to occur through transcriptional activation of heat shock factor 1 (HSF-1) and through Raf-1 and PKA-dependent pathways [17, 18]. However, the effect of arsenite on CFTR expression has not been investigated. Based on our past research on CFTR with regulators of Pgp, we postulated that this compound would affect expression of CFTR. The goal of the study was to test this hypothesis and if found valid, elucidate the mechanism involved. This study demonstrates that trivalent arsenite can suppress CFTR expression and function in T-84 cells in a manner that is independent of the regulatory pathway/s that alter Pgp expression by the same compound.

Materials and Methods

Cell Culture and treatments

Human colon adenocarcinoma T-84 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/F-12 media (Life Technology Inc., Rockville, MD) containing L-glutamine supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (Life Technologies, Rockville, MD). For experimental purposes, cells were plated out at a concentration of 500,000 cells/well on 6-well plates (Coster, Cambridge, MA) and grown to confluence (3-4 days). All drugs were added to cells in media containing serum. Vehicle (water) alone was added to control cells under identical conditions. Most experiments were performed using 25 μM arsenite (trivalent, sodium salt) and cells were treated for 24 hr, unless otherwise specified in figure legend/s. At this drug concentration, in the presence of growth factors, arsenite was not cytotoxic. Viability was routinely monitored using Trypan blue exclusion. All inhibitors of the MAPK pathway were purchased from Calbiochem, La Holla, CA and solubilized in dimethyl sulfoxide (DMSO). The final concentration of DMSO in experiments was kept below 0.1%. Additional chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Immunoblotting

Following drug treatment, cells were washed once with cold PBS, and scraped in a small volume of lysis buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1% NP-40) containing a protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). Proteins were extracted from the samples by incubation on ice (20 min) with intermediate vortexing and frozen at -70°C following removal of insoluble materials by centrifugation. Protein concentration of each sample was quantified by the BCA assay (Pierce, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7% or 4-15% minigels using 50 μg of protein lysate per lane. Proteins were separated and transferred to PVDF membrane (Millipore, Bedford, MA) at 100 mA for 1 hour in Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Following transfer, the blots were blocked and probed sequentially; first with polyclonal A2 rabbit anti-CFTR antibody [19] at a dilution of 1:1000 overnight at 4°C and then, with a horseradish peroxidase-labeled anti-rabbit secondary polyclonal antibody (Amersham, Piscataway, NJ). The antibody was a generous gift of Dr. W. Skach (Oregon Health Sciences Institute). The specificity of this antibody was established in a prior study [20]. For some studies the highly specific, monoclonal antibody to CFTR, M3A7 [21] was used. For detection of Pgp, the monoclonal anti-Pgp antibody F4 was used (Neomarkers, San Diego, CA) at a concentration of 1:1000 using a similar protocol. Membranes were washed 6X in PBST (PBS + 0.3% Tween-20) at room temperature for 10 min each in between. An anti-Vinculin antibody was used for loading control at a 1:1000 dilution (Sigma) and used for normalization through densitometry as described below. All antibodies against Mitogen Activated Protein Kinase (MAPK) proteins and the anti-PKA/PKC-phosphorylated substrate antibody were purchased from Cell Signaling (Beverly, MA) and used

following the manufacturer's instructions. The blots were developed by ECL+ substrate (Amersham) and exposed to film.

Fluorescent chloride efflux assay

Cells were grown to confluence in 24-well plates and loaded with 10 mM N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE, Molecular Probes, Eugene, OR) overnight as described in detail previously [22]. Briefly, cells were incubated in a chloride-containing, nitrate-free buffer for chloride concentration equilibration inside and outside of the cells and background fluorescence was recorded over 3 min. Following this, the buffer was changed to a chloride-free, nitrate-containing buffer. Both buffers contained 200 μM 8-(4-chlorophenylthio)-adenosine 3', 5'-cyclic monophosphate (CPT-cAMP) (Roche Biochemicals) to stimulate CFTR. In the presence of chloride, MQAE is caged and the probe's fluorescence is quenched. Upon changing over to a chloride-free, nitrate-containing buffer, a chloride gradient is established which results in the rapid exchange of chloride for nitrate in the cells. Over the first few minutes, the rate of increase in free MQAE fluorescence is proportional to the number of chloride channels at the membrane. A Cytofluor 2 plate-reader (PerSeptive Biosystems, Framingham, MA) equipped with a 360 nm excitation/460 nm emission filter set was used to measure MQAE fluorescence in the cells.

Calcein-AM Accumulation Assay

In order to assess Pgp function, cellular accumulation of the fluorescent Pgp substrate Calcein-AM was measured. Cells were seeded at 70% confluency and treated for 24 hr with arsenite in 24-well plates. Following this, 20 μM Verapamil (Sigma) was added to some cells for 30 min to block transport of Calcein-AM. After treatment with Verapamil, all wells were loaded with 0.25 ?M Calcein-AM (Molecular Probes) for 30 min. To measure accumulation of Calcein-AM, cells were washed twice with PBS and analyzed using a fluorescent plate reader using a FITC filter set (Cytofluor 2 plate-reader, PerSeptive Biosystems). Five replicate measurements were taken in each well and five separate wells were analyzed for each treatment group. Cells alone, without the dye was used to normalize for background fluorescence, which was typically 1000-fold lower than cells loaded with Calcein-AM.

Software

Densitometric quantification was carried out using Adobe Photoshop (Adobe Software Inc., San Jose CA) and NIH Image (National Institute of Health, Bethesda, MD) softwares on a Lacie Silverscanner III (Lacie Limited, Beaverton, OR). All statistical analysis was performed using Instat and Prism software programs (Graphpad Software, San Diego, CA).

Results

In order to assess the role of arsenite in regulating CFTR and Pgp expression, T-84 cells were treated with increasing amounts of this agent over a 24 hr time course.

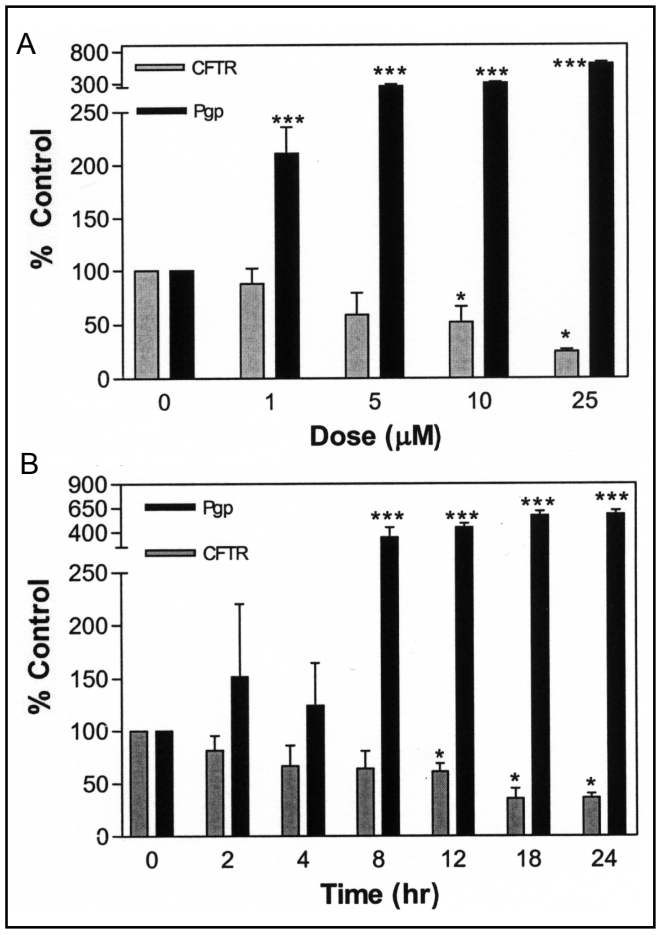


Fig. 1. Effects of arsenite on CFTR and Pgp expression. (A) T-84 cells were treated with increasing amounts of arsenite or vehicle (water). Following incubation for 24 hr, total proteins were isolated and immunoblotted using either the anti-Pgp monoclonal antibody F4 or anti-CFTR antibody M3A7 and digitized blots were analyzed by densitometry. Values reported are Mean + S.E.M. expressed as % untreated control from 3 independent samples. (B) The effect of arsenite on Pgp and CFTR expression over a 24 hr time-course was determined by immunoblot analysis as above. Values reported are Mean + S.E.M. expressed as % untreated control from 3 independent samples. Asterisk/s denotes values that were statistically significant (*, $p < 0.05$; **, $p < 0.01$, ***, $p < .0001$, student's t-test).

Arsenite increased the expression of Pgp and decreased the expression of CFTR proteins in a dose and time dependent manner (Figure 1A, B) as determined by immunoblot analyses. It is worth noting here that in most experiments CFTR is either detected as a single broad band comprising of several minor bands on 7.5% gels, or as two well-separated and distinct bands on high-resolution gradient gels (4-20%) in agreement with literature [21].

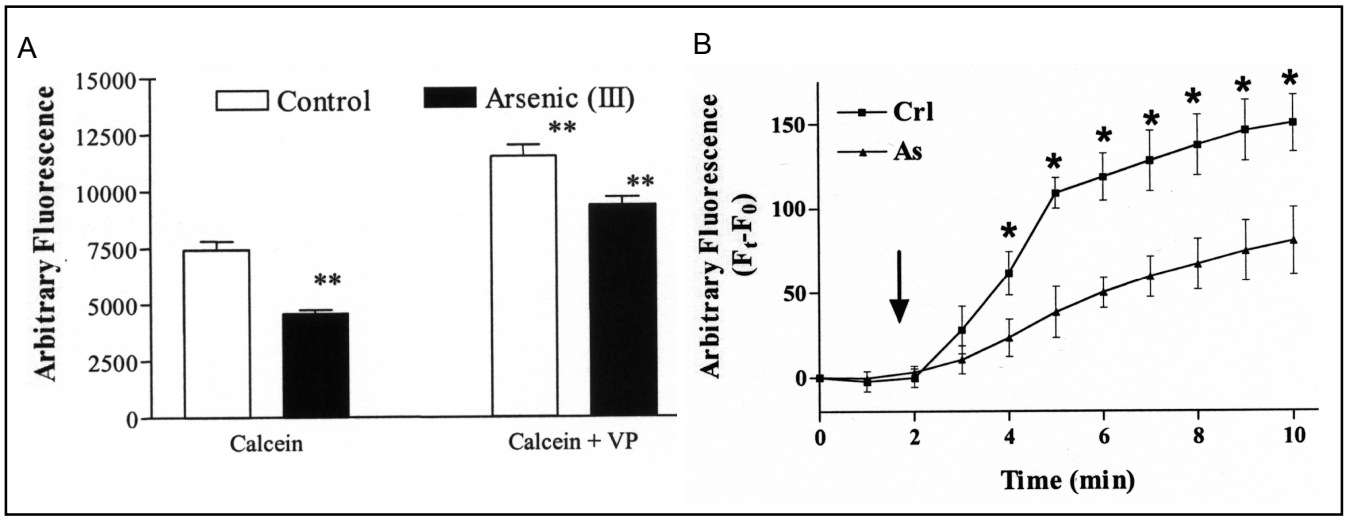


Fig. 2. Effects of arsenite on Pgp and CFTR function. (A) Calcein-AM retention as a measure of Pgp function was evaluated in T-84 cells following treatment with arsenite (black bars) or vehicle (clear bars) for 24 hr. Five independent samples were evaluated for each treatment group. Verapamil (VP) was added to block Pgp function as described above. Mean + S.E.M. has been reported for each group. Asterisks denote values that were statistically significant ($p < 0.01$, student's t-test) from respective control groups. (B) Cpt-cAMP-stimulated increase in MQAE fluorescence as an indicator of functional CFTR expression was measured in T-84 cells following treatment with vehicle (squares) or arsenite (triangles). Five independent samples were evaluated over 10 min in a 12-well plate and corrected for background fluorescence. Values reported for each point is the net change in fluorescence at that time point (F_t) from the fluorescence at the beginning of the experiment (F_0). Mean + S.E.M. has been reported for each time-point. Arrow indicates the time when efflux was initiated. Asterisks denote values that are significantly different between the two groups as determined by student's t-test ($p < 0.05$).

The minor bands on 7.5% gels and the low molecular weight band on gradient gels correspond to the immature, partially glycosylated CFTR molecules at various stages of intracellular processing. Arsenite induced Pgp expression 5- to 6-fold and decreased CFTR expression almost 3-fold compared to vehicle-treated T-84 cells. In all subsequent experiments, cells were treated with 25 μM arsenite for 24 hr as this protocol produced the maximum response in our model system without any detectable cytotoxicity.

The effect of arsenite on Pgp was assessed in the next experiment. Function of Pgp was monitored in T-84 cells using the fluorescent probe Calcein-AM. Pgp rapidly extrudes this compound from cells. As demonstrated in Figure 2A, treatment with arsenite significantly reduced Calcein-AM retention in T-84 cells indicating functional up-regulation of this protein molecule. Blocking Pgp with Verapamil, a well-characterized antagonist, increased Calcein-AM retention in arsenite-treated cells but not to the level of control cells. This can be expected since Verapamil does not block Pgp function completely. As such, a very significant but partial restoration of Pgp function was noted in this experiment. In a complementary experiment, CFTR function was determined in T-84 cells following treatment with arsenite using the chloride probe MQAE. As depicted in Figure 2B, CFTR mediated chloride efflux was significantly reduced in T-84 cells following treatment with arsenite. Thus, these experiments clearly demonstrate a reciprocal effect of arsenite on CFTR and Pgp protein expression and function in T-84 cells.

From a mechanistic standpoint, we wanted to determine whether arsenite-mediated regulation of CFTR and Pgp was transcriptional in nature. Pre-treatment of T-84 cells with DRB, a well-characterized transcriptional inhibitor [23] completely blocked the effects of arsenite on Pgp and CFTR protein expression (Figure 3). This indicated that respective responses of the two genes to arsenite were at the level of gene transcription. However, involvement of altered protein turnover cannot be ruled out completely. Several reports have implicated PKA as the putative molecule involved in up-regulation of Pgp expression following treatment with arsenite [24, 25]. Since both PKA and PKC regulate CFTR expression as well [26, 27], their putative roles, if any, in mediating the response to arsenite in T-84 cells were investigated. Pre-

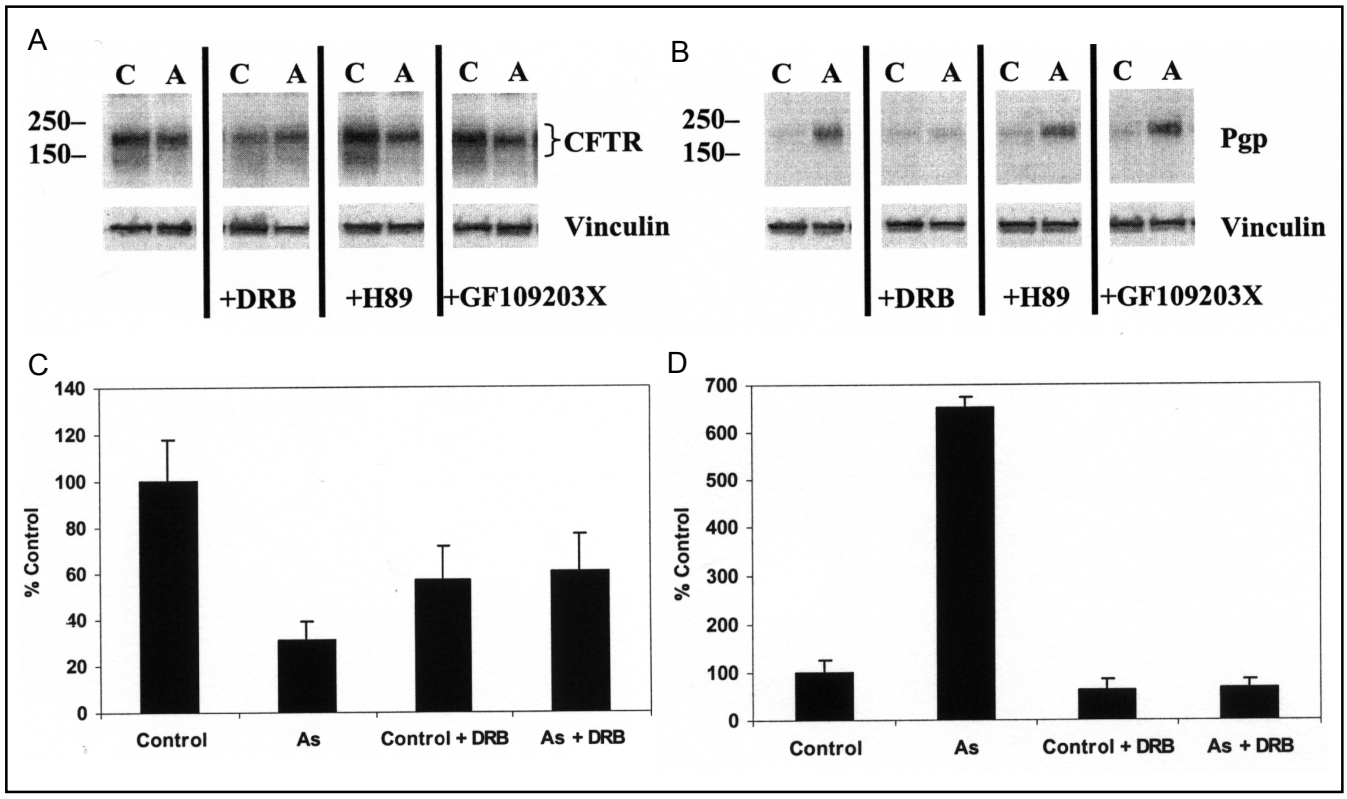


Fig. 3. Arsenite regulates CFTR and Pgp at the level of transcription independent of PKA and PKC. Following pre-treatment for 30 min with DRB (25 μg/ml), H-89 (0.5 μM) or GF 109203X (0.5 μM), cells were treated with arsenite (A) or vehicle control (C). Twenty-four hr post-treatment, cells were lysed and analyzed by immunoblotting using anti-CFTR or anti-Pgp antibodies as described earlier. Vinculin levels were also monitored as a loading control. The inhibitors were in the media throughout the course of the experiment. (A) Effect of specific inhibitors on CFTR. (B) Effect of specific inhibitors on Pgp. (C) Densitometric analysis of CFTR expression following pre-treatment with DRB. (D) Densitometric analysis of Pgp expression following pre-treatment with DRB. Densitometric values are Mean + S.D. expressed as % of control.

treatment of cells with the PKA specific inhibitor H89 or the PKC inhibitor Bisindolylmaleimide 1 (GF 109203X) had no effect on CFTR and Pgp expression (Figure 3A, B, third and fourth panels). The concentration of PKA/PKC inhibitors used in this study was 0.5 μM, a concentration at which both inhibitors are reported to be specific for their substrate enzymes, i.e. PKA [28, 29] and PKC [30]. Furthermore, we also analyzed the level of PKA and PKC substrate-phosphorylation as an indicator of enzyme activity in T-84 cells after treatment with 25 μM arsenite using an antibody that recognizes phosphorylated PKA/PKC substrates in immunoblot assays. As demonstrated in Figure 4, treatment with arsenite did not induce significant phosphorylation of PKA and PKC substrates. As a positive control, cells treated with Calyculin-A were used and demonstrated a robust response. Taken together, these studies indicated that

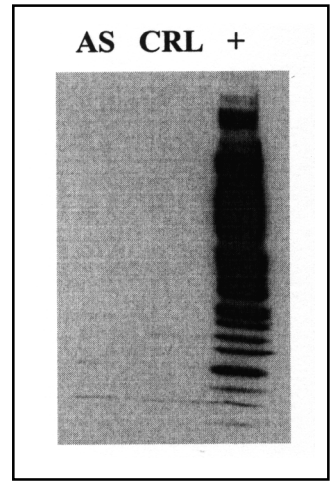


Fig. 4. Lack of phosphorylated PKA/PKC substrates following treatment with arsenite. Cells were treated with arsenite (AS) or vehicle control (CRL) for 30 min, lysed and analyzed by immunoblotting using an anti-PKA-/PKC-phosphorylated substrate polyclonal antibody (1:1000 dilution). A sample from cells treated with Calyculin-A was used as a positive control (+).

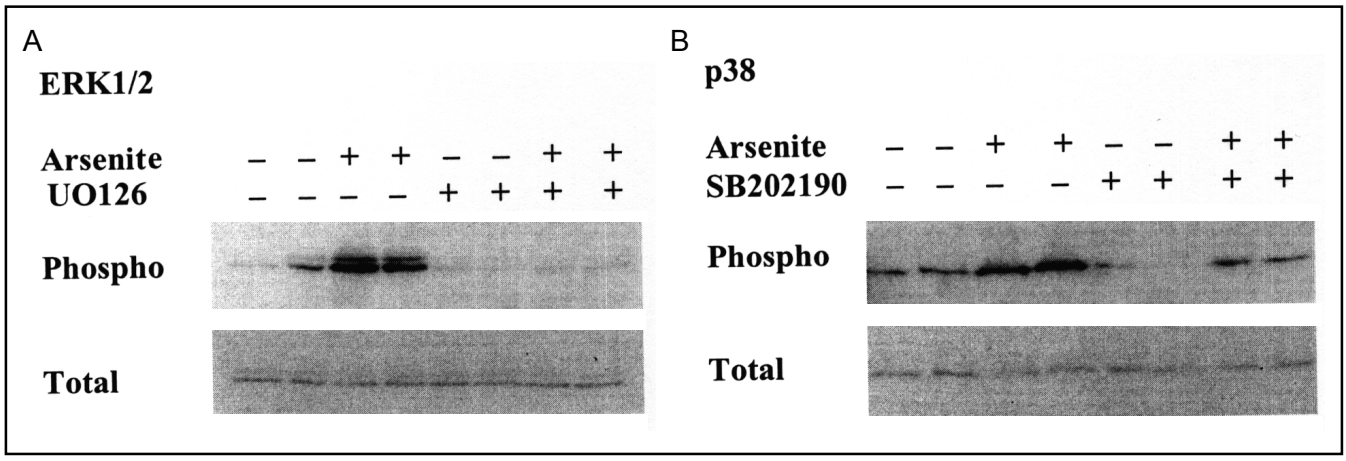


Fig. 5. Effect of arsenite on MAPK phosphorylation. T-84 cells were grown to confluence and treated with 25 μM arsenite or vehicle control as indicated for 30 min. Presence (+) or absence (-) of each compound is indicated. SDS-PAGE was performed with the samples and each MAPK was first detected with an antibody that recognized the phosphorylated form of each MAPK (top panels). The blot was then stripped and re-probed with an antibody that recognized all forms of the same enzyme (bottom panels). (A) Cells were treated as described above or pre-treated with 20 μM ERK specific inhibitor UO126 for 30 min prior to arsenite-treatment. Total proteins were immunoblotted using anti-ERK antibodies as described above. Two independent samples for each treatment group are shown (B) Cells were treated as described above or pre-treated with 20 μM p38 specific inhibitor SB202190 for 30 min prior to arsenite-treatment. Total proteins were immunoblotted using anti-p38 antibodies as described above. Two independent samples for each treatment group are shown.

PKA/PKC enzymes are not important in the regulation of CFTR and Pgp expression by arsenite in T-84 cells under the treatment conditions used in this paper.

Treatment with arsenite has been reported to induce the expression of Mitogen Activated Protein Kinase (MAPK) family members in a several cell types [31]. These kinases represent terminal stages of signaling cascades, initiated by various extra-cellular stimuli such as growth factors, metals, and osmolar stress that result in transcription factor activation and altered gene expression. MAPKs are implicated in the regulation of CFTR and Pgp in response to external stimuli [32, 33]. It was therefore evaluated if MAPKs were activated in T-84 cells following treatment with 25 μM arsenite. There are three major sub-classes of MAPKs. These are p42/p44 extracellular signal-related kinases (ERKs), c-Jun N-terminal protein kinase (JNK)/stress activated protein kinase (SAPK), and p38 MAP kinase (p38). These enzymes are regulated by phosphorylation and dephosphorylation with considerable cross talk between family members [34]. In T-84 cells, arsenite induced the expression of both ERK (Figure 5A) and p38 (Figure 5B), but not SAPK (data not shown). The activation of MAPKs could be blocked by specific inhibitors of each cascade as demonstrated in Figure 5A,B. Since SB202190 inhibits p38 activity, not phosphorylation, only a partial attenuation of the signal was observed, possibly due to inhibition of any subsequent feedback-mediated phosphorylation event. The failure of arsenite to induce phosphorylation of SAPK is not unexpected since the dose of arsenite used in our study did not cause overt cytotoxicity and therefore possibly failed to induce a stress-related response in these cells.

The effects of inhibiting ERK and p38 on CFTR and Pgp were assessed using immunoblotting. As demonstrated in Figure 6A, inhibition of ERK by UO126 blocked the suppression of CFTR by arsenite, whereas, a negative control compound, U0124, had no influence on this effect. By contrast, inhibition of p38 by SB202190 had no effect on suppression of CFTR by arsenite (Figure 6B). These studies clearly indicated that signaling through the ERK pathway was important in the suppression of CFTR expression by arsenite. Interestingly, neither compound had any significant effect on enhanced expression of Pgp. Additionally, pre-treatment of cells with the anti-oxidant N-acetyl cysteine (NAC) had no effect on expression of CFTR or Pgp (Figure 6C). Thus, reactive oxygen species (ROS)-mediated signaling through the NFκB [35] or SAPK pathway [36] was not involved in this response. This observation agrees with lack of

Fig. 6. Effects of MAPK inhibitors on CFTR and Pgp expression. (A) Following pre-treatment of some experimental groups with 20 μM UO126 or UO124 for 30 min, cells were treated with 25 μM arsenite or vehicle for 24 hr. Cells were lysed and equal amounts of total proteins were subjected to SDS-PAGE. Pgp and CFTR levels were sequentially detected by immunoblotting as described earlier. Vinculin levels were also monitored as loading control on the same blot. A representative blot from 3 independent experiments is shown and presence (+) or absence (-) of each compound is indicated. (B) Following pre-treatment of some experimental groups with 20 μM SB202190 or SB202474 for 30 min, cells were treated with 25 μM arsenite or vehicle for 24 hr. Cells were lysed and equal amounts of total proteins were subjected to SDS-PAGE. Pgp and CFTR levels were sequentially detected by immunoblotting as described earlier. Vinculin levels were also monitored as a loading control. A representative blot is shown and presence (+) or absence (-) of each compound is indicated. (C) Some cells were pre-treated with 5 mM NAC overnight or vehicle. Next morning, cells were treated with 25 μM arsenite or vehicle for 24 hr. Cells were lysed and equal amounts of total proteins were subjected to SDS-PAGE. Pgp and CFTR levels were detected by immunoblotting as described earlier. Vinculin levels were also monitored as a loading control. A representative blot is shown and presence (+) or absence (-) of each compound is indicated.

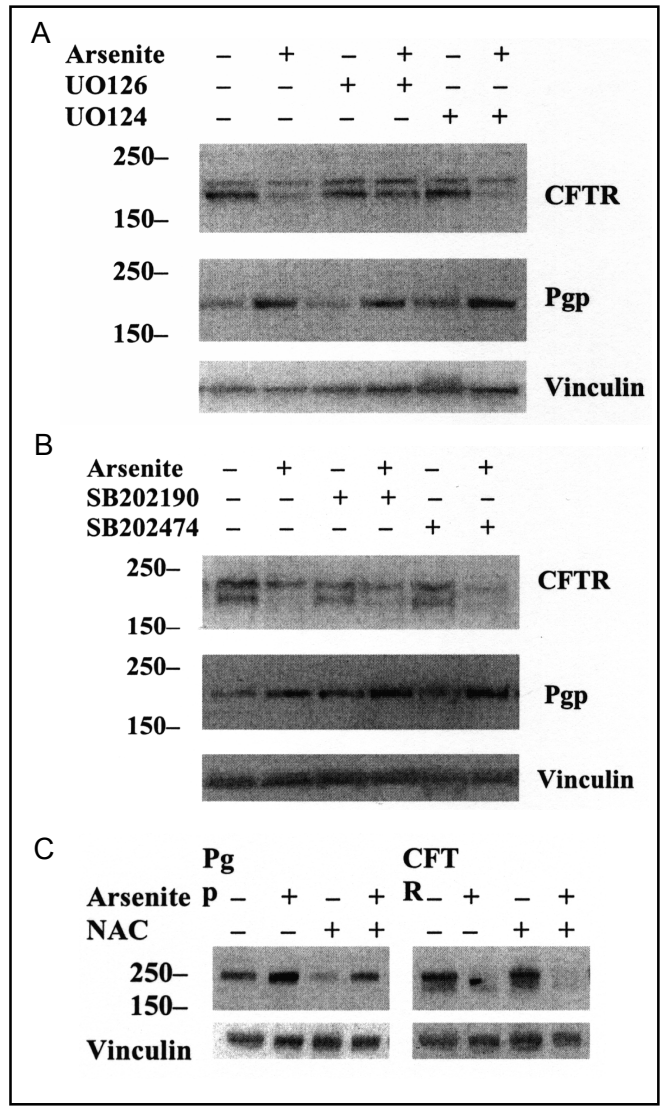
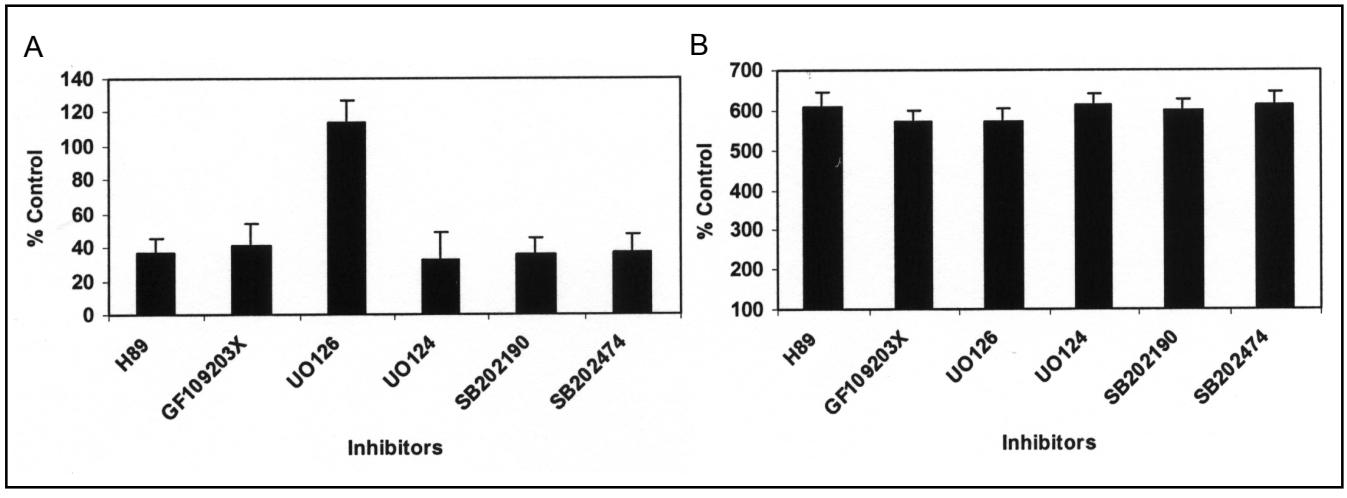


Fig. 7. Summarized effects of various inhibitors on Pgp and CFTR expression. (A) Effects of various inhibitors on CFTR expression following treatment with arsenite. Densitometric values are Mean + S.D. expressed as % of matched control from 3 independent samples. (B) Effects of various inhibitors on Pgp expression following treatment with arsenite. Densitometric values are Mean + S.D. expressed as % of matched control from 3 independent samples.



phosphorylation of SAPK noted in our system following treatment with arsenite as stated above. Taken together, through these studies we conclude that transcriptional down-regulation of CFTR expression by treatment with arsenite in T-84 cells is mediated by ERK family of MAPKs independent of Pgp up-regulation (Figure 7).

Discussion

The aim of these studies was to determine whether arsenite is capable of regulating expression of CFTR and shed mechanistic insight into the process. The effects of arsenite on Pgp have been reported in the past. However, here for the first time, we report that CFTR expression is suppressed by arsenite. Past studies that investigated coordinated expression of these two genes had either focused on long term effects of chemotherapeutic entities on expression of CFTR and Pgp, or on developmental patterns of expression *in vivo*. We used T-84 cells as our model system for these studies. This model has the advantage of endogenously expressing both CFTR and Pgp. Thus, this model is perhaps more physiologically relevant in understanding the effects of various stimuli on Pgp and CFTR. Our data demonstrate that arsenite is capable of reciprocally regulating CFTR and Pgp in T-84 cells at a non-cytotoxic dose (25 μM, 24 hr). This regulation was both time- and dose-dependent (Figure 1). While maximum response was noted at the highest dose of arsenite tested (25 μM), even a relatively low, non-cytotoxic, environmentally relevant dose (5 μM), elicited such a response in these cells. This pattern of regulation, while transcriptional in nature (Figures 3), ultimately resulted in increased functional expression of Pgp and decreased functional expression of CFTR in T-84 cells (Figure 2).

Past studies had indicated that the effects of arsenite on Pgp expression were mediated in part by PKA and Raf-1. CFTR is regulated by PKA and PKC as well. Hence, we conducted studies to investigate if PKA and PKC inhibitors were involved in arsenite-stimulated regulation of CFTR and Pgp. Pre-incubation of T-84 cells with the PKA inhibitor H-89 (0.5 μM), or the PKC inhibitor GF 109203X (0.5 μM) had no effect on arsenite-induced regulation of CFTR and Pgp (Figures 3, 7). Also, elevated levels of phosphorylated PKA and PKC substrates were not observed (Figure 4) in these cells, indicating that PKA/PKC enzyme activities were not significantly altered following treatment with 25 μM arsenite. These pathways do not appear to be important in mediating the effect of arsenite on CFTR and Pgp expression in T-84 cells under our experimental conditions.

In agreement with past reports, certain members of the MAPK family were found to be elevated in these cells following treatment with arsenite (Figure 5). Inhibition of ERK with the specific inhibitor UO126 blocked the down-regulation of CFTR in these cells (Figures 6A, 7). By contrast, the p38 inhibitor SB202190 had no effect on down-regulation of CFTR (Figures 6B, 7). Pre-treatment of cells with NAC to block ROS mediated signaling independently, or through the SAPK pathway, had no effect on CFTR and Pgp (Figure 6C). Interestingly, none of these treatments had any significant effect on up-regulated expression of Pgp. Clearly, divergent signaling events regulate the two genes in response to arsenite in T-84 cells. In literature, reciprocal regulation of these two genes is associated with rate of proliferation and cellular differentiation with up-regulated expression of Pgp noted in proliferating cells and higher levels of CFTR reported in differentiated cells [9, 37]. Arsenite can have both proliferative and apoptotic effects on cells in a dose-dependent fashion. For example, in rat epithelial cells, low-dose arsenite increases proliferation through activation of ERK whereas higher doses induce apoptosis through the JNK pathway [38]. We saw no evidence of JNK activation in our system but ERK activation was noted (Figure 5). Furthermore, the ERK pathway was found to be involved in suppression of CFTR but not activation of Pgp (Figures 6, 7). While the expression of these two genes following treatment with arsenite can be pegged to molecular phenotypes, such as, proliferation or differentiation, from a mechanistic standpoint different pathways seem to be necessary. In any event, these data clearly support the idea that Pgp is an arsenite-inducible gene. Future studies will need to focus on the identification of molecules upstream and downstream of ERK involved in the regulation of CFTR in these cells by treatment with arsenite. Additionally, pathways involved in the induction of Pgp by arsenite in T-84 cells remain to be discovered.

There are practical implications associated with these studies. CFTR has been associated with the pathophysiology of polycystic kidney disease [39, 40]. CFTR is a regulator of fluid volume in cysts and is over-expressed in patients suffering from this disorder. Over-stimulation of CFTR by bacterial enterotoxins is also implicated in secretory diarrhea [41]. This disease is the second leading cause of infant mortality in the developing

world, causing 3 million deaths per year [42] in children under the age of three. Thus, identification of pharmacological agents and pathways that can regulate CFTR expression may be useful in understanding and treating these disorders.

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References

1 Roepe PD, Wei LY, Hoffman MM, Fritz F: Altered drug translocation mediated by the MDR protein: direct, indirect, or both? J Bioenerg Biomembr 1996;28:541-555.

2 Sheppard DN, Welsh MJ: Structure and function of the CFTR chloride channel. Physiological Reviews 1999;79:S23-45.

3 Zielenski J, Tsui LC: Cystic fibrosis: genotypic and phenotypic variations. Annu Rev Genet 1995;29:777-807.

4 Roninson IB: The role of the MDR1 (P-glycoprotein) gene in multidrug resistance in vitro and in vivo. Biochem Pharmacol 1992;43:95-102.

5 Broeks A, Gerrard B, Allikmets R, Dean M, Plasterk RH: Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode Caenorhabditis elegans. EMBO J 1996;15:6132-6143.

6 Bates SE, Currier SJ, Alvarez M, Fojo AT: Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate. Biochemistry 1992;31:6366-6372.

7 Ohga T, Uchiumi T, Makino Y, Koike K, Wada M, Kuwano M, Kohno K: Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance 1 gene. J Biol Chem 1998;273:5997-6000.

8 Breuer W, Slotki IN, Ausiello DA, Cabantchik IZ: Induction of multidrug resistance downregulates the expression of CFTR in colon epithelial cells. Am J Physiol 1993;265:C1711-1715.

9 Trezise AE, Romano PR, Gill DR, Hyde SC, Sepulveda FV, Buchwald M, Higgins CF: The multidrug resistance and cystic fibrosis genes have complementary patterns of epithelial expression. EMBO J 1992;11:4291-4303.

10 Trezise AE, Ratcliff R, Hawkins TE, Evans MJ, Freeman TC, Romano PR, Higgins CF, Colledge WH: Co-ordinate regulation of the cystic fibrosis and multidrug resistance genes in cystic fibrosis knockout mice. Hum Mol Genet 1997;6:527-537.

11 Johannesson M, Nordqvist AC, Bogdanovic N, Hjelte L, Schalling M: Polymorphic expression of multidrug resistance mRNA in lung parenchyma of nonpregnant and pregnant rats: a comparison to cystic fibrosis mRNA expression. Biochem Biophys Res Commun 1997;239:606-611.

12 Lallemand JY, Stoven V, Annereau JP, Boucher J, Blanquet S, Barthe J, Lenoir G: Induction by antitumoral drugs of proteins that functionally complement CFTR: a novel therapy for cystic fibrosis? Lancet 1997;350:711-712.

13 Wei LY, Stutts MJ, Hoffman MM, Roepe PD: Overexpression of the cystic fibrosis transmembrane conductance regulator in NIH 3T3 cells lowers membrane potential and intracellular pH and confers a multidrug resistance phenotype. Biophys J 1995; 69: 883-895

14 Shibata Y, Morita M, Fuwa K: Selenium and arsenic in biology: their chemical forms and biological functions. Adv Biophys 1992;28:31-80.

15 Costa M: Carcinogenic metals. Sci Prog 1998;81(Pt 4):329-339.

16 Zhang P: The use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia. J Biol Regul Homeost Agents 1999;13:195-200.

17 Kim SH, Yeo GS, Lim YS, Kang CD, Kim CM, Chung BS: Suppression of multidrug resistance via inhibition of heat shock factor by quercetin in MDR cells. Exp Mol Med 1998;30:87-92

18 Vilaboa NE, Galan A, Troyano A, de Blas E, Aller P: Regulation of multidrug resistance 1 (MDR1)/P-glycoprotein gene expression and activity by heat-shock transcription factor 1 (HSF1). J Biol Chem 2000;275:24970-24976

19 Xiong X, Bragin A, Widdicombe JH, Cohn J, Skach WR: Structural cues involved in endoplasmic reticulum degradation of G85E and G91R mutant cystic fibrosis transmembrane conductance regulator. J Clin Invest 1997;100:1079-1088.

20 Maitra R, Shaw CM, Stanton BA, Hamilton JW: Increased functional cell surface expression of CFTR and DeltaF508-CFTR by the anthracycline doxorubicin. Am J Physiol Cell Physiol 2001;280:C1031-C1037.

21 Kartner N, Riordan JR: Characterization of polyclonal and monoclonal antibodies to cystic fibrosis transmembrane conductance regulator. Methods in Enzymology 1998;292:629-652.

22 West MR, Molloy CR: A microplate assay measuring chloride ion channel activity. Anal Biochem 1996;241:51-58.

23 Yamaguchi Y, Wada T, Handa H: Interplay between positive and negative elongation factors: drawing a new view of DRB. Genes Cells 1998;3:9-15.

24 Kim SH, Lee SH, Kwak NH, Kang CD, Chung BS: Effect of the activated Raf protein kinase on the human multidrug resistance 1 (MDR1) gene promoter. Cancer Lett 1996;98:199-205.

25 Rohlff C, Glazer RI: Regulation of the MDR1 promoter by cyclic AMP-dependent protein kinase and transcription factor Sp1. Int J Oncol 1998;12:383-386.

26 Fischer H, Illek B, Machen TE: Regulation of CFTR by protein phosphatase 2B and protein kinase C. Pflugers Arch 1998;436:175-181.

27 Chao AC, de Sauvage FJ, Dong YJ, Wagner JA, Goeddel DV, Gardner P: Activation of intestinal CFTR Cl-channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase. EMBO J 1994;13:1065-1072.

28 Hidaka H, Hagiwara M, Chijiwa T: Molecular pharmacology of protein kinases. Neurochem Res 1990;15:431-434.

29 Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H: Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 1990;265:5267-5272.

30 Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C: Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem 1993;268:9194-9197.

31 Bernstam L, Nriagu J: Molecular aspects of arsenic stress. J Toxicol Environ Health B Crit Rev 2000;3:293-322.

32 Ratner AJ, Bryan R, Weber A, Nguyen S, Barnes D, Pitt A, Gelber SE, Cheung,A, Prince A: Cystic fibrosis pathogens activate Ca{super2+}-dependent MAPK signaling pathways in airway epithelial cells. J Biol Chem 2001;276:19267-19275.

33 Wartenberg M, Ling FC, Schallenberg M, Baumer AT, Petrat K, Hescheler J, Sauer H: Down-regulation of intrinsic p-glycoprotein expression in multicellular prostate tumor spheroids by reactive oxygen species. J Biol Chem 2001;276:17420-17428.

34 Winston LA, Hunter T: Intracellular signalling: putting JAKs on the kinase MAP. Curr Biol 1996;6:668-671.

35 Barchowsky A, Roussel RR, Klei LR, James PE, Ganju N, Smith KR, Dudek EJ: Low levels of arsenic trioxide stimulate proliferative signals in primary vascular cells without activating stress effector pathways. Toxicol Appl Pharmacol 1999;159:65-75.

36 Chakraborti S, Chakraborti T: Oxidant-mediated activation of mitogen-activated protein kinases and nuclear transcription factors in the cardiovascular system: a brief overview. Cell Signal 1998;10:675-683.

37 Mylona P, Glazier JD, Greenwood SL, Sides MK, Sibley CP: Expression of the cystic fibrosis (CF) and multidrug resistance (MDR1) genes during development and differentiation in the human placenta. Mol Hum Reprod 1996;2:693-698.

38 Lau AT, Li M, Xie R, He QY, Chiu JF: Opposed arsenite-induced signaling pathways promote cell proliferation or apoptosis in cultured lung cells. Carcinogenesis 2004;25:21-28.

39 Sullivan LP, Wallace DP, Grantham JJ: Epithelial transport in polycystic kidney disease. Physiological Reviews 1998;78:1165-1191.

40 Persu A, Devuyst O, Lannoy N, Materne,R, Brosnahan G, Gabow PA, Pirson Y, Verellen-Dumoulin C: CF gene and cystic fibrosis transmembrane conductance regulator expression in autosomal dominant polycystic kidney disease. J Am Soc Nephrol 2000;11:2285-2296.

41 Kirk KL: New paradigms of CFTR chloride channel regulation. Cell Mol Life Sci 2000;57:623-634.

42 Akabas MH: Cystic fibrosis transmembrane conductance regulator. Structure and function of an epithelial chloride channel. J Biol Chem 2000;275:3729-3732.