Glutathione Release from an Airway Epithelial Cell Line

Colin A. Chang

Department of Physiology
McGill University, Montreal PQ
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ABSTRACT

Glutathione (GSH) is the most abundant antioxidant in the lung and plays an important protective role. The level of GSH in the airway surface liquid (ASL) is significantly reduced in cystic fibrosis (CF) patients, which may exacerbate the oxidative stress in the airway lumen induced by inflammation. GSH release may also be harmful to surface cells lining the airways if it reduces intracellular levels. The mechanisms of glutathione transport by airway epithelia are not known.

GSH release from airway epithelial cells was measured using a colorimetric assay in which oxidized sample glutathione is converted to its reduced form by glutathione reductase in the presence of NADPH. To examine the role of CFTR, glutathione release was studied using an IB3 cell line transfected with CFTR under the control of an inducible promoter. A low rate of glutathione release was measured under control conditions and this was increased by cAMP agonists, however the response was independent of CFTR expression. Hypotonic stress, which causes release of many large organic molecules, also increased glutathione release from IB3 cells irrespective of CFTR. The mechanisms and signaling pathways influencing GSH efflux under isoosmotic and hypotonic conditions were investigated using inhibitors and activators. Brefeldin A, an inhibitor of vesicle trafficking, inhibited hypotonic GSH release. Inhibitors of PI3-kinase and tyrosine kinases elevated GSH release under isoosmotic conditions. Similar GSH effluxes were observed during hypotonic challenge in the presence of PI3-kinase, PKC and tyrosine kinase inhibitors. By contrast, modulators of purinergic and MAP kinase pathways had no effect on hypotonic GSH release. These

experiments demonstrate a cAMP stimulated, CFTR-independent glutathione release mechanism in the airway surface epithelial cell line IB3. The pharmacology of GSH efflux is compatible with a vesicular mechanism, which may be tonically down-regulated by PKC and tyrosine kinases.

RÉSUMÉ

Le glutathion (GSH) est l'antioxydant le plus abondant dans les poumons et joue un rôle important de protection. Le niveau de GSH dans le liquide de surface des voies aériennes est significativement réduit chez les patients atteints de fibrose kystique, ce qui peut exacerber le stress oxidatif dans la lumière des voies aériennes induit par l'inflammation. La libération de GSH peut être également nuisible aux cellules de surface bordant les voies aériennes si le niveau intracellulaire de GSH est réduit. Les mécanismes de transport du glutathion par l'épithelium des voies aériennes ne sont pas connus.

La libération de GSH par les cellules épithéliales des voies aériennes a été mesurée en utilisant un essai colorimétrique dans lequel l'échantillon de glutathion oxydé est converti en sa forme réduite par la glutathion réductase, en présence de NADPH. Afin d'examiner le rôle de CFTR, la libération de glutathion a été étudiée dans la lignée cellulaire IB3 transfectée avec le gène cftr sous le contrôle d'un promotteur inductible. Une faible libération de glutathion a été mesurée dans les conditions de contrôle et était plus élevée en présence des agonistes de l'AMPc, et ceci indépendamment du niveau d'expression de CFTR. Le stress hypotonique, responsable de la libération de plusieurs molécules organiques de grandes tailles, augmentait également la libération de glutathion des cellules IB3 indépendamment de l'expression de CFTR. Les mécanismes et les voies de signalisation influençant l'efflux de GSH dans des conditions isoosmotiques et hypotoniques ont été examinés en utilisant des inhibiteurs et activateurs. La bréfeldine A, un inhibiteur du trafic vésiculaire, inhibait la libération hypotonique de GSH. Les

inhibiteurs de kinase PI3 et des tyrosine kinases induisaient une augmentation de la libération de GSH dans des conditions isoosmotiques. Des efflux similaires de GSH ont été observés lors d'un stress hypotonique en présence des inhibiteurs de la kinase PI3, de la PKC et des tyrosine kinases. A l'inverse, les modulateurs des voies de signalisation purinergique et de la kinase MAP n'ont pas eu d'effet sur la libération hypotonique de GSH. Ces expériences démontrent un mécanisme de libération de glutathion stimulé par l'AMPc et indépendant de CFTR dans la lignée cellulaire épithéliale des voies aériennes IB3. La pharmacologie de transport de GSH semble être liée à un mécanisme vésiculaire, qui pourrait être régulée négativement de façon permanente par la PKC et les tyrosine kinases.

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LIST OF ABBREVIATIONS

CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
ABC	ATP-binding cassette
MRP	Multidrug resistance protein
SUR	Sulfonylurea receptor
MSD	Membrane spanning domain
NBD	Nucleotide binding domain
R domain	Regulatory domain
PKA	Protein kinase A
PKC	Protein kinase C
CaCC	Calcium-activated chloride channel
CPAE cells	Calf pulmonary artery endothelial cells
AE	Anion exchanger
HEK cells	Human embryonic kidney cells
AQP	Aquaporin
CHO cells	Chinese hamster ovary cells
ORCC	Outwardly rectifying chloride channel
ENaC	Epithelial sodium channel
NHERF	Sodium/hydrogen exchanger regulatory factor
ERM	Ezrin-radixin-moeisin
EBP	Ezrin-radixin-moesin-binding phosphoprotein
CAL	CFTR-associated ligand
CAP	CFTR-associated protein
E3KARP	Exchanger 3 kinase A regulatory protein
t-SNARE	Target-membrane-associated-soluble N-ethylmaleimide fusion protein attachment protein (SNAP) receptor
N-terminus	Amino (NH_2) terminus
C-terminus	Carboxy (COOH) terminus
ER	Endoplasmic reticulum
F508	Phenylalanine at amino-acid position 508
GSH	Reduced glutathione
GSSG	Oxidized (disulfide) glutathione
NFκB	Nucleotide factor κB
T_H1 immune response	T-helper cell 1 immune response
T_H2 immune response	T-helper cell 1 immune response
cMRP	Canalicular multidrug resistance protein
cMOAT	Canalicular multispecific organic anion transporter

ASL	Airway surface liquid
PI3-kinase	Phosphatidyl-inositol-3-kinase
PDGF	Platelet derived growth factor
HTC	Hepatoma tissue culture
PIP-kinase	Phosphoinositide phosphate kinase
DNA-PK	DNA-dependent protein kinases
ATM-kinase	Ataxia telangiectasia mutated kinase
ATR-kinase	Ataxia telangiectasia related kinase
DNA	Deoxyribose nucleic acid
mTOR	Mammalian target of rapamycin
SGLT	Sodium/glucose cotransporter
GTP	Guanosine triphosphate (GTP)
G-protein	GTP-binding protein
PLC	Phospholipase C
PLD	Phospholipase D
$cPLA_2$	Cytosolic phospholipase A_2
MAP-kinase/MAPK	Mitogen-activated protein kinase
MEK	MAPK-kinase
MEKK	MEK-kinase
JNK	c-Jun-NH_2-terminal kinases
ERK	Extracellular signal-regulated kinase
ARF	ADP-ribosylation factor
COP	Coat protein complex
JAK	Janus kinase
SYK	Spleen tyrosine kinase
Abl	Abelson tyrosine kinase
Src	Sarcoma (a tyrosine kinase)
Csk	C-terminal Src kinase
FAK	Focal adhesion kinase
Itk	Inducible T-cell kinase
BAL fluid	Bronchoalveolar lavage fluid
HBSS	Hank's balanced salt solution
IBMX	3-isobutyl-1-methyl-xanthine
DMSO	Dimethyl sulfoxide
ANOVA	Analysis of variance
DOX	Doxycycline
TSA	Trichostatin A
FBS	Fetal bovine serum
DMEM	Dulbecco's modified Eagle's medium
F12	Ham's nutrient mixture F12
DTNB	5,5'-dithiobis-2-nitrobenzoate (Ellman's reagent)
EDTA	Ethylenediaminetetraacetic acid
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced
BCA	Bicinchoninic acid
LDH	Lactate dehydrogenase

RIPA buffer	Radioimmunoprecipitation buffer
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
ECL	Enhanced chemiluminescence
db-cAMP	Dibutyryl-cAMP
hypo	Hypotonic HBSS (i.e. 70%HBSS)
γ-GT	Gamma-glutamyl transpeptidase
RVD	Regulatory volume decrease
HMWG	High molecular weight glycoprotein
Chel	Chelerythrine
BFA	Brefeldin A
TRE	Tetracycline-responsive element
mRNA	Messenger ribonucleic acid
GABA	Gamma-amino butyric acid
MDCK cells	Madin-Darby canine kidney cells

CHAPTER 1: INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is a disease that affects the respiratory, digestive and reproductive systems. It is one of the most common genetically inherited diseases, and is believed to affect approximately 60,000 people worldwide (Blau, 2003). Diagnosis is usually made in early childhood, with the median life expectancy currently about 30 years of age. It is caused by a defect in the ability to transport anions across epithelial cell membranes, causing the production of thickened mucus in various exocrine tissues throughout the body. While initially CF patients are able to cope with pathogens and clear mucus, with time this ability decreases as recurrent inflammation causes irreversible damage to the lungs (Ratjen *et al.*, 2003). Other epithelial, particularly exocrine organs, such as the pancreas, sweat glands, and liver are also affected. Chronic pathogenic colonization of the lung occurs, most commonly by opportunistic bacteria such as *Pseudomonas aeruginosa* (Lyczak *et al.*, 2002), which ultimately causes respiratory failure and death.

While the most common and severe defect is the delta F508 mutation, there are hundreds of other mutations that have been documented to cause CF (Zielenski, 2000). Mutations can cause varying degrees of altered CFTR transport activity, due to misfolding and retention in the endoplasmic reticulum as well as decreased cell surface expression, resulting in a broad spectrum of CF phenotypes (Kulczyczki *et al.*, 2003). For instance, a patient carrying two non-functional CFTR alleles may exhibit more severe symptoms such as pancreatic enzyme insufficiency, chronic pulmonary obstruction and

infections, or male infertility due to blockage of the vas deferens. However, a patient carrying only one allele that encodes a partially functional CFTR channel may exhibit milder symptoms and retain most pancreatic function.

1.1.1 CFTR (Cystic Fibrosis Transmembrane Conductance Regulator)

CFTR is a membrane glycoprotein which functions as an ATP dependent, cAMP-regulated chloride channel. It is a member of the ATP-binding cassette (ABC) superfamily that includes, among others, MRP (multidrug resistance protein), P-glycoprotein and the sulfonylurea receptor SUR. There are currently 48 human genes known to encode for ABC transporters, and these have been divided into 7 subfamilies named ABCA to ABCG (Dean *et al.*, 2001). ABC transporters are found in most cell types, where they actively transport a wide variety of organic compounds and ions across membranes.

1.1.2 CFTR structure

CFTR is composed of a pair of hydrophobic membrane spanning domains (MSDs), each containing six transmembrane α-helical domains, and two cytosolic NBDs (Nucleotide Binding Domains) that bind ATP (Figure 1). In addition, CFTR contains a unique R (Regulatory) domain that has multiple phosphorylation sites. The exact tertiary structure for the entire CFTR protein is not known, however crystal structures have been solved for bacterial NBDs (e.g. Hung *et al.*, 1998; Diederichs *et al.*, 2000; reviewed by Hanrahan *et al.*, 2002) and the entire *E.coli* ABC transporter MsbA (Schmitt, 2002). It is

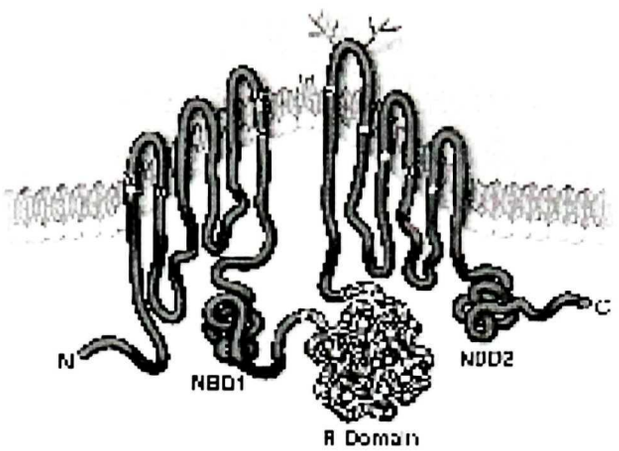


Figure 1: Structure of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Schematic diagram of CFTR in a cell membrane showing transmembrane α-helical domains, amino (N) and carboxy (C) termini, nucleotide-binding domains 1 (NBD1) and 2 (NBD2), and the regulatory domain (R-Domain). (Picture credit: Hanrahan *et al.*, 2003)

thought that NBD1 and NBD2 form a head-to-tail dimer, and that conformational changes are transmitted to the channel by the cytoplasmic loops between the membrane spanning segments.

1.1.3 CFTR function

CFTR gating occurs through two main processes, ATP interactions at the NBDs and phosphorylation of the R domain. There are multiple phosphorylation sites on the R domain for cAMP-dependent PKA and PKC (Sheppard *et al.*, 1999). While some phosphorylation sites have been found to inhibit CFTR activation (serine 737 and 768; Wilkinson *et al.*, 1997), open probability generally increases as more sites are phosphorylated (Akabas *et al.*, 2000). It has been suggested that CFTR is regulated via a "ball and chain" mechanism, similar to that proposed in Shaker K^+ channels (Sheppard *et al.*, 1999). In this scheme, unphosphorylated R domain acts as a physical barrier occluding the channel, whereby phosphorylation of the R domain causes release of the block and opening of the pore entrance. However, recent studies in this laboratory suggest that phosphorylation of the R domain enhances its interaction with the N terminal half of CFTR (Irvine, 2002). Phosphorylation of the R domain presumably allows coupling of conformational changes at the NBDs to channel gating, however the mechanism is not understood.

1.1.4 CFTR interaction with other proteins

In addition to its own function as a chloride channel, CFTR is known to affect the activity of other ion transporters. In two CFTR-expressing airway epithelial cell lines, forskolin administration increased intracellular calcium levels (Walsh *et al.*, 2000), which

could then affect the activity of other calcium signaling-dependent membrane transporters. Calcium-activated chloride channels (CaCCs) were also recently found to be inhibited by CFTR, as CFTR expression in bovine pulmonary artery endothelial cells (CPAE) caused a decrease in CaCC-mediated currents (Wei *et al.*, 1999). Activation of CFTR prior to CaCC stimulation caused a further decrease in CaCC currents. CFTR has been reported to affect Cl^-/HCO_3^- anion exchangers (AEs), as cAMP activation of CFTR stimulated AE activity in transfected NIH-3T3 and HEK-293 cells (Lee *et al.*, 1999). CFTR can also affect the activity of aquaporins, as stimulated CFTR increased water permeability via aquaporin 3 (AQP3) in *Xenopus* oocytes and Chinese hamster ovary (CHO) cells (Schreiber *et al.*, 1999). Other membrane proteins reportedly affected by CFTR include outwardly rectifying chloride channels (ORCC) (Schwiebert *et al.*, 1995), amiloride-sensitive ENaC sodium channels (Stutts *et al.*, 1995), ROMK2 (McNicholas *et al.*, 1996) and $K_{ir}6.1$ potassium channels (Ishida-Takahashi *et al.*, 1998). The mechanisms of these regulatory effects are not known but are likely to be indirect.

CFTR interacts with several proteins that contain PDZ domains such as the Na^+/H^+ exchanger regulatory factor (NHERF), also known as the ezrin-radixin-moeisin (ERM)-binding phosphoprotein of 50 kDa, EBP50 (Kleizen *et al.*, 2000). Other such PDZ-containing proteins include the CFTR-associated ligand (CAL) (Cheng *et al.*, 2002), the CFTR-associated protein (CAP70; Wang *et al.*, 2000) and E3KARP (Nilius *et al.*, 2003). These proteins bind to a highly conserved PDZ-interacting domain in the C-terminus of CFTR and may help localize CFTR at the apical membrane of polarized epithelial cells (Moyer *et al.*, 1999). However, a recent pulse-chase study suggests that CFTR is initially inserted randomly into both apical and basolateral membranes, without

the help of PDZ domains (Swiatecka-Urban *et al.*, 2002). CFTR is then selectively retained at the apical membrane through its interaction with PDZ domain proteins that increase its half-life in the apical membrane by enhancing the efficiency of endocytic recycling. t-SNARE proteins such as syntaxin 1A have also been reported to interact directly with CFTR. Syntaxin 1A has been found near the apical membrane surface in human and mouse airway and intestinal epithelial cells in close physical association with the N-terminus of CFTR (Naren *et al.*, 2000). In *Xenopus* oocyte experiments, this binding of syntaxin 1A to CFTR is thought to decrease cAMP-activated Cl^- currents by disrupting interactions between the N terminus and the R domain (Naren *et al.*, 1998).

1.1.5 CFTR biogenesis/trafficking/degradation

CFTR is synthesized by ribosomes and translocated to the lumen of the endoplasmic reticulum (ER), where it may be localized through various mechanisms involving PDZ domain-containing proteins and SNARE (Kleizen *et al.*, 2000). However, even wild-type CFTR processing is inefficient, with only 25% of immature CFTR being converted into the mature glycosylated form and exported from the ER. CFTR with the delta F508 mutation is synthesized but then trapped in the ER, where it eventually becomes polyubiquinated and degraded by cytosolic proteasomes (Akabas, 2000). However, patch clamp studies suggest that ER-localized delta F508 CFTR protein has cAMP-regulated chloride channel activity (Pasyk *et al.*, 1995). In light of these results, one therapeutic modality being pursued is to "rescue" delta F508 CFTR from the ER by enhancing its trafficking to the apical membrane, with the hope that this protein can restore at least partial functionality to epithelia (Riordan, 1999). A large proportion of

CF patients have mutations that allow normal processing and targeting of CFTR protein to the plasma membrane, indicating altered functional properties (Welsh *et al.*, 1993).

1.1.6 CFTR permeation properties

Reversal potential experiments using a range of sodium chloride concentrations showed that CFTR channels are selective for anions over cations (Tabcharani *et al.*, 1997). CFTR is permeable to polyatomic anions in a lyotropic sequence of NO_3^->Cl^- >HCO_3^->formate>acetate (Linsdell *et al.*, 1997). Based on the size of the largest permeant anion, the size of the CFTR pore is believed to be a minimum of 5.3 angstroms in diameter (Linsdell *et al.*, 1997), however, large organic anions up to 13 angstroms in diameter are slightly permeant from the intracellular side of CFTR (Linsdell *et al.*, 1998). Other ions, water and urea are reported to permeate through CFTR. ATP permeation has been controversial, but most studies indicate that CFTR is impermeable to ATP (reviewed by Akabas, 2000). The large organic anion glutathione, both oxidized (GSSG) and reduced (GSH) forms, were suggested to permeate through CFTR based on electrophysiological data (Linsdell *et al.*, 1998). Recordings made with inside-out excised membrane patches revealed currents carried by anions flowing from nominally Cl^--free GSH solutions, and macroscopic Cl^- current through CFTR was blocked reversibly by intracellular GSH and GSSG, consistent with an open-channel block mechanism from the intracellular end of the pore. In addition, patch clamp studies under biionic conditions indicate that intracellular gluconate block of CFTR is partly relieved by increasing the extracellular Cl^- concentration, suggesting that external Cl^- can bind in the pore and destabilize binding of internal gluconate (Linsdell *et al.*, 1997).

1.2 Glutathione

Glutathione (γ-Glu-CysH-Gly) is a low molecular weight tripeptide found in most living organisms. It is involved in many processes within the body, with key roles in metabolic pathways, transport of compounds and metabolites, immune system modulation, and neutralization of oxidants and toxic compounds (Meister, 1988; see below). The –SH functional group accounts for the antioxidant properties of glutathione, as it neutralizes free radicals by acting as an electron donor.

1.2.1 Glutathione synthesis and metabolism

Glutathione is synthesized inside the cell through the action of γ-glutamylcysteine and GSH synthetases (Meister, 1988). It is degraded by the action of γ-glutamyltranspeptidase, an enzyme attached to the outer leaflet of the plasma membrane, particularly in cells of the kidney and liver. This enzyme uses the γ-glutamyl portion of glutathione to form γ-glutamyl amino acids, which can be transported into the cell (Cantin *et al.*, 1991).

1.2.2 Glutathione functions

Glutathione has several important and varied roles in the body:

1) Conjugation: GSH conjugates with various compounds such as cytotoxins and carcinogens so that they can be safely excreted from the body. In addition, glutathione conjugation with leukotriene A_4 is essential for formation of leukotrienes C_4 and D_4, which are important mediators of inflammation and vascular permeability (Samuelsson, 1983).

2) Immune responses: Intracellular GSH levels play an important role in modifying the immune response. Inactivating γ-glutamylcysteine synthetase to deplete cells of GSH interferes with activation of NFκB signaling (Dröge *et al.*, 1994) and inhibits the proliferation of human T-lymphocytes (Suthanthiran *et al.*, 1990). In addition, glutathione levels in antigen-presenting cells such as macrophages, dendritic cells and B-lymphocytes help determine the equilibrium between Th1 and Th2 immune responses (Peterson *et al.*, 1998).

3) Transport: Various electrophilic compounds, metabolites and xenobiotics are transported across membranes as glutathione S-conjugates (Keppler, 1999) by multidrug resistance proteins (MRPs). Export of oxidized (disulfide) glutathione by MRP1 and MRP2 has also been observed (Leier *et al.*, 1996). The role of MRP in GSH transport is discussed in more detail below. In addition, breakdown of extracellular glutathione releases a γ-glutamyl group which can combine with amino acids such as L-cysteine and L-glutamine. These form γ-glutamyl acids, which are easily transported across the membrane via the γ-glutamyl cycle for use by the cell (Meister, 1988).

4) Antioxidant: GSH is an important antioxidant which removes potentially dangerous oxidizing species such as peroxides (Cantin *et al.*, 1991). In a healthy cell, most glutathione is in the reduced form to ensure that adequate electron donors are available for neutralization of free radicals and other toxic oxidants.

1.2.3 MRP and glutathione transport

Based on studies of rat heart sarcolemma vesicles (Ishikawa, 1989) and rat erythrocyte inside-out membrane vesicles (Heijn *et al.*, 1992), it has been known for many years that glutathione S-conjugates are transported across plasma membranes by an ATP-dependent active transport system. This transporter was later shown to be the multidrug resistance related protein 1 (MRP1) (Leier *et al.*, 1994; Jedlitschky *et al.*, 1994), so named because its upregulation in cancer cells by one chemotherapeutic agent confers a resistance to a wide variety of other drugs. MRP1 is widely expressed in erythrocytes, hepatocytes, and cardiac cells (Keppler, 1999), and in the plasma membrane of hepatocytic carcinoma cells (Nies *et al.*, 2001). It belongs to the ABC transporter superfamily (Keppler *et al.*, 1997) like CFTR, but has an additional N-terminal membrane domain consisting of four to five helices. Substrates transported with high affinity by MRP1 include bilirubin glucuronide conjugates in the liver (Jedlitschky *et al.*, 1997) and glutathione S-conjugates such as leukotriene C_4 (Ishikawa *et al.*, 1990). It also transports oxidized glutathione (GSSG), albeit with much lower affinity (Leier *et al.*, 1996).

The isoform MRP2 also transports glutathione conjugates and is known as the canalicular multidrug resistance protein (cMRP), or canalicular multispecific organic anion transporter (cMOAT). Initially thought to be the same protein, MRP2 is in fact structurally distinct from MRP1, sharing only 49% amino acid sequence homology (Keppler *et al.*, 1997). Immunofluorescence staining and confocal laser scanning microscopy indicate that MRP2 is found in liver canalicular apical membrane (Büchler *et al.*, 1996). Despite considerable divergence in amino acid sequence, MRP2 is an ATP-requiring active transporter with substrate specificity similar to that of MRP1. When the

MRP2 gene is mutated, humans can develop Dubin-Johnson syndrome, an inherited disorder of the liver which causes defective transport of these conjugated anions across the hepatocyte canalicular membrane and into the bile for elimination (Paulusma *et al.*, 1996).

Other human MRP isoforms, including MRP3, MRP4, MRP5, MRP6, MRP7, MRP8 and MRP9 have also been discovered, but their physiological functions remain to be fully clarified. MRP3 is localized to the liver, small intestine and colon (Kiuchi *et al.*, 1998), and transports glucuronide conjugates, glutathione S-conjugates and monoanionic bile salts (Zeng *et al.*, 2000). MRP4, which is widely expressed but is most abundant in prostate cells (Lee *et al.*, 1998), transports cyclic nucleotides and estradiol 17-β-D-glucuronide (Chen *et al.*, 2001). MRP5 is also widely expressed, but with high expression localized to the skeletal muscle and brain (Kool *et al.*, 1997). RNase protection assays indicate MRP6 is highly expressed in liver and kidney, with very low expression elsewhere in the body (Kool *et al.*, 1999). The physiological role of MRP6 has not been determined, although there is speculation that it plays a role in detoxification based on its high expression in the excretory organs. It does not contribute to drug resistance in the cell lines analyzed and apparently does not transport organic anion conjugates (Lai *et al.*, 2002). Little is known about MRP7, MRP8 and MRP9 as they have only recently been discovered (Hopper *et al.*, 2001; Tammur *et al.*, 2001).

1.2.4 Glutathione: Role in the airways

The airway lumen is unique in that it consists of a large surface area which is continually exposed to the environment and potentially damaging substances such as free radicals and other oxidizing species. These substances may originate from various sources such as air pollutants, or radicals and peroxides released from alveolar macrophages during inflammatory episodes. Several antioxidant molecules are present on the epithelial surface which serve to protect the cells, including the free-radical scavenger glutathione. The physiological concentration of GSH inside the cell is relatively high (1-10mM; Kogan *et al.*, 2001), but it is also the most abundant extracellular antioxidant in the lung, and may play an important protective role in the airway surface liquid (ASL). GSH in the extracellular lining fluid of normal nonsmokers is ~300μM, much higher than in plasma and other extracellular fluids (Cantin *et al.*, 1987). Many lung cells secrete glutathione, including alveolar macrophages, fibroblasts, lymphocytes and epithelial cells (Cantin *et al.*, 1991). One explanation for the high level of GSH in the lung may be the low level of γ-glutamyl transpeptidase in this organ. This enzyme, which is responsible for catabolizing GSH, is at about 300-fold lower levels than in kidney (Cantin *et al.*, 1991). Emphysema, asthma, hyperoxia, pulmonary fibrosis and low levels of glutathione in the airway surface liquid are associated with abnormally high oxidant/antioxidant ratios (Comhair *et al.*, 2002).

1.3 Signaling pathways involved in cellular stress responses

Many signaling pathways can be activated when cells are exposed to stressful chemical and osmotic environments.

1.3.1 PI3-kinase pathway

Phosphatidyl-inositol-3-kinases (PI3-kinases) are a group of lipid kinases involved in various aspects of cellular regulation such as cell growth, survival, intracellular trafficking, immune responses and cellular motility. The downstream targets of PI3-kinases include phospholipase C-γ and protein kinases B and C. There are three main types which have been classified according to sequence homology, functional characteristics and substrate specificity (Stein, 2001). In humans, class I enzymes are subdivided into Ia (p110α, p110β, p110δ) and Ib (p110γ). The classical p110α and p110β are expressed ubiquitously throughout the body, while p110δ and p110γ are found mostly in leucocytes. Class Ia PI3-kinases are activated by most tyrosine kinase coupled trans-membrane receptors, such as those for insulin and platelet derived growth factor (PDGF) (Wymann *et al.*, 1998). By contrast, the class Ib enzyme p110γ is activated by the βγ subunits of heterotrimeric G proteins (Stein, 2001). Little is known about class II PI3-kinases, although they are probably activated by tyrosine kinase-coupled receptors (Arcaro *et al.*, 2000). Finally, class III PI3-kinases are constitutively active and involved in intracellular protein trafficking in mammalian cells (Stein, 2001).

The PI3-kinase pathway may mediate diverse cellular stress responses, since it is activated by hypotonic stress in hepatocytes (Krause *et al.*, 1996) and by shear stress in

human umbilical vein endothelial cells (Dimmeler *et al.*, 1999). Inhibiting PI3-kinase decreases hypotonically-induced ATP efflux from HTC hepatoma cells (Feranchak *et al.*, 1998), suggesting a role in cell volume regulation.

Two drugs commonly used to inhibit PI3-kinase are LY294002 and wortmannin. LY294002 is derived from the broad-spectrum kinase inhibitor quercetin, whereas wortmannin is a compound originally isolated from *Penicillium funiculosum* (Stein, 2001). LY294002 is a pure competitive inhibitor of ATP binding whereas wortmannin irreversibly alkylates the PI3-kinase ATP-binding site, while both drugs appear to display little selectivity between classes of the PI3-kinase family (Stein, 2001). The specificity of these compounds has been explored and they are reasonably selective, however at a minimum concentration of 6.9μM, LY294002 does inhibit casein kinase 2, an enzyme involved in many cell processes (Davies *et al.*, 2000). LY294002 was also found to inhibit the PI3-kinase related protein kinases (Stein, 2001). The PI3-kinase related protein kinases are a group of enzymes whose kinase domains closely resemble those of PI3-kinases. These include: PI4 kinases and phosphoinositide phosphate (PIP)-kinases (involved in maintaining the phosphoinositide cycle), DNA-dependent protein kinases (DNA-PK), ataxia telangiectasia mutated (ATM) and ataxia telangiectasia related (ATR) kinases (collectively involved in functionally linking DNA repair machinery to cell cycle control), and mTOR (involved in regulation of protein synthesis). Wortmannin was also found to inhibit the PI3-kinase related protein kinases but with lower potency.

1.3.2 Protein kinase C (PKC) pathway

Protein kinase C is activated in response to many extracellular hormones, neurotransmitters and growth factors (Nishizuka, 1992). These agonists initiate membrane phospholipid hydrolysis through phospholipase C, D and A_2-mediated pathways, producing diacylglycerol and arachidonic acids which activate PKC. Eleven PKC isozymes have been defined in mammalian tissues which differ in their tissue-specific expression, intracellular location, and catalytic properties (Nishizuka, 1992; Schenk *et al.*, 1999). PKC plays a role in many body functions such as cell cycle regulation, where PKC is known to influence both G1 progression and the transition between G2 and mitosis phases in mammalian cells (Livneh *et al.*, 1997). PKC is also involved as an intermediate of the signal transduction pathways leading to cellular apoptosis (Lavin *et al.*, 1996) and degranulation of mast cells involved in inflammatory processes (Nechushtan *et al.*, 2001). PKC activation plays a role in several membrane transport processes such as Na^+/H^+ exchanger function (Saxena *et al.*, 1993), sodium glucose cotransporter (SGLT1) expression via regulated exocytosis (Wright *et al.*, 1997), and mediation of catecholamine exocytosis in bovine chromaffin cells (Borges *et al.*, 2002). Cell swelling is also known to activate PKC in hepatocytes (Roman *et al.*, 1998), and protein kinase C inhibitors such as the commonly used drug chelerythrine reduced the osmotically-induced release of aspartate, glutamate, taurine and glycine in rat cardiac cells (Song *et al.*, 1998). However, the role of PKC in swelling activated release of osmolytes is uncertain, as other studies have raised the possibility that chelerythrine is in fact not a potent PKC inhibitor (Lee *et al.*, 1998; Eckly-Michel, 1997).

1.3.3 Purinergic pathway

Adenosine triphosphate (ATP) is an important high-energy molecule involved in active ion transport, cellular metabolism and motility. ATP efflux occurs constitutively at a slow rate from some cells (Lazarowski *et al.*, 2000), and has been suggested that this event occurs via conductive ATP release channels, via non-conductive ATP transporters, permeases or flippases, or via exocytosis of ATP-containing vesicles (Schwiebert *et al.*, 2001). Once outside the cell, ATP and its degradation products ADP and adenosine can act as autocrine/paracrine signaling molecules. ATP and ADP bind to purinergic receptors, either G-protein coupled P2Y receptors or ionotropic P2X receptor channels, whereas adenosine binds to G-protein coupled P1 receptors (Schwiebert *et al.*, 2001). P2Y receptors are expressed in most epithelial cells, and activate phospholipases PLC, PLD and $cPLA_2$, as well as protein kinases A and C (Insel *et al.*, 2001). P2X receptors are expressed in multiple cell types including epithelial, endothelial, and neuronal cells (North, 2002). They are non-selective cation channels that open in response to ATP binding, which is subsequently followed by plasma membrane depolarization and influx of calcium. Seven subtypes have been cloned thus far, which include P2X1 through P2X7 (Khakh *et al.*, 2001), and it is thought that a functional receptor consists of three or more of these subunits (North, 2002). Finally, adenosine-binding P1 receptors are found in many cell types such as neuronal cells, arterial cells and lymphocytes (Klinger *et al.*, 2002). There are four subtypes, which are generally subdivided into stimulatory and inhibitory groups. The inhibitory receptors include A_1 and A_3 receptors, which activate phospholipase Cβ and can modulate the activity of both K^+ and Ca^{2+} channels (Fredholm *et al.*, 2000). By contrast, the stimulatory receptors include A_{2a} and A_{2b} receptors, which

generate cAMP through activation of adenylate cyclase. Signaling initiated by ATP can affect many cellular functions, such as epithelial chloride transport and cell volume regulation. ATP is released in response to intracellular cAMP (Schwiebert *et al.*, 1995), mechanical stress (Grygorczyk *et al.*, 1997) and osmotic stress (Guyot *et al.*, 2002; Wang *et al.*, 1996), all of which are consistent with a role in cell volume regulation.

1.3.4 MAP kinase pathway

Mitogen-activated protein kinase (MAPK) signaling pathways are implicated in a broad array of cell processes including proliferation, differentiation, motility and apoptosis. MAP kinases are activated via a three-tiered cascade that involve phosphorylation by upstream MAPK kinases (MEKs), which in turn are phosphorylated by MEK kinases (MEKKs) further upstream. This signaling pathway ultimately results in phosphorylation of terminal MAP kinase substrates. There are three subgroups of MAPKs, c-Jun-NH_2-terminal kinases (JNKs), p38 MAPKs, and the extracellular signal-regulated kinases (ERKs). MAP kinases are activated by many stimuli including mitogens, differentiation factors and various stress signals (Schramek, 2002). In particular, cell swelling and mechanical stress activate p38 MAPKs and ERKs. For example, p38 MAP kinases and Erk 1 and 2 are activated by hypoosmotic stress in rat hepatocytes (Kim *et al.*, 2000; vom Dahl *et al.*, 2001). In addition, fluid shear stress has been shown to activate Erk 1 and 2 in human umbilical vein endothelial cells (Surapisitchat *et al.*, 2001). A variety of inhibitors are available for studying the role of stress-activated signal pathways. These include SB203580, which specifically inhibits p38 MAP kinase (Cuenda *et al.*, 1995), as well as the MEK1-specific inhibitor PD98059, which prevents downstream activation of Erk 1 and 2 (Alessi *et al.*, 1995).

1.3.5 Exocytosis

The plasma membrane separates the cellular contents from the extracellular environment and is in a state of flux, as membrane proteins and lipids are continually added and removed from the membrane by vesicular insertion and retrieval (Park *et al.*, 2000). This balance can often be altered by hormones, cytokines, protein kinases and metabolites to accommodate the changing requirements of a cell (Royle *et al.*, 2003). Processes that involve intracellular motility, such as vesicular transport and plasma membrane recycling, occur via myosin and kinesin/dynein motors that run along a network of either actin filaments or microtubules, respectively (Park *et al.*, 2000). Two protein families, known as ARF (ADP-ribosylation factor) and COP (coat protein complex), are integral parts of the secretory pathway (Lippincott-Schwartz *et al.*, 1998). As cargo is being prepared for export in the ER, COPII assembles onto the activated ER membrane. This causes migration of protein and lipids to the COPII-bound region, and after ER membrane blebbing, they become enveloped in a COPII-coated vesicle. As the vesicle moves towards the Golgi complex, the COPII-coating gradually gets replaced by COPI proteins and becomes pre-Golgi intermediates. ARFI is essential in mediating this process, along with the subsequent recruitment and fusing of the vesicle/COPI/ARFI complex to the *cis* face of the Golgi complex. Indeed, drugs such as brefeldin A, which inhibits ARFI activation, have been found to prevent vesicular transport between the ER and Golgi because COPI no longer associates to Golgi membranes (Donaldson *et al.*, 1990). Membrane trafficking processes, such as exocytotic apical plasma membrane insertion in renal epithelial cells, are known to be activated by hypoosmotic stress (Reid *et al.*, 2000). In *Xenopus* oocytes, brefeldin A also inhibits ATP release (Maroto *et al.*, 2001), which is known to occur in response to osmotic stress. This raises the possibility

of exocytosis as a general mechanism for extruding macromolecules during osmotic stress.

1.3.6 Protein tyrosine kinase pathway

Protein tyrosine kinases are crucial for conveying extracellular signals to the intracellular compartment. There are two groups of protein tyrosine kinases, the receptor class and the non-receptor class. Members of the receptor class are comprised of four major domains; a ligand-binding extracellular domain, a membrane spanning transmembrane domain, a tyrosine kinase domain that mediates biological responses, and a juxtamembrane domain that contains autophosphorylated regulatory domains and links the tyrosine kinase and transmembrane domains (Cadena *et al.*, 1996). Receptor class kinases have been organized into several subgroups based on differences in structural motifs, especially in the ligand-binding domains to which many different hormones and signaling factors attach themselves. Tyrosine kinases activate many downstream signaling pathways already discussed, including PI3-kinase and MEK. Non-receptor class protein tyrosine kinases do not have an extracellular-facing ligand-binding domain. They are classified into several subgroups with varying localization and expression patterns, and consist of the JAK, SYK, Abl, Src, Csk, FAK, Fps and Itk families (Twamley *et al.*, 1996). Protein tyrosine kinases may be inhibited by genistein, a drug that likely attaches to a common, highly conserved sequence in the region of the ATP-binding site of the enzyme (Nedeljković *et al.*, 2001). It was found that swelling-induced efflux of $^{125}I^-$ and $^{86}Rb^+$ in intestine 407 cells could be inhibited using genistein, indicating that osmoregulation involves activation of protein tyrosine kinases (Tilly *et al.*, 1993). In addition, genistein reduced effluxes of aspartate, glutamate, taurine and

phosphoethanolamine, suggesting the possibility of tyrosine kinase-regulated efflux of macromolecules (Song *et al.*, 1998).

1.4 Rationale

Glutathione levels are markedly lower in the bronchoalveolar lavage (BAL) fluid from cystic fibrosis patients than from normal individuals (Roum *et al.*, 1993). Glutathione may play an important role in the pathophysiology of CF, and glutathione aerosol therapy has been shown to elevate GSH in the airway surface liquid (ASL) and reduce the oxidant burden in CF patients (Roum *et al.*, 1999). While the metabolism of GSH has been studied in detail (Dickinson *et al.*, 2002; Meister, 1988), the mechanisms and regulation of its release by cells remain poorly understood. The goal of this study was to examine the mechanism of GSH release from airway epithelial cells and to investigate its possible role in CF. Since CFTR is an ABC transporter and is closely related to the glutathione conjugate transporter MRP1, it has been hypothesized that decreased ASL glutathione may reflect diminished glutathione efflux through CFTR. Indeed, a patch clamp study by Linsdell *et al.* (1998) suggested that CFTR mediates glutathione currents in excised baby hamster kidney (BHK) cell membrane patches, and a study by Gao *et al.* (1999) suggested that a lack of wild-type CFTR in CFT1 airway cells might be responsible for a decrease in apical medium GSH content. However, the mechanism of release remains unknown, and GSH release can be restored in CF cells by expressing a different chloride conductance, suggesting it is not mediated by CFTR (Gao *et al.*, 2001). This study investigates whether glutathione transport varies with CFTR expression in a cell line transfected with CFTR in an inducible expression cassette. Possible signalling pathways and alternative release mechanisms involved in glutathione transport are also explored.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals

All chemicals were from Sigma-Aldrich (Oakville, ON) unless otherwise stated. Hank's balanced salt solution (HBSS) without phenol red was used as efflux buffer. Dibutyryl-cyclic AMP (db-cAMP) was prepared in HBSS and used at a final concentration of 200μM. Forskolin and IBMX stocks were dissolved in DMSO and used at final concentrations of 10μM and 1mM, respectively. The maximum DMSO concentration of 0.2% during studies with db-cAMP/IBMX/forskolin had no effect on GSH release in control experiments (Figure 4c). DMSO concentration was maintained at 0.1% when added with other drugs (LY294002, LY303511, wortmannin, chelerythrine, genistein, daidzein). Various pharmacological agents were prepared as stock solutions, and added to efflux buffer at the following final concentrations: 20μM LY294002, 20μM LY303511, 20nm wortmannin (from *Penicillium funiculosum*), 5μg/ml brefeldin A, 10μM chelerythrine, 100μM genistein, 100μM daidzein, 500μM MgATP, 1U/ml apyrase, 0.2mg/ml acivicin. No biohazardous materials were used in this study.

2.2 Statistics

Two-way analysis of variance (ANOVA) was performed on most experimental data, with criteria for significant difference set to 95% confidence. One-way ANOVA was performed for long-term (24h) GSH efflux experiments to compare control, vehicle and cAMP treatments. Bonferroni post-hoc analysis was performed on experiments with three treatment groups to determine significant differences between groups. Independent t-tests were performed to compare efflux values between different cell types (i.e. Figures

5a, 5b, 6), and Bonferroni adjustments were made to these p values to compensate for the multiple t-tests. Data were expressed as means ±S.D., with the exception of the summary graphs where individual data points from all experiments were shown along with minimum, maximum and mean values for the data set. While variation between replicate data points for a given experimental condition on a single day was small, there was often considerable variability in results for an experiment performed on different days despite efforts to keep all conditions identical. In those experiments with small day-to-day variation in data points, data were pooled and represented as a single graph. In those experiments where there was variation between days, a representative experiment (in triplicate) on a single day is shown along with a summary graph that shows all the data from experiments performed on different days.

2.3 Cell culture

Two variants of IB3 human bronchial epithelial cells were generously provided by Drs. J.Hu and L.Ye (The Hospital for Sick Children, Toronto, ON). IB3-837 cells were transfected with a doxycycline (DOX)-inducible pTRE18iTEC expression vector containing CFTR cDNA. IB3-TA8 cells were transfected with pTRE18iTEC control vector lacking CFTR. Doxycycline (1mg/ml) was added to the medium for 24 hours to induce CFTR expression (Ye *et al.*, 2001). TA8 cells had no CFTR expression detectable by Western blotting, either in the presence or absence of DOX. Both IB3 cell clones were grown in (serum-free) LHC-8 media (Biofluids, Rockville, MD, USA) supplemented with 5% TSA-FBS, 1% penicillin/streptomycin, 300μg/ml G418, along with 150μg/ml hygromycin B for the IB3-837 cells only.

Human embryonic kidney (HEK) cells stably transfected with MRP1 (HEK-MRP1) were from Dr. P. Gros (Department of Biochemistry, McGill University), and were compared with the untransfected parental cell line (HEK-293) which lacks MRP1. Both HEK clones were grown in Dulbecco' s Modified Eagle' s Medium and Ham's nutrient mixture F12 (DMEM/F12) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS, 1% penicillin-streptomycin, along with 100ug/ml hygromycin added to the HEK-MRP1 cells only.

Baby hamster kidney (BHK) cells and BHK cells stably transfected with wild-type CFTR (BHKwtCFTR) were grown in DMEM/F12 (Gibco BRL, Grand Island, NY, USA) supplemented with 5% FBS, 1% penicillin-streptomycin, along with 500μM methotrexate added to the BHKwtCFTR cells only.

Cells were cultured to confluence in six-well plates (Sarstedt Inc., Montreal, Canada) and incubated in a water-jacketed CO_2 incubator (Forma Scientific, Marietta, OH, USA) set at 37°C and 5% CO_2.

2.4 Sampling protocol

Growth medium was removed on the day of the experiment and replaced with 1ml of HBSS. Three samples were taken at thirty minute intervals to establish a baseline rate of glutathione efflux, then osmotic or pharmacological interventions were applied and the entire volume (1ml) was sampled and immediately replaced by fresh solution every thirty minutes. Between samples, the cells were kept in an incubator filled with room air

(ambient CO_2) at 37°C (Boekel Industries, Feasterville, PA, USA). Samples were centrifuged at 6,000 rpm for 1 minute before GSH assays to pellet any cell debris.

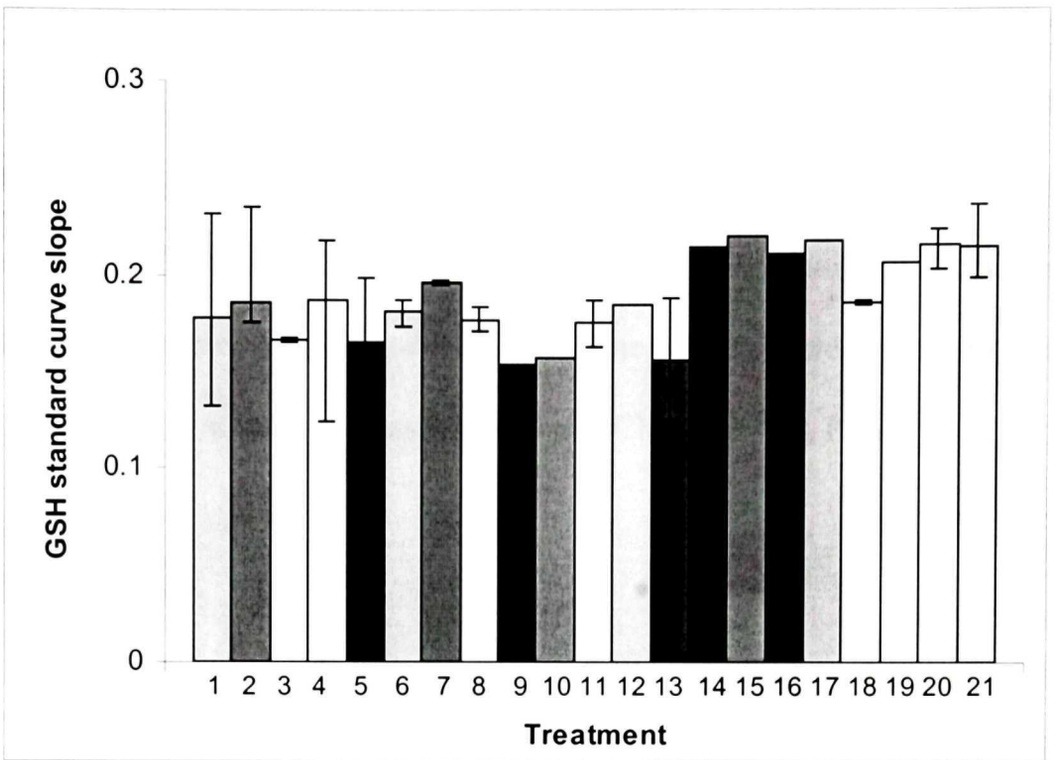
2.5 Glutathione assay

Total glutathione (GSH+GSSG) was detected using a modification of the method described by Tietze (1969), in which Ellman's reagent (DTNB) reacts with reduced glutathione in the presence of NADPH to form a colored species that absorbed light at 412nm. After centrifugation, 400ul of sample was added to 600µl of 0.163M potassium phosphate buffer, pH 7.4, containing 8.65mM EDTA, 417µM DTNB, 0.83U/ml glutathione reductase, and 365µM NADPH (all reagents from Sigma-Aldrich, Oakville, ON), and the rate of reduction of DTNB was recorded at 412nm using a DU-64 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Standard curves using known concentrations of GSSG (0.25-8µM) in corresponding efflux buffers were used to determine sample glutathione concentrations for each experimental condition.

Various treatments caused relatively minor variations in the standard curves compared to control, and the treatment slope means remained within +0.058 and -0.024 of the control slope mean (Figure 2a). In any case, GSH values for each drug treatment were calculated from standard curves that used the same respective drug in the buffer, serving as an internal adjustment for any possible drug effects on the assay and allowing for determination of actual drug effects on the cells. The GSH standard curves were also linear, with the curve only slightly displaced from the origin (Figure 2b).

Figure 2: GSH standard curves. A. Summary graph comparing GSH standard curve slope means for control and treatment solutions, with bars representing range of slope values obtained. Treatment key: 1-control HBSS buffer, 2-HBSS+cAMP/IBMX/forskolin, 3-HBSS+DMSO, 4-HBSS70%, 5-HBSS70%+acivicin, 6-HBSS70%+LY294002, 7-HBSS70%+LY303511, 8-HBSS70%+wortmannin, 9-HBSS+wortmannin, 10-HBSS+genistein, 11-HBSS70%+genistein, 12-HBSS70%+daidzein, 13-HBSS70%+chelerythrine, 14-HBSS+PD98059, 15-HBSS70%+PD98059, 16-HBSS+SB203580, 17-HBSS70%+SB203580, 18-HBSS70%+ATP, 19-HBSS70%+apyrase, 20-HBSS+brefeldinA, 21-HBSS70%+brefeldinA. **B.** Representative graph showing GSH standard curves for control and hypotonic solutions.

A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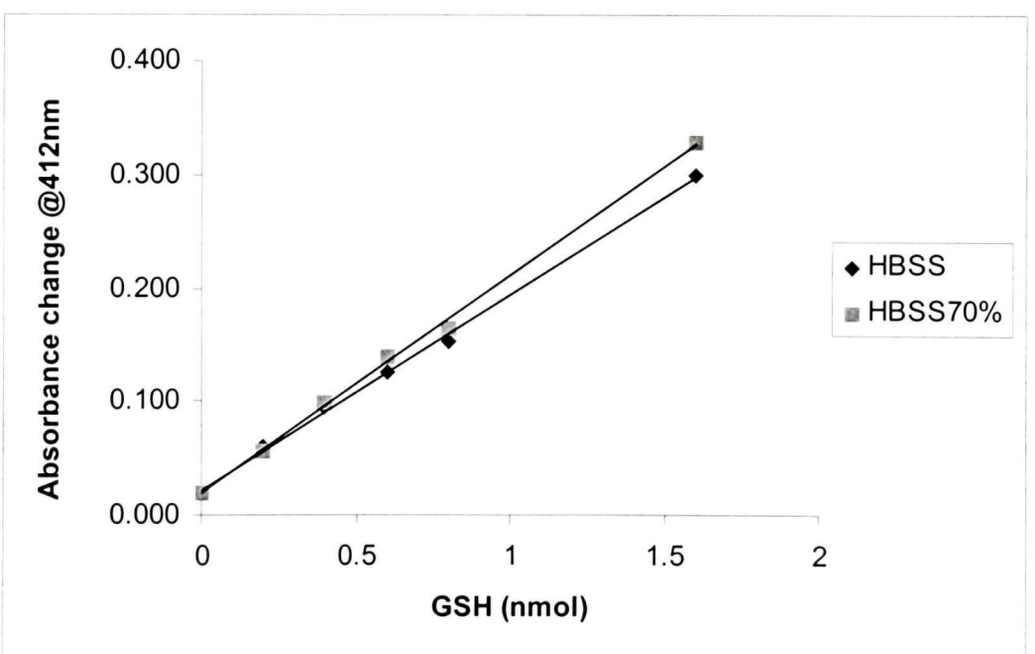
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Treatment

B


Absorbance change @412nm
0.400
0.300
0.200
0.100
0.000
0
0.5
1
1.5
2
GSH (nmol)
HBSS
HBSS70%

2.6 Protein assay

Cells were scraped using a rubber policeman into 1ml of HBSS, then centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellets saved at -20°C. To assay protein, pellets were lysed in 1ml of 1M NaOH, stirred with a magnetic stir bar for 1 hour at 4°C, and passed through a 33½ gauge needle using a 3cc syringe to ensure homogenization of the cells. Protein levels were determined using the Pierce bicinchoninic acid (BCA) assay kit (Pierce Chemical Co., Rockford, IL, USA).

2.7 Iodide effluxes

IB3-837 cells were cultured to confluence and stimulated with doxycycline for 24 hours. Culture medium was replaced with 2ml of iodide loading buffer (136mM NaI, 3mM KNO_3, 2mM $Ca(NO_3)_2$, 11mM glucose, 20mM HEPES, pH of 7.4) for one hour at 37°C, then cells were rinsed ten times with HBSS efflux buffer. To sample iodide efflux, cells were covered with 1ml of HBSS, which was removed and replaced with fresh HBSS at 1 minute intervals. Following collection of all the samples, iodide concentration was determined using an iodide-sensitive electrode (Orion Research Inc., Boston, MA). The amount of iodide released during each one minute interval was determined after calibrating the electrode meter using known concentrations of iodide (range 1-100μM NaI).

2.8 LDH assay

A lactate dehydrogenase (LDH) kit from Sigma (St.Louis, MO), which measures cytosolic LDH released as a result of cell damage, was used to measure LDH in the efflux

samples. Two wells were used for each LDH assay. One well contained doxycycline-induced IB3-837 cells expressing CFTR exposed to control HBSS throughout the experiment whilst the other contained similarly treated IB3-837 cells that had been exposed to HBSS70% for one hour. 1ml samples were collected and replaced every thirty minutes. From the 1ml sample, 600µl were used to assay LDH activity and the other 400µl was used to measure GSH. As a positive control for cell lysis, a third well was incubated with 1ml distilled water. An LDH standard curve using known concentrations of LDH was constructed to allow determination of sample LDH levels.

2.9 DNA amplification

pcDNA6MRP4 was a generous gift of Dr. T. Tan (National University of Singapore, Singapore) and used to transform TOP10 *E.coli.* Plasmid DNA suitable for use in cell transfections was then isolated and purified using a QIAfilter plasmid midi kit (Qiagen Inc., Mississauga, ON, Canada).

2.10 Transient transfections

Subconfluent HEK-293 cells were transiently transfected with pcDNA6MRP4 using the calcium phosphate coprecipitation method (Sambrook *et al.*, 2001) or using FuGENE 6 following the manufacturers protocol (Roche Diagnostics, Indianapolis, IN).

2.11 Western blot

HEK or IB3 cells were washed twice with ice-cold PBS, harvested by scraping, and lysed on ice for ten minutes in RIPA buffer supplemented with protease inhibitor

cocktail. The lysate was centrifuged (10,000g, 10 minutes @4°C) and an aliquot of supernatant was assayed for protein using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Loading buffer was added to an equal volume of supernatant containing 20μg of CFTR protein or 100μg of MRP4 protein, subjected to 7.5% SDS-PAGE, and transferred to a nitrocellulose membrane. The monoclonal anti-CFTR antibody M3A7 (Kartner & Riordan, 1998) or monoclonal anti-V5 antibody (Invitrogen, Burlington, ON) was used as the primary antibody. The secondary antibody (goat anti-mouse conjugated to peroxidase; Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) was detected using the enhanced chemiluminescence (ECL) kit from Amersham Pharmacia Biotech.

CHAPTER 3: RESULTS

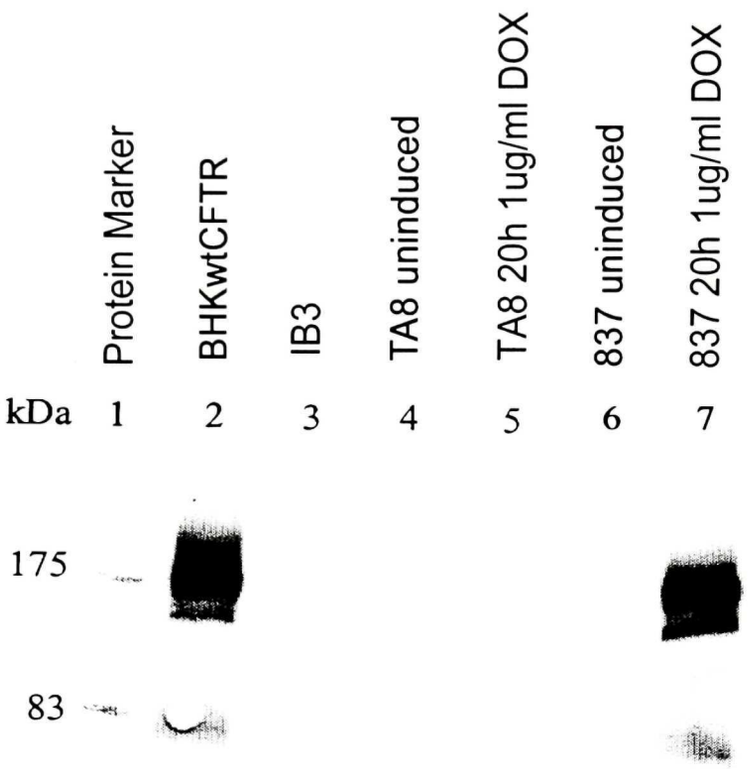
3.1 cAMP elevates GSH efflux in IB3 cells independently of CFTR expression.

Initially, we were interested in determining whether CFTR could transport glutathione, since levels of glutathione were found to be decreased in the airway surface liquid of CF patients. To test this hypothesis, we used a human bronchial epithelial cell line transfected with a doxycycline-inducible vector containing CFTR, so that CFTR expression levels could be varied. Western blots revealed high levels of doxycycline-induced CFTR expression in IB3-837 cells, with negligible CFTR expression in vector-only control IB3-TA8 cells (Figures 3a, 3b).

To test whether activation of CFTR could modulate GSH efflux, we measured effluxes in CFTR-expressing cells both in the presence or absence of CFTR agonists. We found that in IB3-837 cells induced to express CFTR (i.e. +DOX), cAMP/IBMX/forskolin caused an elevation of glutathione efflux over a two-hour period compared to baseline GSH efflux levels (Figure 4a). Further examination confirms that the change in GSH release (ΔGSH) with CFTR-agonists is significantly greater than the ΔGSH seen with control efflux buffer (Figure 4b, $p<0.01$). As a control, we verified that GSH effluxes were not altered by 0.2% DMSO (Figure 4c, $p>0.05$). Again, CFTR agonists caused an elevated GSH efflux over a two-hour period compared to both baseline and DMSO-control effluxes ($p<0.01$). While it would have been preferable to use the same sample interval for both the control and treatment periods, this was not done in this experiment as it was necessary to take multiple time points before stimulation in order to establish the basal rate of GSH accumulation. Since the sample intervals were

Figure 3: Induction of CFTR expression in human airway IB3 cells. A. Cells were grown in the presence or absence of doxycycline (1μg/ml) for 20h. Cells were lysed and 20μg of protein was loaded in each lane. The Western blot was probed using an M3A7 antibody against the C-terminus of CFTR. BHK cells expressing wild-type CFTR served as a positive control. Lane 1 - protein marker; lane 2 - BHK cells; lane 3 - IB3 cells; lane 4 - uninduced (-DOX) IB3-TA8 cells; lane 5 – induced (+DOX) IB3-TA8 cells; lane 6 – uninduced (-DOX) IB3-837 cells; lane 7 – induced (+DOX) IB3-837 cells. This blot courtesy of Dr. A. Guyot. **B.** IB3-837 and IB3-TA8 cells were grown in the presence of doxycycline (1μg/ml) for 24h. Cells were lysed and 20μg of protein was loaded in each lane, and the Western blot was performed as above. Lane 1 – induced (+DOX) IB3-837 cells; lane 2 – induced (+DOX) IB3-TA8 cells; lane 3 – BHK cells.

A


Protein Marker
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TA8 20h 1ug/ml DOX
837 uninduced
837 20h 1ug/ml DOX
kDa
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2
3
4
5
6
7
175
83

B

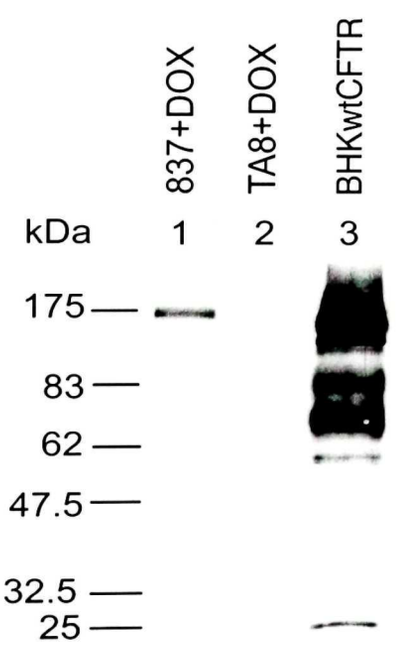
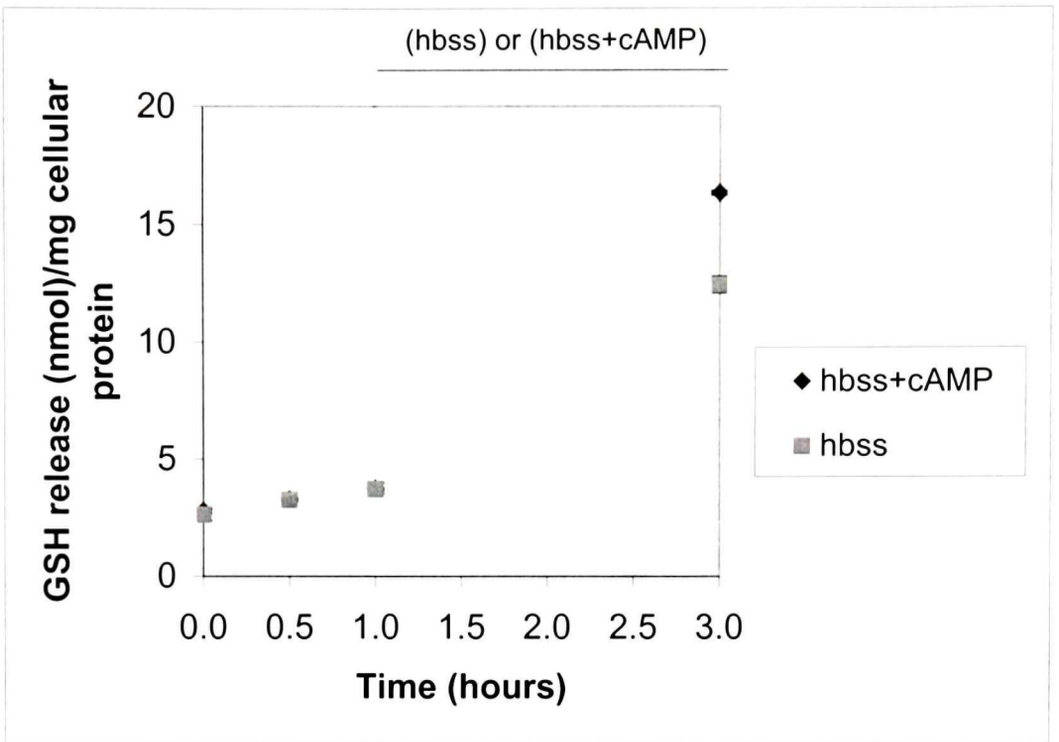
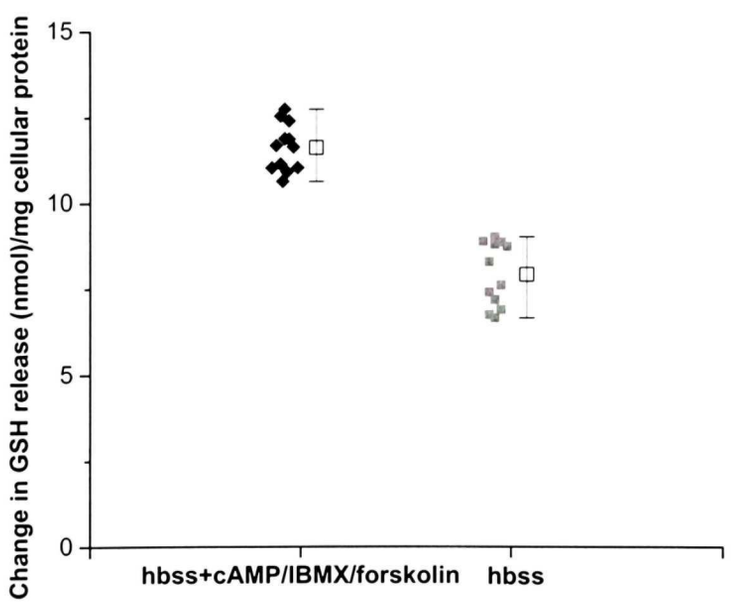

837+DOX
TA8+DOX
BHKwtCFTR
kDa
1
2
3
175
83
62
47.5
32.5
25

Figure 4: Acute effects of CFTR agonists on GSH efflux from CFTR-expressing IB3 cells. Monolayers of CFTR-expressing (+DOX) IB3-837 cells were cultured and baseline GSH efflux was established prior to adding 200μM db-cAMP, 10μM forskolin and 1mM IBMX at t=1.0h. Bar represents duration of treatment with CFTR agonists. **A.** Representative experiment showing GSH effluxes in CFTR-expressing IB3-837 cells, with and without CFTR agonist stimulation (n=3 for each data set). **B.** Summary of GSH efflux changes from t=1.0h to 3.0h in IB3-837 cells expressing CFTR, with or without CFTR agonist stimulation (n=12 for each data set). Graph represents individual data points from multiple experiments. **C.** Comparison of GSH effluxes in CFTR-expressing IB3-837 cells, exposed to control, vehicle or CFTR agonists (n=4 for each data set).

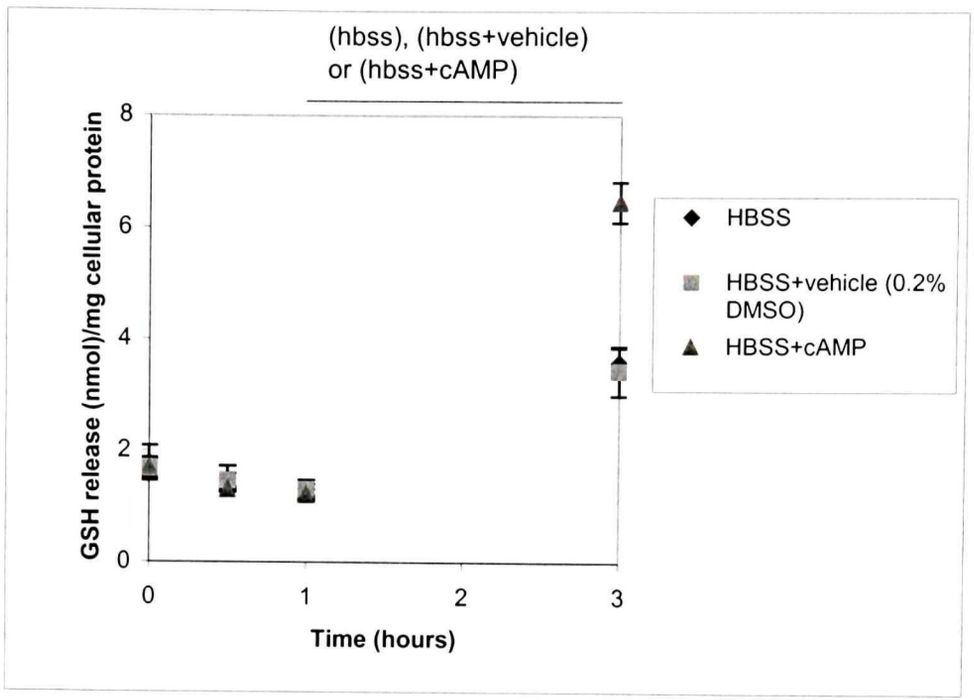
A


(hbss) or (hbss+cAMP)
GSH release (nmol)/mg cellular protein
20
15
10
5
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hbss+cAMP
hbss

B


Change in GSH release (nmol)/mg cellular protein
15
10
5
0
hbss+cAMP/IBMX/forskolin
hbss

C

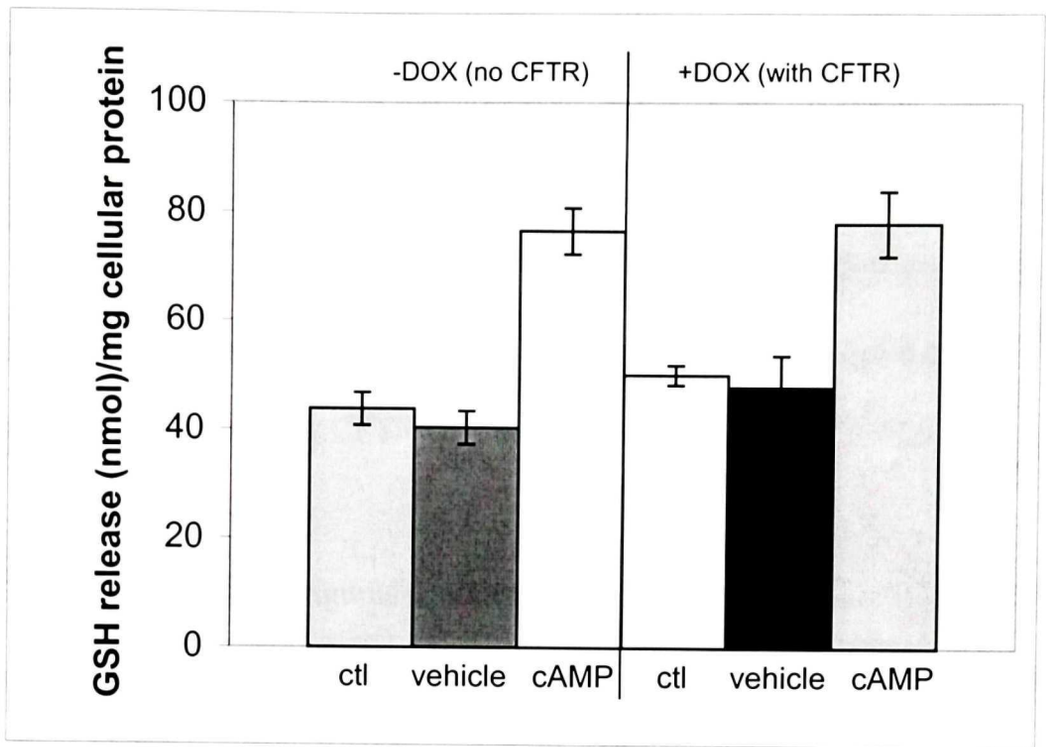

(hbss), (hbss+vehicle)
or (hbss+cAMP)
GSH release (nmol)/mg cellular protein
8
6
4
2
0
0
1
2
3
Time (hours)
HBSS
HBSS+vehicle (0.2% DMSO)
HBSS+cAMP

unequal, the data could not be converted to rates of GSH accumulation since other factors, in addition to efflux, would come into play, such as extracellular degradation (which would be greater with longer intervals). This could potentially affect GSH accumulation, and might explain the small apparent decline in efflux rate between one and three hours.

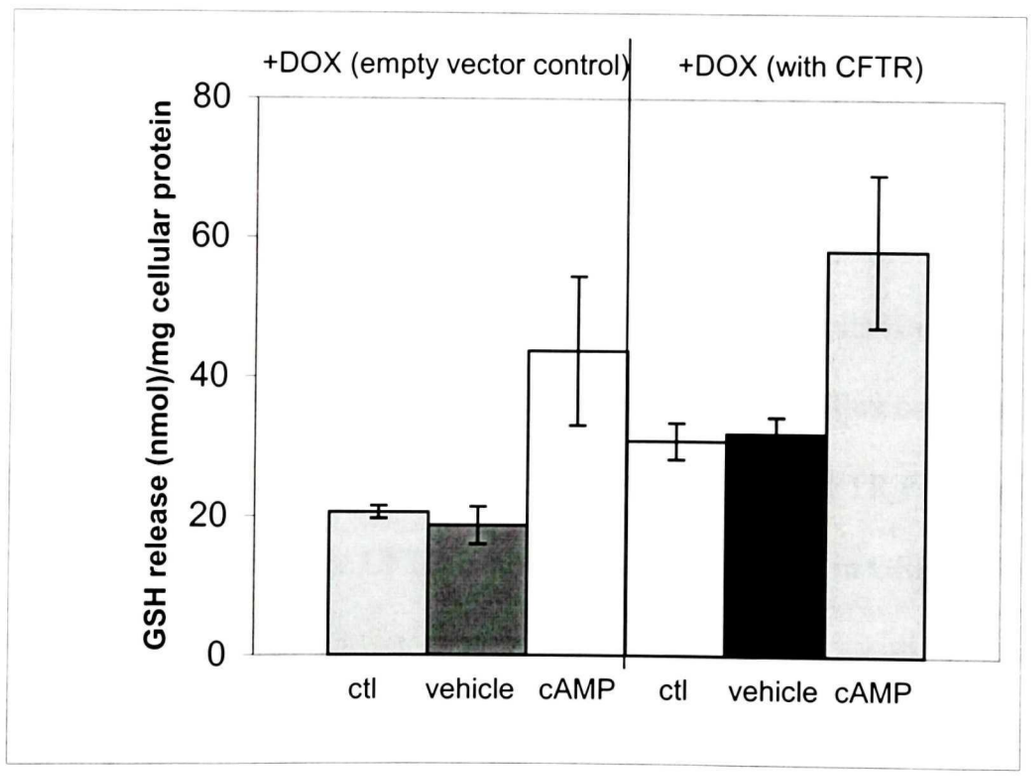
To test whether the presence of CFTR could affect GSH efflux, we compared effluxes in cells expressing or lacking CFTR, both in the presence or absence of CFTR agonists. The experiment was performed over a longer time period of twenty-four hours in order to amplify any differences between agonist and control groups, and to perform the experiment with cell-specific culture media in a CO_2-controlled incubator. Comparison of DOX-induced and DOX-uninduced IB3-837 cells (Figure 5a) indicates that CFTR expression had a small but significant effect on control GSH efflux ($p<0.05$), but did not alter release during exposure to cAMP ($p>0.05$) or vehicle ($p>0.05$). The vehicle group, which consisted of control media with 0.2% DMSO, was used to verify that the DMSO in which the CFTR agonists were dissolved were not having a large effect on GSH transport. GSH effluxes in vehicle solution were not significantly different compared to control solution for IB3-837 cells with DOX ($p>0.05$), while vehicle GSH efflux were marginally lower than control in IB3-837 cells without DOX ($p<0.05$). Nevertheless, cAMP elicited a significantly larger increase in GSH release compared to both vehicle ($p<0.01$) and control ($p<0.01$) groups. Importantly, the elevation in GSH efflux was seen in cells expressing CFTR and in those lacking CFTR, suggesting it does not mediate glutathione efflux from these IB3 cells. When DOX-induced IB3-837 cells expressing CFTR were compared with vector control IB3-TA8 cells lacking CFTR

Figure 5: Long-term effects of CFTR agonists on GSH efflux from CFTR-expressing IB3 cells. Monolayers of CFTR-expressing cells (IB3-837, +DOX), non-CFTR expressing cells (IB3-837, -DOX), and empty vector control cells (+DOX, IB3-TA8) were cultured. 200μM db-cAMP, 10μM forskolin and 1mM IBMX were added to culture medium, then incubated for 24 hours, with DMSO used as a negative control. **A.** Comparison of GSH effluxes in IB3-837 cells expressing and lacking CFTR, with and without CFTR agonists or DMSO (n=4 for each data set). **B.** Comparison of GSH effluxes in IB3-837 and IB3-TA8 cells expressing and lacking CFTR, respectively (n=4 for each data set).

A


-DOX (no CFTR)
+DOX (with CFTR)
GSH release (nmol)/mg cellular protein
100
80
60
40
20
0
ctl
vehicle
cAMP
ctl
vehicle
cAMP

B


+DOX (empty vector control)
+DOX (with CFTR)
GSH release (nmol)/mg cellular protein
80
60
40
20
0
ctl
vehicle
cAMP
ctl
vehicle
cAMP

(Figure 5b), there was no significant difference in the cAMP-mediated GSH release ($p>0.05$). However, both control ($p<0.01$) and vehicle-mediated ($p<0.01$) GSH effluxes were significantly lower in cells lacking CFTR. When comparing GSH effluxes in vehicle solution to control solution, there was no significant difference in both IB3-837 cells ($p>0.05$) and IB3-TA8 cells ($p>0.05$). In summary, cAMP elicited a significant increase in GSH release compared to vehicle ($p<0.01$) and control ($p<0.01$) GSH effluxes in cells expressing or lacking CFTR despite different baseline GSH effluxes.

It was noted that in Figures 5a and 5b, there was some variability in the baseline GSH accumulation. The cells used for each figure had different passage numbers; Figure 5a (showing higher baseline GSH fluxes) used cells at passage 26, whilst Figure 5b (showing lower GSH fluxes) used cells at passage 10. While it is possible that intracellular levels increase with passage number, this was not directly assessed.

GSH release was also measured using CFTR-expressing or nonexpressing baby hamster kidney (BHK) cells (Figure 6), since the electrophysiological evidence for GSH permeation through CFTR was previously obtained using these cells (Linsdell *et al.*, 1998). Addition of cAMP agonists significantly elevated GSH efflux compared to control buffer alone in both cells with or without high levels of CFTR expression ($p<0.01$), again confirming that CFTR is not playing a direct role in GSH trafficking from the cell.

We focused on extracellular GSH accumulation in our experiments for a couple of reasons. One advantage is that extracellular GSH can be measured at multiple time points

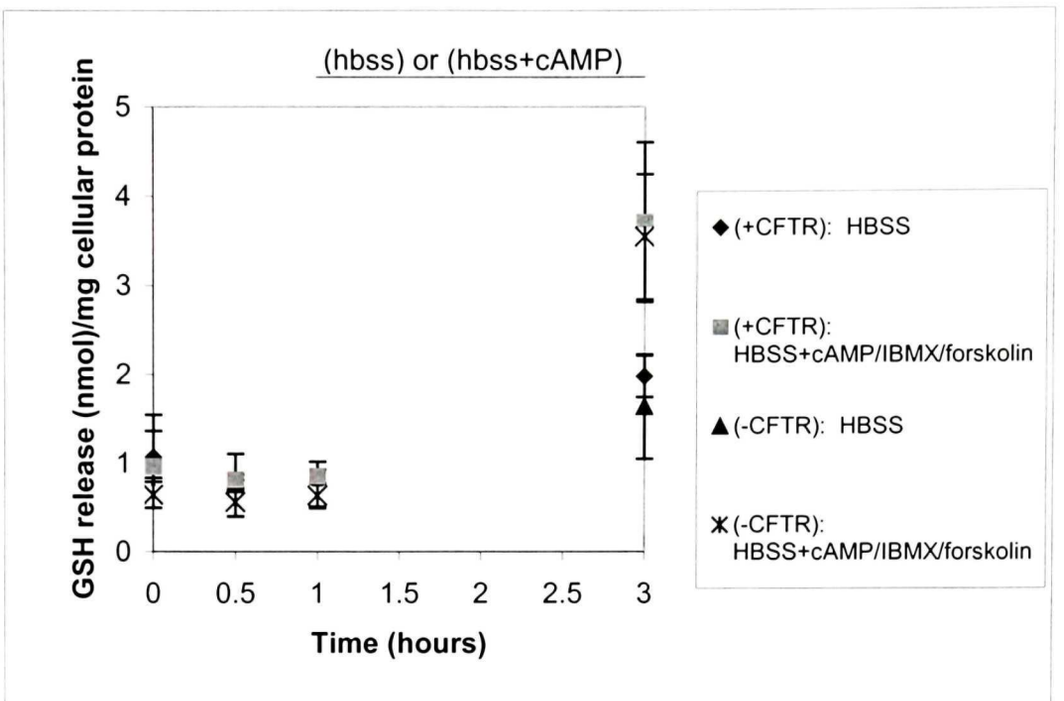
using the same cells. In addition, since extracellular glutathione levels are normally low compared to intracellular levels, effluxes may be expected to cause lower relative changes in extracellular glutathione. Indeed, intracellular glutathione levels in DOX-induced, CFTR-expressing IB3-837 cell monolayers were calculated in two experiments to be 31 and 33 nanomoles, respectively. Since baseline effluxes over thirty minutes were typically in the low nanomole range and represent only a few percent of the intracellular pool, this small change in intracellular glutathione might not be detectable, and thus it was more feasible to measure extracellular glutathione accumulation.

3.2 Hypotonic stress causes transient elevation of GSH efflux in CFTR-expressing IB3 cells.

Hypotonicity stimulates the release of many large organic solutes such as ATP (Boudreault *et al.*, 2002) and the amino acids taurine, glutamate, aspartate and glycine (Estevez *et al.*, 1999). To assess whether glutathione might also be released by cell swelling, we measured GSH release after diluting the HBSS by 30%. The application of 70% HBSS to CFTR-expressing IB3-837 cells increased GSH efflux more than 2-fold (Figures 7a, 7b; $p<0.01$). This effect was transient, with most release occurring within the first half-hour. GSH efflux returned almost to baseline levels within one hour. To examine the possibility that glutathione release was elevated during hypotonic stress (Figure 8a) due to lysis of some cells, lactate dehydrogenase (LDH) assays were performed (Figures 8b, 8c). LDH activity was below the limit of detection until cell lysis was induced as a positive control by replacing HBSS with distilled water. To examine

Figure 6: Acute effects of CFTR agonists on GSH efflux from BHK cells with and without CFTR. A. Comparison of GSH effluxes in CFTR-expressing or non-expressing BHK cells, both with and without CFTR agonist stimulation (200μM db-cAMP, 10μM forskolin and 1mM IBMX; n=8 for each control data set, n=16 for each agonist data set). **B.** Summary of GSH efflux changes from t=1.0h to 3.0h in response to CFTR agonists, compared to baseline GSH efflux, in both CFTR-expressing and non-expressing BHK cells (n=8 for each control data set, n=16 for each agonist data set).

A


(hbss) or (hbss+cAMP)
GSH release (nmol)/mg cellular protein
0
1
2
3
4
5
0
0.5
1
1.5
2
2.5
3
Time (hours)
(+CFTR): HBSS
(+CFTR): HBSS+cAMP/IBMX/forskolin
(-CFTR): HBSS
(-CFTR): HBSS+cAMP/IBMX/forskolin

B

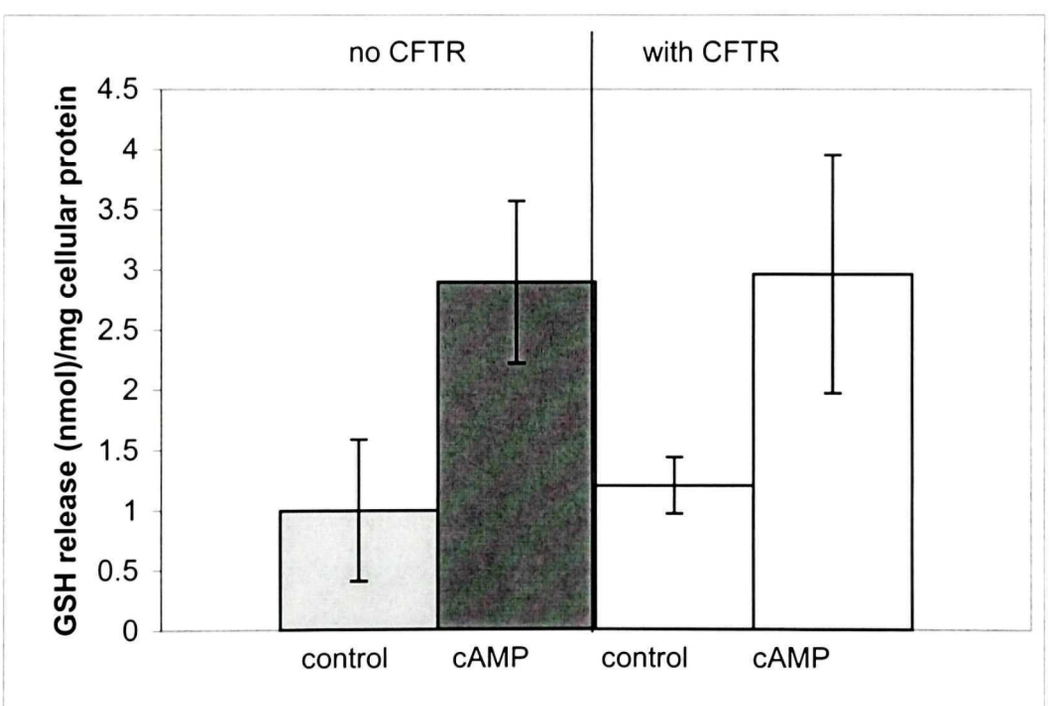
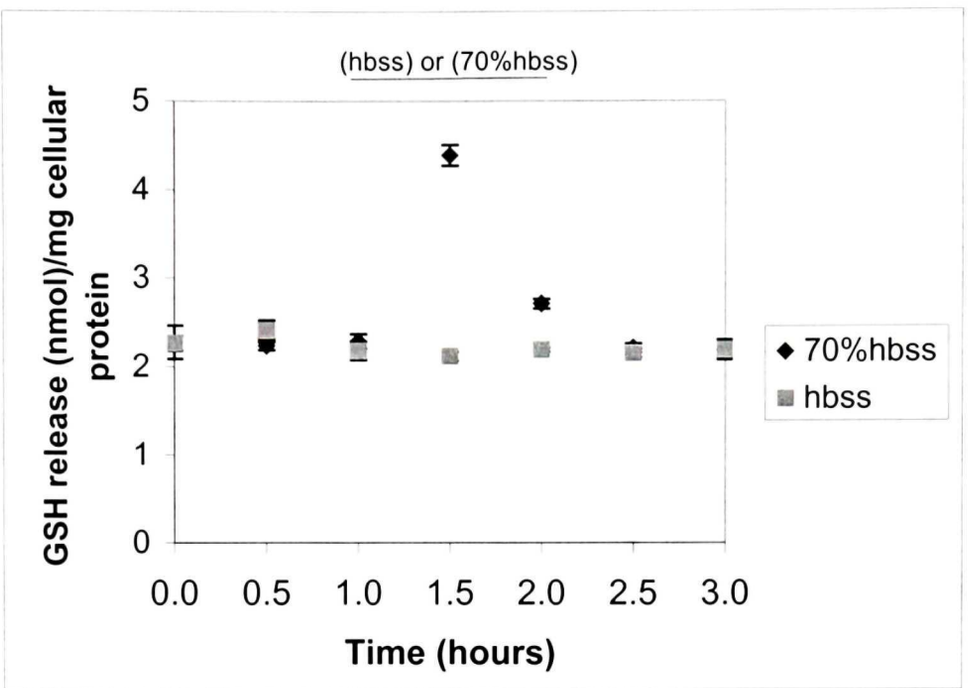

no CFTR
with CFTR
GSH release (nmol)/mg cellular protein
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
control
cAMP
control
cAMP

Figure 7: GSH release from CFTR-expressing IB3 cells induced by 70% HBSS. Monolayers of CFTR-expressing cells (IB3-837, +DOX) were cultured, and baseline GSH efflux was established prior to adding 70%HBSS. Bar represents duration of hypotonic treatment. **A.** Representative experiment showing GSH effluxes in IB3-837 cells expressing CFTR, with and without hypotonic stimulation (n=3 for each data set). **B.** Summary of GSH efflux changes from t=1.0h to 1.5h in IB3-837 cells expressing CFTR, with or without hypotonic stimulation (n=12 for each data set). Graph represents individual data points from multiple experiments.

A


(hbss) or (70%hbss)
GSH release (nmol)/mg cellular protein
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
70%hbss
hbss

B

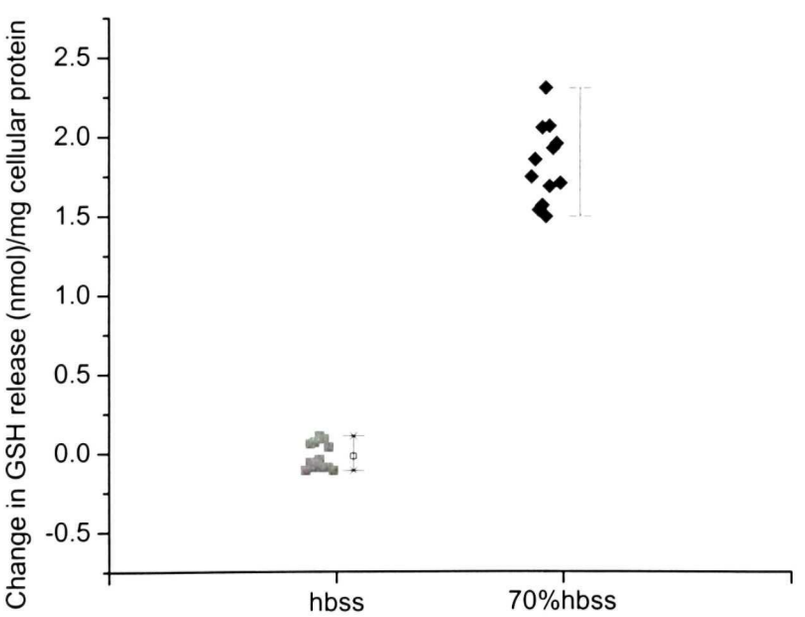
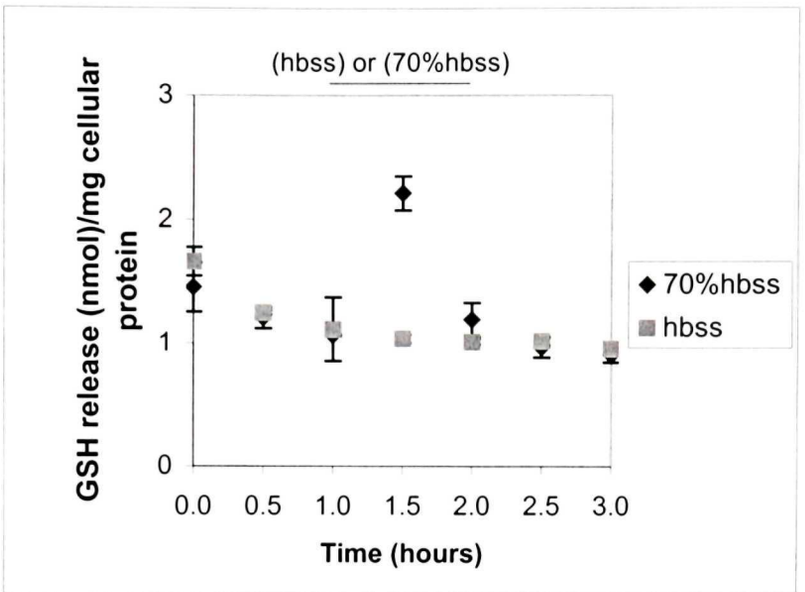
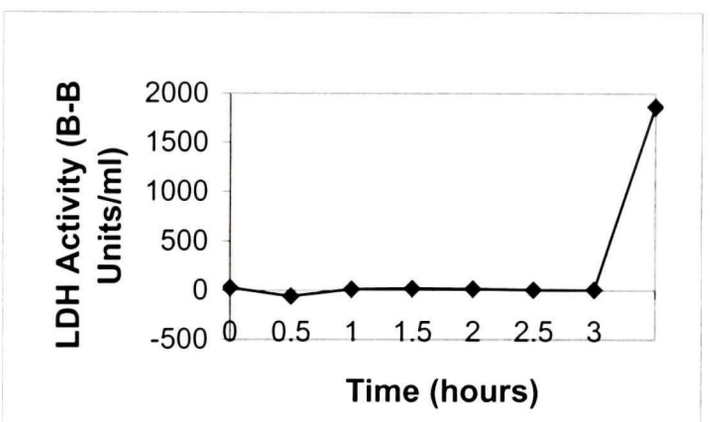

Change in GSH release (nmol)/mg cellular protein
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
hbss
70%hbss

Figure 8: Comparison of the effect of hypotonic solution on GSH release and lactate dehydrogenase (LDH) activity, a marker of cellular lysis. A. Comparison of GSH effluxes in CFTR-expressing IB3-837 cells, with and without hypotonic stimulation (n=3 for each data set). **B.** LDH activity detected in efflux buffer samples collected every 30 minutes from CFTR-expressing cells exposed to 70%HBSS solution (n=1 for each data set). ddH_2O was added to cells for one hour to serve as positive controls for cellular lysis, which was verified by observation under light microscope. **C.** LDH activity detected in efflux buffer samples collected every 30 minutes from CFTR-expressing cells exposed to control HBSS solution (n=1 for each data set). ddH_2O was again added to cells for one hour to serve as positive controls for cellular lysis.

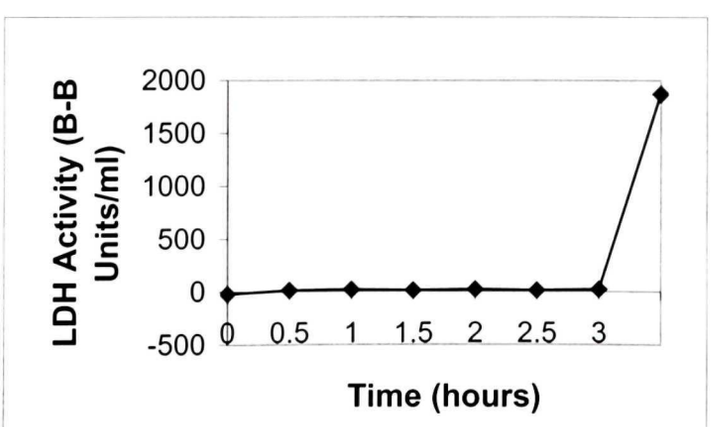
A


(hbss) or (70%hbss)
GSH release (nmol)/mg cellular protein
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
70%hbss
hbss

B


LDH Activity (B-B Units/ml)
2000
1500
1000
500
0
-500
0
0.5
1
1.5
2
2.5
3
Time (hours)

C


LDH Activity (B-B Units/ml)
2000
1500
1000
500
0
-500
0
0.5
1
1.5
2
2.5
3
Time (hours)

whether hypotonic solution might inhibit extracellular GSH degradation by γ-glutamyltranspeptidase (γ-GT, a ubiquitous GSH-degrading enzyme), the γ-GT inhibitor acivicin (0.2mg/ml) was present throughout some experiments. As evident from Figure 9, acivicin had no effect on baseline or hypotonically-stimulated GSH efflux. Taken together, the results suggest that glutathione is transiently released in response to hypotonicity.

3.3 Signaling pathways

Several signaling mechanisms have been implicated in hypotonically-induced solute efflux as part of a regulatory volume decrease (RVD) mechanism, and might be relevant to GSH release under these conditions. These include PI3-kinases, tyrosine kinases, protein kinase C, and MAP kinases. These pathways could be stimulated directly or by some autocrine factor such as ATP which is released in response to hypotonicity. Finally, cAMP and hypotonic shock stimulate exocytosis in various cell types, raising the possibility that GSH may be released by cAMP- or hypotonically-induced fusion of vesicles with the plasma membrane. DOX-induced CFTR-expressing IB3-837 cells were used to explore the signaling pathways involved in GSH release.

3.3.1 PI3-kinase pathway

The effects of two widely used PI3-kinase inhibitors, LY294002 (Vlahos *et al.*, 1994) and the fungal metabolite wortmannin (Powis *et al.*, 1994), were investigated for their effects on hypotonically-induced glutathione efflux, because PI3-kinase has been reported to mediate volume regulation in rat hepatocytes (Krause *et al.*, 1996). In

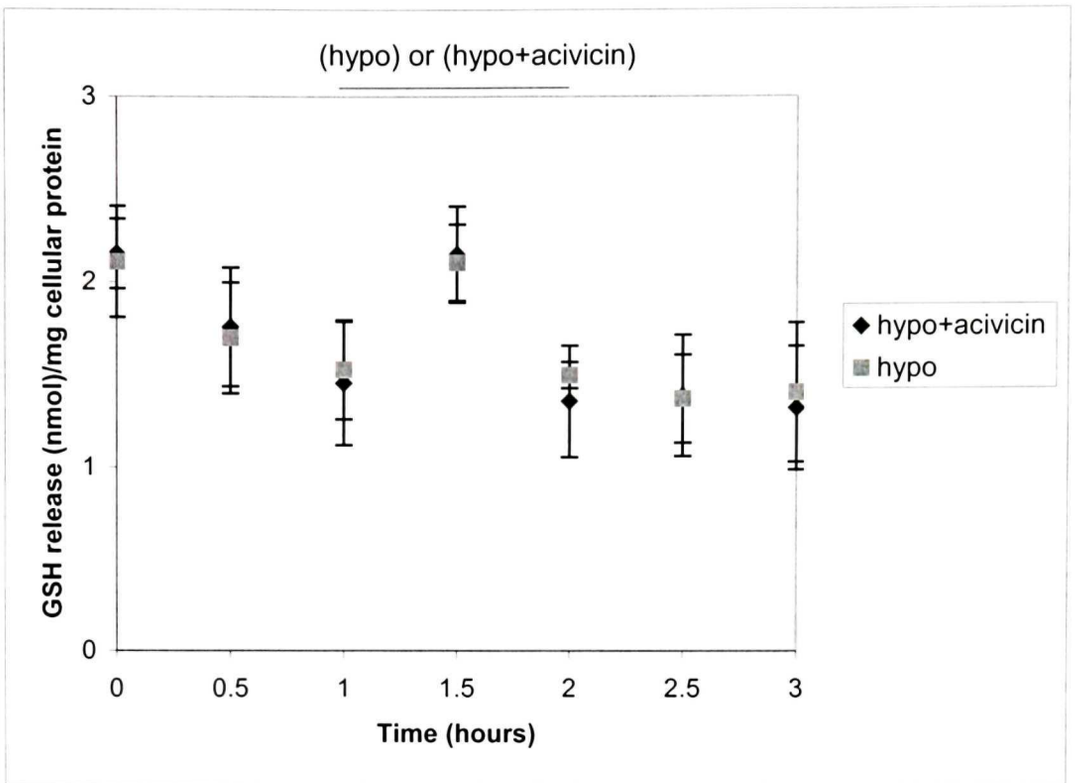


Figure 9: Comparison of GSH efflux response in IB3 cells, with and without acivicin. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline level of GSH efflux was established prior to adding 0.2mg/ml acivicin at t=1.0 hours. Bar represents duration of treatment with acivicin (n=9 for each data set). Data was pooled from multiple experiments.

addition, we also used LY303511, an inactive analogue of LY294002, as a negative control. When LY294002 (20μM) was added to cells along with hypotonic stress, it dramatically enhanced the GSH efflux as compared to that seen with hypotonic stress alone (Figure 10a; $p<0.01$). This potentiating effect is summarized for nine monolayers in Figure 10b. Surprisingly, when the inactive congener LY303511, which does not inhibit PI3-kinase, was tested under the same conditions (i.e. simultaneously with hypotonic stress), it caused a similar elevation of GSH efflux (Figures 11a, 11b). These results suggest that neither hypotonicity-induced GSH release nor the potentiating effect of LY294002 on GSH release were due to PI3-kinase activity. This was confirmed by examining the effect on hypotonically-induced GSH release of wortmannin, another inhibitor of PI3-kinase that is chemically unrelated to LY294002. Wortmannin (20nM) had no effect on hypotonically-induced GSH release (Figure 12a; $p>0.05$) or when added alone (Figure 12b; $p>0.05$). To ensure that similar swelling and volume regulatory responses were evoked in the presence of LY294002 and wortmannin, iodide efflux was measured under control conditions and with inhibitors present (Figure 13). Neither drug induced iodide efflux under isotonic conditions, nor did they alter hypotonically-induced iodide efflux. These results suggest that PI3-kinase is not involved in GSH release, and that the potentiating effect of LY294002 on hypotonically-stimulated release must reflect some other action of the drug.

There was some variability noted in the hypotonic induced effluxes from Figures 7, 10 and 11, but since all the cells used in these experiments were used between 7 and 11 days after passaging, it is unlikely that cell senescence caused these differences. Cell

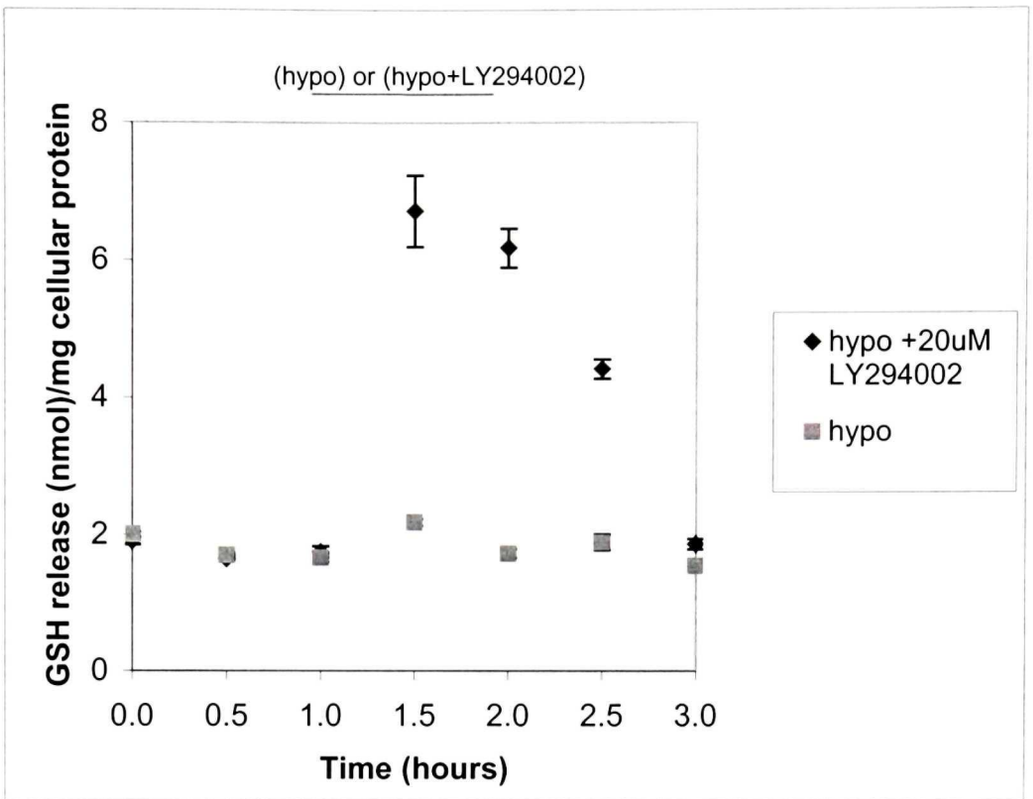
passage was also not a factor since all cells were of a similar passage number (22-23, albeit from different thawed batches of IB3-837 cells). Cell protein levels were higher in the experiments with lower hypotonically-activated effluxes (i.e. Figures 10 and 11), but the reason for this inverse relationship is not known. With respect to possible variability in the iodide efflux assays upon doxycycline induction of CFTR expression in Figure 13, experiments were not designed here to test this, however similar experiments were carried out by Ye *et al.* (2001) (see their Figure 6), and they reported extremely low variability in the response of iodide efflux in IB3-837 cells after doxycycline induction. Also, because the experiment in Figure 13 was designed to test the effect of pharmacological inhibitors on hypotonically-activated iodide efflux, the same CFTR-expressing IB3-837 cells were used in the presence or absence of the various drugs. Focusing on the role of CFTR in swelling activated iodide efflux would indeed require controls with untransfected IB3 cells, but this topic was not addressed here.

3.3.2 Pharmacological studies on the role of protein tyrosine kinases

Tyrosine kinases are activated by many stresses including heat, free radicals and osmotic shock (Gatsios, 1998), and are implicated in cell volume regulation. To examine their role in GSH release, we tested the effects of genistein, a commonly used broad-spectrum tyrosine kinase antagonist (Akiyama *et al.*, 1987). Genistein (100μM) stimulated GSH release approximately 2.5-fold under isoosmotic (Figures 14a, 14b) and also hypotonic conditions (Figures 14c, 14d). Thus genistein-sensitive tyrosine kinases do not contribute to hypotonically-stimulated GSH release. These results do suggest however that GSH may be tonically suppressed by tyrosine phosphorylation. To further

Figure 10: Response of hypotonic GSH efflux to PI3-kinase inhibitor LY294002. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline GSH efflux was established prior to adding 20μM LY294002. Bar represents duration of treatment. **A.** Representative experiment showing hypotonic-induced GSH effluxes in IB3-837 cells expressing CFTR, with and without LY294002 (n=3 for each data set). **B.** Summary of hypotonic-induced GSH efflux changes from t=1.0h to 1.5h in IB3-837 cells expressing CFTR, with and without LY294002 (n=9 for each data set). Graph represents individual data points from multiple experiments.

A


(hypo) or (hypo+LY294002)
GSH release (nmol)/mg cellular protein
8
6
4
2
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo +20uM LY294002
hypo

B

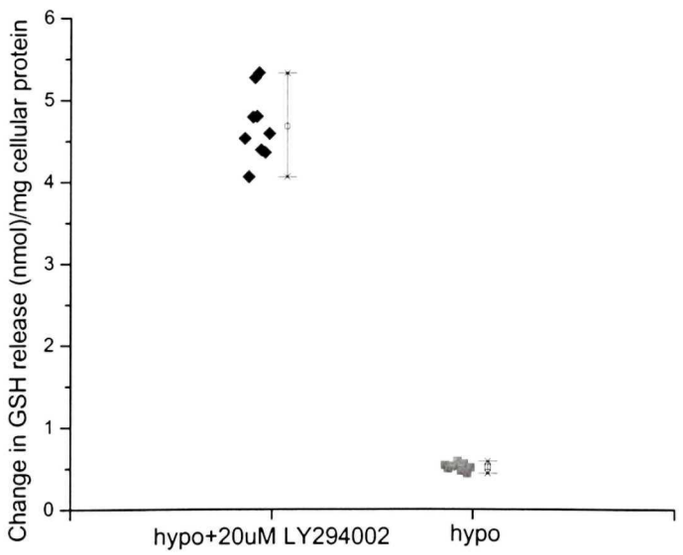
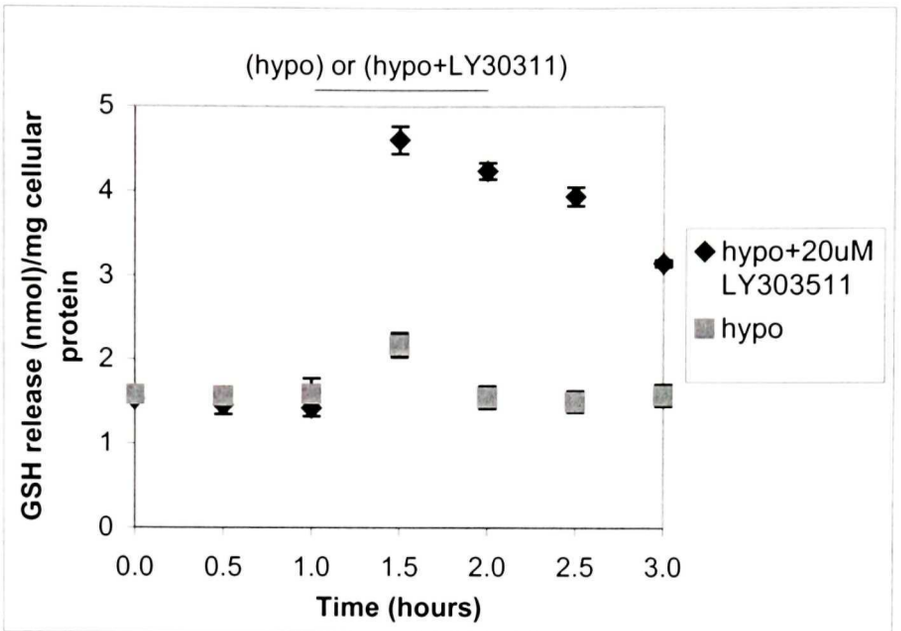

Change in GSH release (nmol)/mg cellular protein
6
5
4
3
2
1
0
hypo+20uM LY294002
hypo

Figure 11: Response of hypotonic GSH efflux to LY303511, an inactive analogue of the PI3-kinase inhibitor LY294002. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline GSH efflux was established prior to adding 20μM LY303511. Bar represents duration of exposure to LY303511. **A.** Representative experiment showing hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without LY303511 (n=3 for each data set). **B.** Summary of hypotonically-induced GSH efflux changes from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without LY303511 (n=6 for each data set). Graph represents individual data points from multiple experiments.

A


(hypo) or (hypo+LY30311)
GSH release (nmol)/mg cellular protein
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo+20uM LY303511
hypo

B

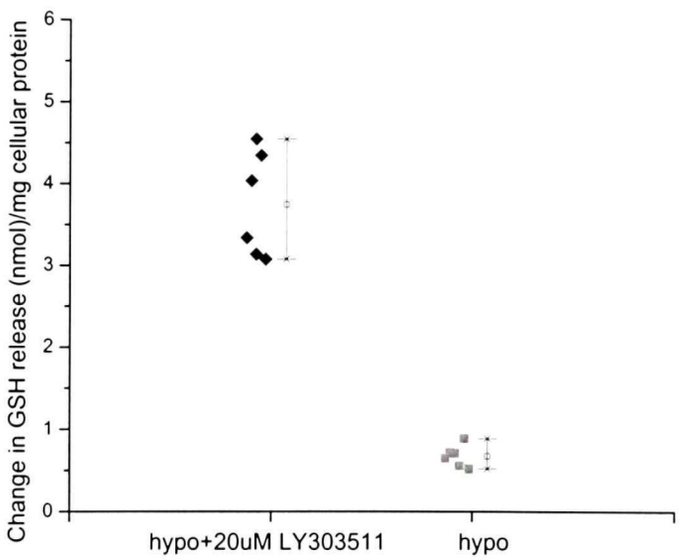
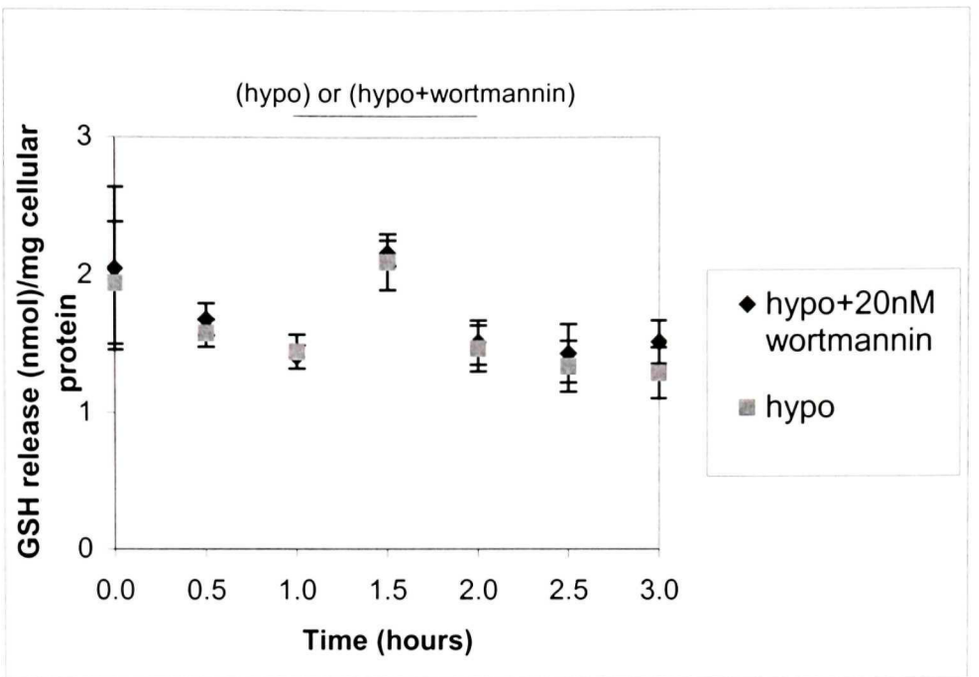
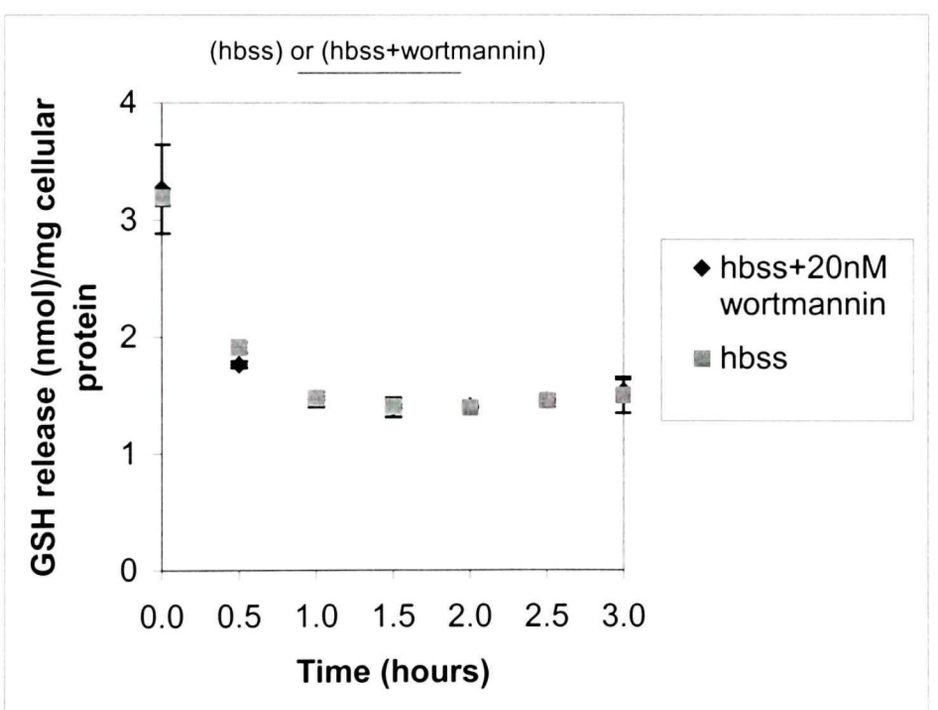

Change in GSH release (nmol)/mg cellular protein
6
5
4
3
2
1
0
hypo+20uM LY303511
hypo

Figure 12: Response of hypotonic GSH efflux to PI3-kinase inhibitor wortmannin. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline GSH efflux was established prior to adding 20nM wortmannin. Bar represents duration of treatment with wortmannin. **A.** Hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without wortmannin (n=6 for each data set). Data points was combined from multiple experiments. **B.** GSH effluxes in CFTR-expressing IB3-837 cells, with and without wortmannin (n=3 for each data set). Data was combined from multiple experiments.

A


(hypo) or (hypo+wortmannin)
GSH release (nmol)/mg cellular protein
0
1
2
3
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo+20nM wortmannin
hypo

B


(hbss) or (hbss+wortmannin)
GSH release (nmol)/mg cellular protein
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hbss+20nM wortmannin
hbss

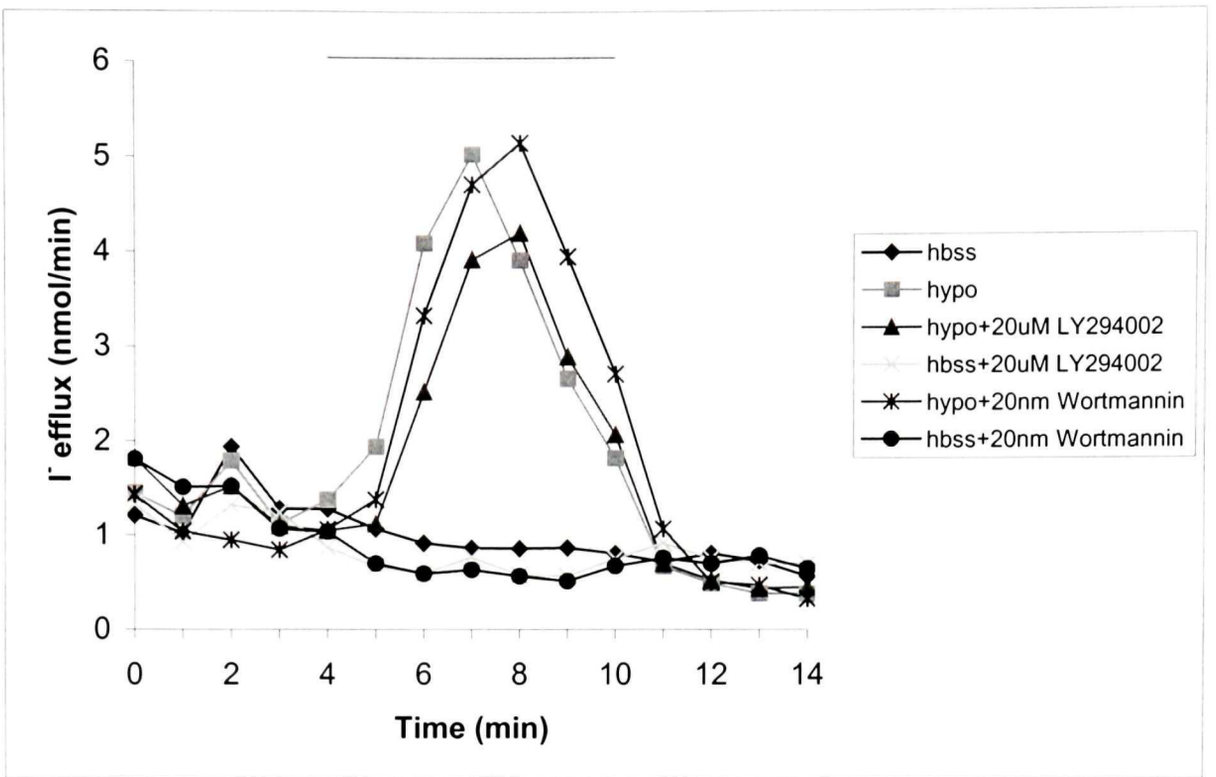


Figure 13: Iodide efflux response to hypotonic stress, with and without PI3-kinase inhibitors. Monolayers of CFTR-expressing IB3-837 cells were cultured, and incubated with 136mM NaI loading buffer for one hour at 37°C. Cells were then rinsed ten times with HBSS efflux buffer, and a baseline iodide efflux was established prior to stimulation with HBSS alone (negative control), hypotonic stress alone, LY294002 alone, wortmannin alone, hypotonic stress and LY294002, or hypotonic stress and wortmannin (n=2 for each data set), with bar representing treatment duration.

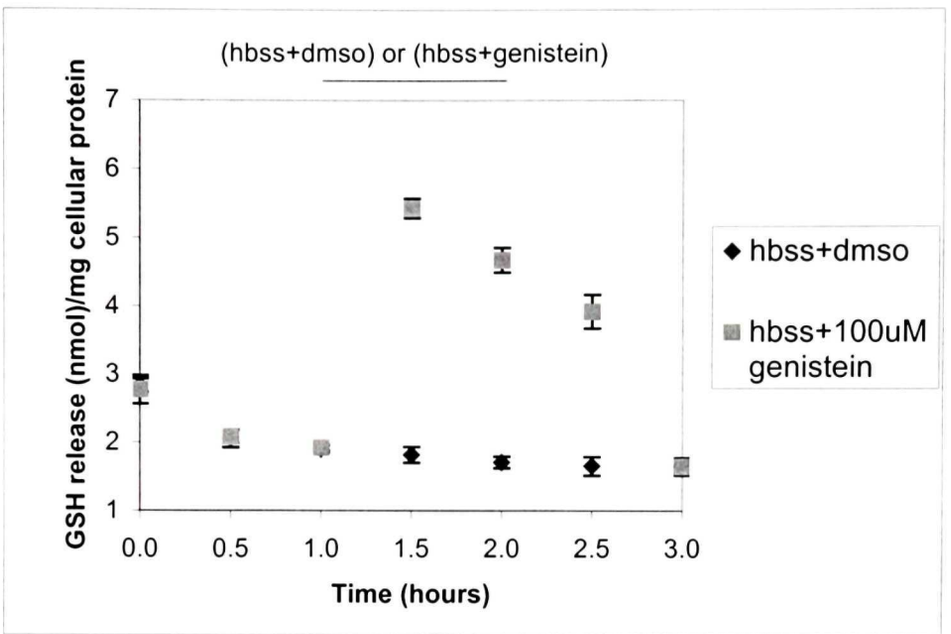
assess if genistein stimulation involves inhibition of tyrosine kinases, the inactive genistein analogue daidzein (100μM) was also tested for its effect on hypotonically-induced GSH release. Although the daidzein effect was approximately 40% smaller than that for genistein, daidzein nevertheless increased the hypotonic GSH response significantly (Figures 15a, 15b; $p<0.01$). If daidzein is ineffective against tyrosine kinases as is generally assumed, these results suggest that some other signaling pathway might regulate GSH release.

3.3.3 Protein kinase C pathway

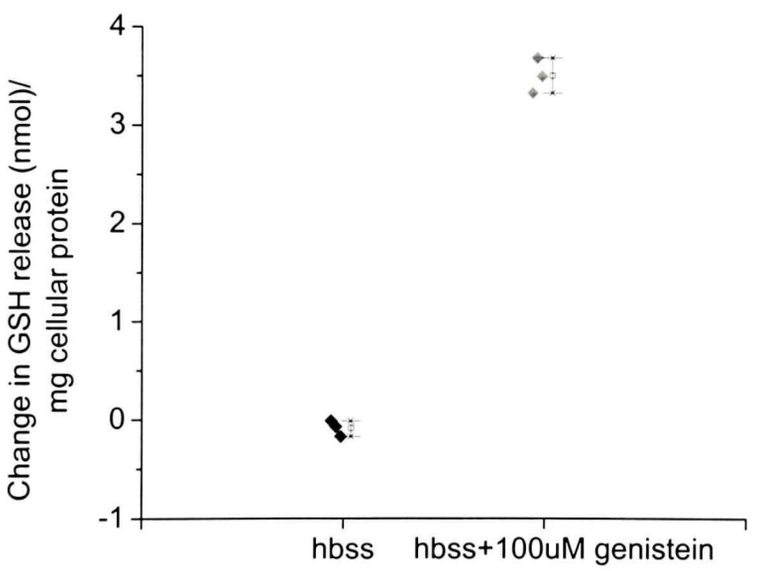
Protein kinase C (PKC) is activated by hypoosmotic stress in some cells and stimulates amino acid release from rat cardiac myocytes (Song *et al.*, 1998) and secretion of high molecular weight glycoprotein (HMWG) mucus from hamster tracheal epithelial cells (Kai *et al.*, 1994). To assess the role of PKC, cells were exposed to hypotonic solution containing chelerythrine (10μM), a PKC inhibitor. Chelerythrine caused an approximately 4.5-fold elevation of GSH efflux when compared to hypotonic stress alone (Figures 16a, 16b; $p<0.05$). This may indicate relief of tonic inhibition by PKC of GSH efflux, although apparent enhancement of the hypotonically-induced response could also reflect inhibition of the regulatory volume decrease (RVD), since that would potentiate cell swelling and therefore the stimulus for GSH release.

Figure 14: Response of basal and hypotonic GSH effluxes to tyrosine kinase inhibitor genistein. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline GSH efflux was established prior to adding 100μM genistein. Bar represents duration of exposure to genistein. **A.** Representative experiment showing hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without genistein (n=3 for each data set). **B.** Summary of hypotonically-induced GSH efflux change from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without genistein (n=6 for each data set). Graph represents individual data points from multiple experiments. **C.** Representative experiment showing basal GSH effluxes in IB3-837 cells expressing CFTR, with and without genistein (n=3 for each data set). **D.** Summary of basal GSH efflux changes from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without genistein (n=4 for each data set). Graph represents individual data points from multiple experiments.

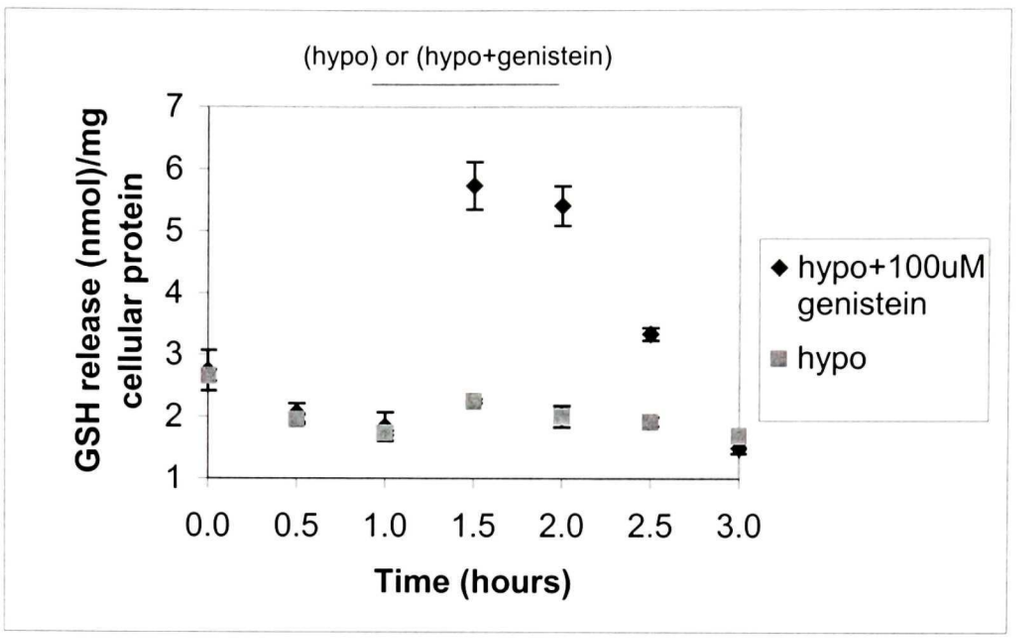
A


(hbss+dmso) or (hbss+genistein)
GSH release (nmol)/mg cellular protein
7
6
5
4
3
2
1
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hbss+dmso
hbss+100uM genistein

B


Change in GSH release (nmol)/ mg cellular protein
4
3
2
1
0
-1
hbss
hbss+100uM genistein

C


(hypo) or (hypo+genistein)
GSH release (nmol)/mg cellular protein
7
6
5
4
3
2
1
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo+100uM genistein
hypo

D

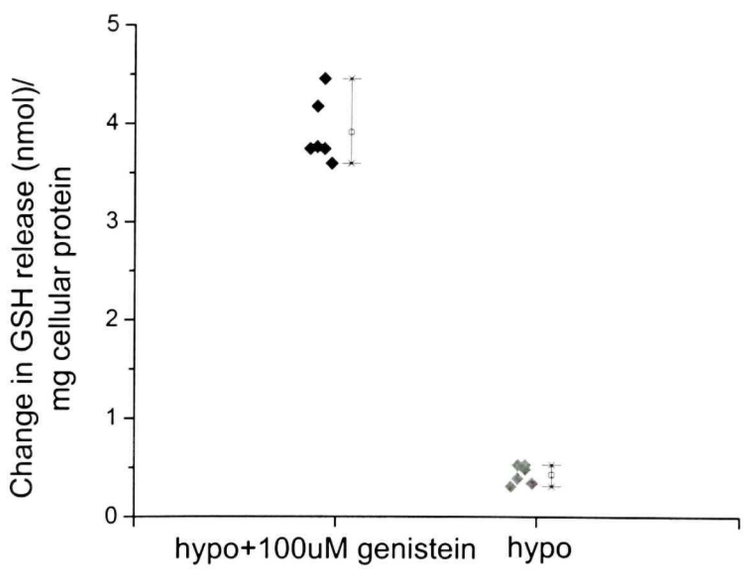
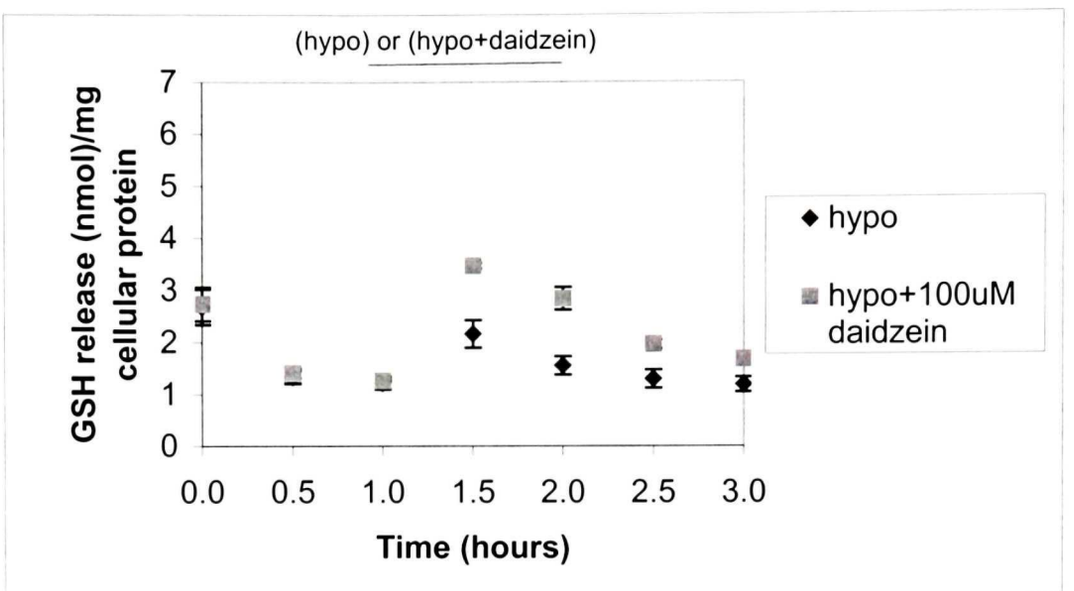

Change in GSH release (nmol)/ mg cellular protein
5
4
3
2
1
0
hypo+100uM genistein
hypo

Figure 15: Response of hypotonic GSH efflux to daidzein, an inactive analogue of the tyrosine kinase inhibitor genistein. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline GSH efflux was established prior to adding 100μM daidzein. Bar represents duration of exposure to daidzein. **A.** Comparison of hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without daidzein (n=3 for each data set). **B.** Summary of hypotonically-induced GSH efflux changes from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without daidzein (n=3 for each data set).

A


(hypo) or (hypo+daidzein)
GSH release (nmol)/mg cellular protein
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo
hypo+100uM daidzein

B

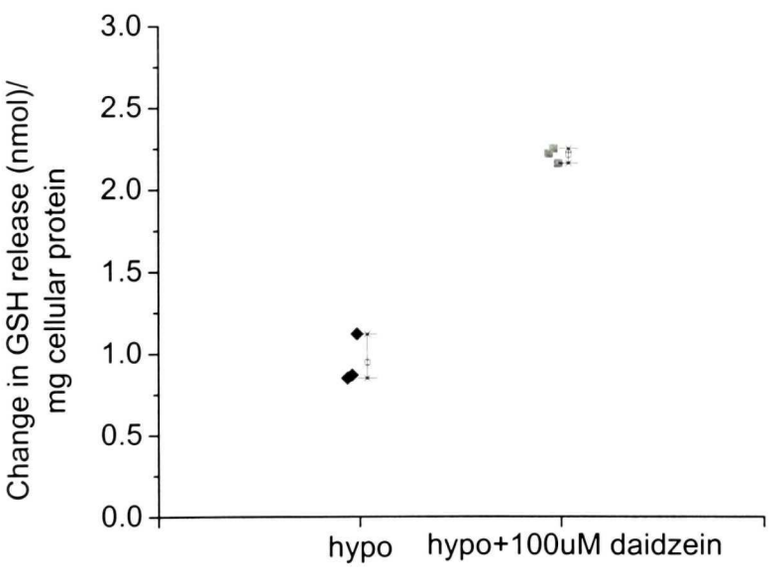
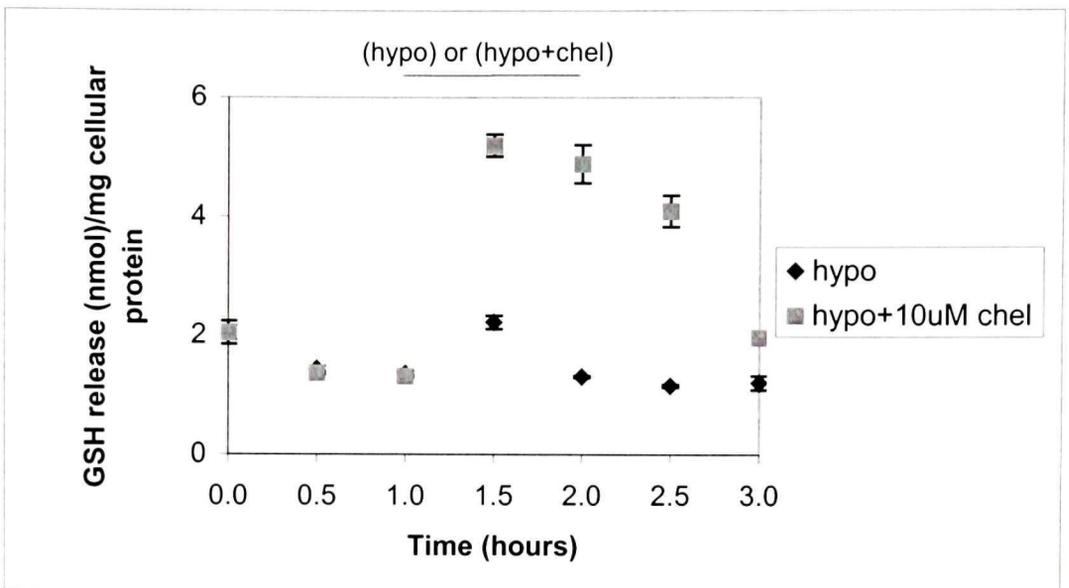
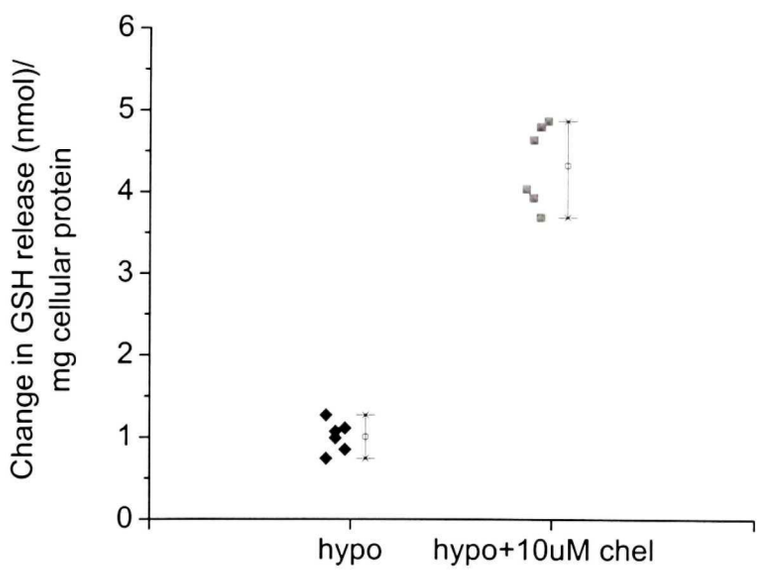

Change in GSH release (nmol)/ mg cellular protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
hypo
hypo+100uM daidzein

Figure 16: Response of hypotonic GSH efflux to protein kinase C inhibitor chelerythrine. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline GSH efflux was established prior to adding 10μM chelerythrine. Bar represents duration of exposure to chelerythrine. **A.** Representative experiment showing hypotonic GSH effluxes in CFTR-expressing IB3-837 cells, with and without chelerythrine (n=6 for each data set). **B.** Summary of hypotonically-induced GSH efflux changes from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without chelerythrine (n=6 for each data set). Graph represents individual data points from multiple experiments.

A


(hypo) or (hypo+chel)
GSH release (nmol)/mg cellular protein
6
4
2
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo
hypo+10uM chel

B


Change in GSH release (nmol)/ mg cellular protein
6
5
4
3
2
1
0
hypo
hypo+10uM chel

3.3.4 MAP kinase pathway

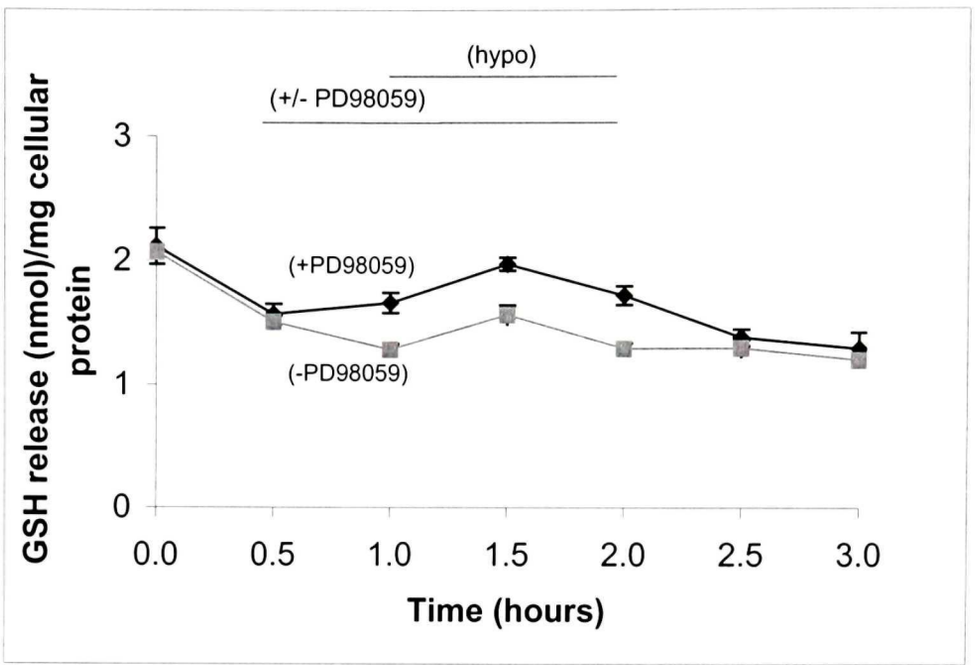
Mitogen-activated protein (MAP) kinases are activated by cell swelling and other stresses in rat hepatocytes (Kim *et al.*, 2000), renal epithelial A6 cells (Niisato *et al.*, 1999) and human intestine 407 cells (Tilly *et al.*, 1996), and therefore are candidates to mediate hypotonically-induced GSH release. Two key steps in stress-activated MAP kinase pathways are the upstream MAP kinase kinase (MEK) and p38 MAP kinase. Both these enzymes were inhibited individually to assess their roles in GSH efflux. Preincubation of cells with PD98059 (50μM), a specific MEK inhibitor (Alessi *et al.*, 1995), followed by application of hypotonic stress in the presence of PD98059 did not alter the magnitude of GSH release induced by hypotonic stress ($p>0.05$), although the drug did cause a modest increase in basal GSH release levels compared to control buffer alone at t=1.0h ($p<0.05$; Figures 17a, 17b). The same protocol was used to test SB203580, a specific inhibitor of p38 MAP kinase (Cuenda *et al.*, 1995). SB203580 (10μM) marginally decreased the GSH response to hypotonic stress ($p<0.05$; Figure 17c, 17d), although this might have been due to the small sample size and influence of outliers among the hypotonic stress samples. Like PD98059, SB203580 also caused a modest increase in basal GSH release compared to control buffer alone at t=1.0h ($p<0.05$).

3.3.5 Purinergic pathway

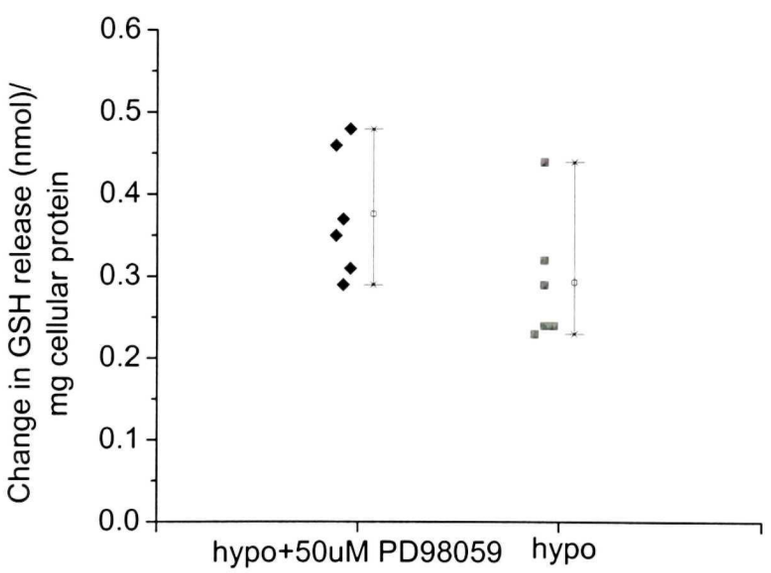
ATP is an important autocrine signaling molecule which is released during exposure to hypotonic solution. Indeed, one previous study suggested that such ATP release is essential for cell volume regulation in liver cells (Feranchak *et al.*, 1998). To examine the possible role of ATP autocrine signaling in GSH release, cells were

Figure 17: Response of hypotonic GSH efflux to MEK inhibitor PD98059 and p38 MAP kinase inhibitor SB203580. Monolayers of CFTR-expressing IB3-837 cells were cultured, and cells were preincubated with 50μM PD98059 or 10μM SB203580 for thirty minutes. Bar represents duration of exposure to PD98059 or SB203580. **A.** Representative experiment showing hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without PD98059 (n=3 for each data set). **B.** Summary of hypotonically-induced GSH efflux change from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without PD98059 (n=6 for each data set). Graph represents individual data points from multiple experiments. **C.** Representative experiment showing hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without SB203580 (n=3 for each data set). **D.** Summary of hypotonically-induced GSH efflux change from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without SB203580 (n=6 for each data set). Graph represents individual data points from multiple experiments.

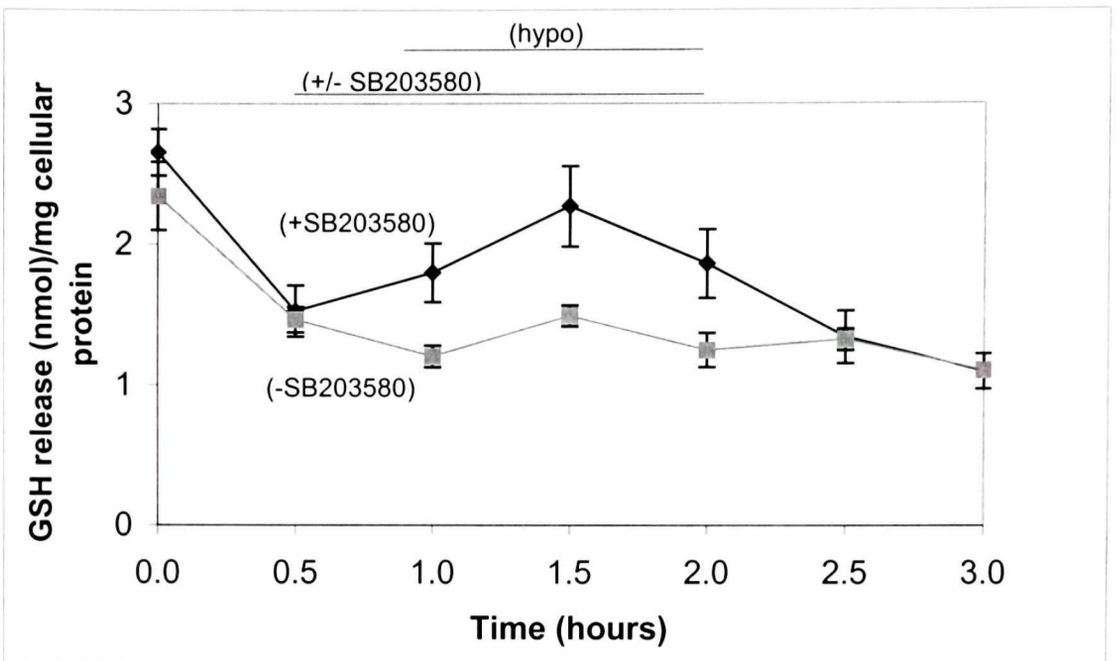
A


(hypo)
(+/- PD98059)
GSH release (nmol)/mg cellular protein
3
2
1
0
(+PD98059)
(-PD98059)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)

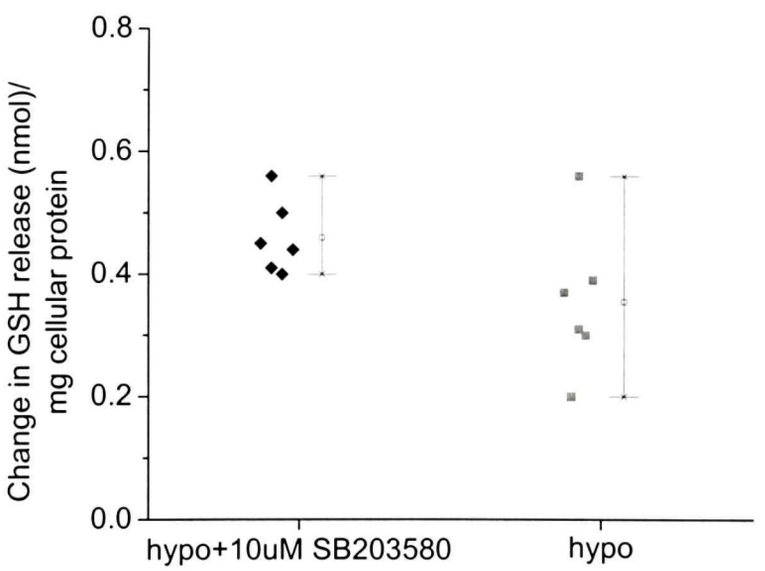
B


Change in GSH release (nmol)/ mg cellular protein
0.6
0.5
0.4
0.3
0.2
0.1
0.0
hypo+50uM PD98059
hypo

C


(hypo)
(+/- SB203580)
GSH release (nmol)/mg cellular protein
(+SB203580)
(-SB203580)
0
1
2
3
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)

D


Change in GSH release (nmol)/ mg cellular protein
0.0
0.2
0.4
0.6
0.8
hypo+10uM SB203580
hypo

challenged with hypotonic solution containing either ATP (500μM) or apyrase (1U/ml). Adding excess ATP (Figure 18a, 18b) or clamping ATP at low levels with apyrase (Figure 18c, 18d) had no effect on modifying hypotonic GSH efflux ($p>0.05$), suggesting GSH release is not secondary to autocrine activation of purinoceptors. Further evidence for this comes from the finding that low-Ca^{2+} buffer did not adversely affect hypotonically-induced GSH efflux (unpublished results).

Examination of candidate efflux pathways for GSH

3.3.6 Exocytotic pathway

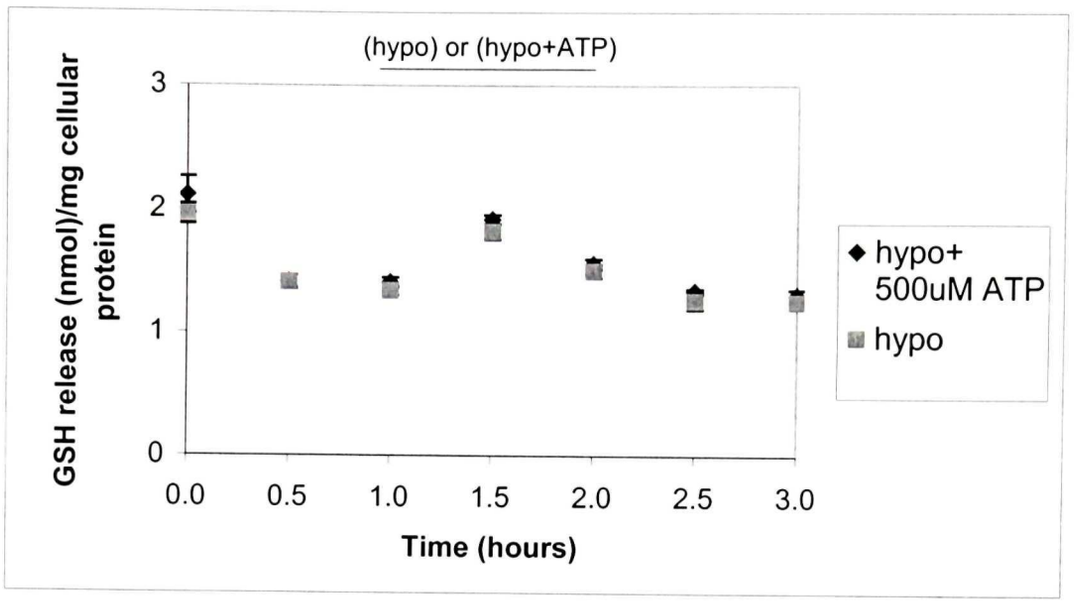
It has been proposed that ATP and other macromolecules can be released from intestine 407 cells through an exocytotic pathway (van der Wijk *et al.*, 2000). To assess the contribution of exocytosis to GSH release, cells were preincubated with brefeldin A (BFA; 5ug/ml), a well-known inhibitor of vesicular transport, for one hour and then exposed to hypotonic stress in the continued presence of BFA (Figures 19a, 19b). Brefeldin A caused a marked increase in basal GSH release under isoosmotic conditions compared to control buffer alone at t=1.0h ($p<0.05$), and partially inhibited hypotonically-induced GSH release ($p<0.05$).

3.3.7 Overexpression of MRP1 or MRP4 does not affect hypotonically-induced GSH efflux

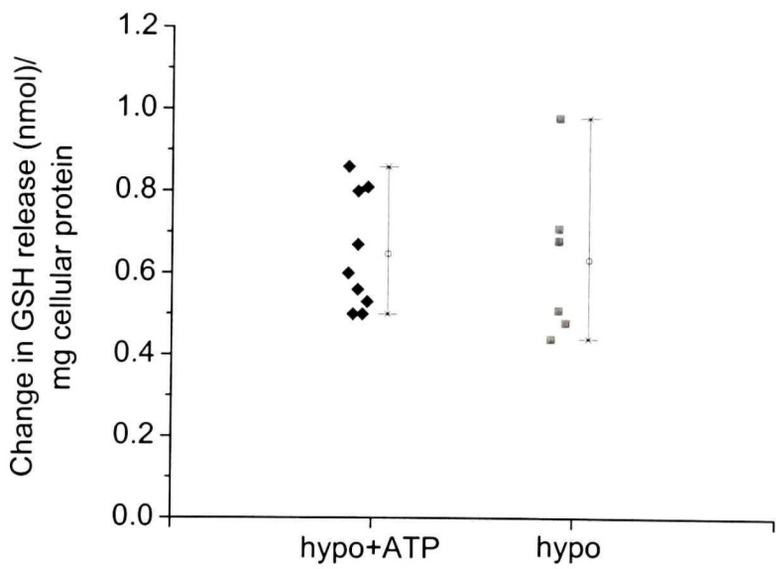
MRP1 transports hydrophobic molecules with GSH, therefore we examined whether MRP1 overexpression in human embryonic kidney (HEK) cells enhances

Figure 18: Response of hypotonic GSH efflux to ATP addition or depletion. Monolayers of CFTR-expressing IB3-837 cells were cultured, and baseline GSH efflux was established prior to adding 500μM ATP or 1U/ml apyrase. Bar represents duration of exposure to ATP or apyrase. **A.** Representative experiment showing hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without ATP (n=3 for each data set). **B.** Summary of hypotonically-induced GSH efflux changes from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without ATP (n=6 for each data set). Graph represents individual data points from multiple experiments. **C.** Representative experiment showing hypotonic GSH effluxes in CFTR-expressing IB3-837 cells, with and without apyrase (n=3 for each data set). **D.** Summary of hypotonically-induced GSH efflux change from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without apyrase (n=6 for each data set). Graph represents individual data points from multiple experiments.

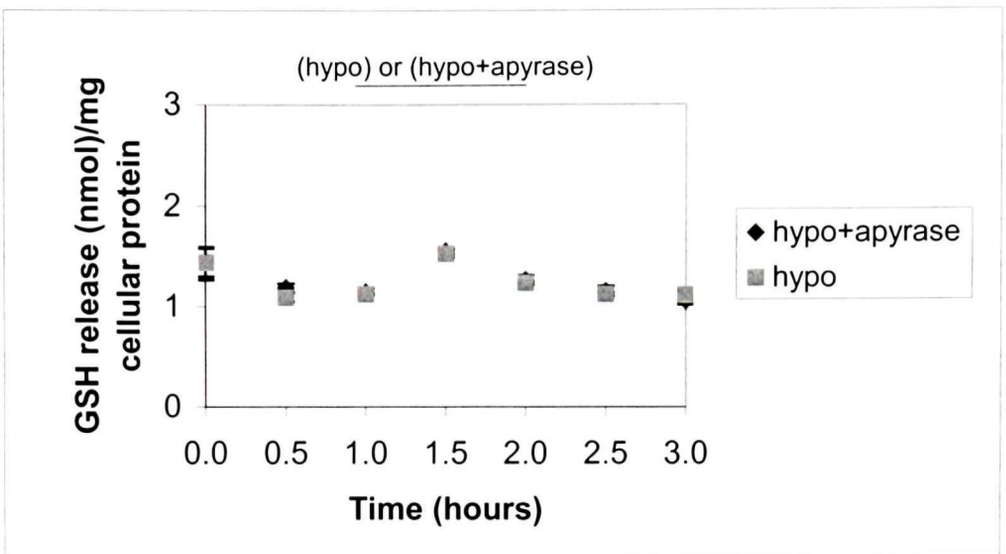
A


(hypo) or (hypo+ATP)
GSH release (nmol)/mg cellular protein
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo+ 500uM ATP
hypo

B


Change in GSH release (nmol)/ mg cellular protein
1.2
1.0
0.8
0.6
0.4
0.2
0.0
hypo+ATP
hypo

C


(hypo) or (hypo+apyrase)
GSH release (nmol)/mg cellular protein
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)
hypo+apyrase
hypo

D

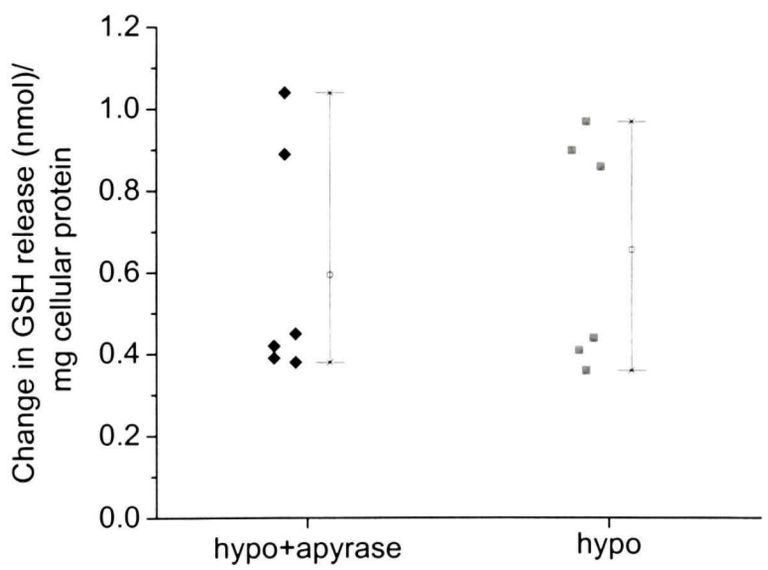
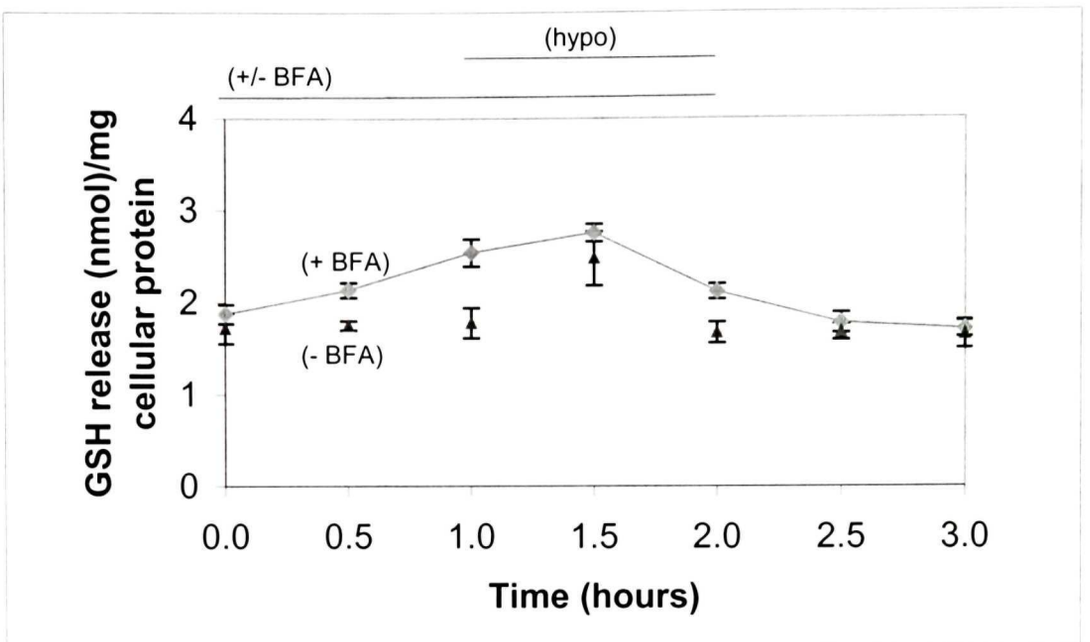
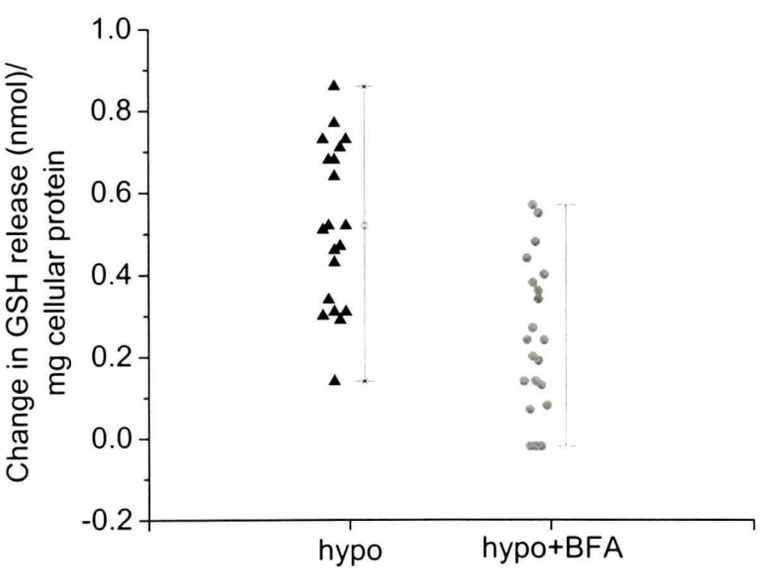

Change in GSH release (nmol)/ mg cellular protein
1.2
1.0
0.8
0.6
0.4
0.2
0.0
hypo+apyrase
hypo

Figure 19: Response of hypotonic GSH efflux to vesicular transport inhibitor brefeldin A. Monolayers of CFTR-expressing IB3-837 cells were cultured, and cells were preincubated with 5μg/ml brefeldin A for one hour. Bar represents duration of exposure to brefeldin A. **A.** Representative experiment showing hypotonically-induced GSH effluxes in CFTR-expressing IB3-837 cells, with and without brefeldin A (n=3 for each data set). **B.** Summary of hypotonically-induced GSH efflux change from t=1.0h to 1.5h in CFTR-expressing IB3-837 cells, with and without brefeldin A (n=20 for each data set). Graph represents individual data points from multiple experiments.

A


(hypo)
(+/- BFA)
GSH release (nmol)/mg cellular protein
4
3
2
1
0
(+ BFA)
(- BFA)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)

B


Change in GSH release (nmol)/ mg cellular protein
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
hypo
hypo+BFA

hypotonically-induced GSH efflux. Interestingly, the basal GSH efflux rate was much higher in MRP-transfected cells than in control 293 cells ($p<0.01$; Figure 20), indicating that MRP does contribute to constitutive GSH release. However, no hypotonically-stimulated GSH release was detected (Figure 20), therefore it is unlikely that MRP1 plays a role in swelling-induced efflux in HEK-293 or IB3 epithelial cells.

A recent study by Lai *et al.* (2002) demonstrated that MRP4 transports reduced glutathione. To examine the effect of overexpressing MRP4 on swelling-induced GSH release, pcDNA6MRP4 obtained from Dr.T.Lai was transiently transfected into HEK-293 cells using FuGENE 6. The MRP4 had a V5 epitope at its C-terminus so that its expression could be confirmed by Western blotting (Figure 21a). Transient expression of MRP4 did not enhance hypotonically-induced GSH efflux when compared to 293 and 293pcDNA3 vector-transfected controls (Figure 21b). Similar negative results were obtained when cells were transfected using a calcium phosphate transient transfection protocol (data not shown).

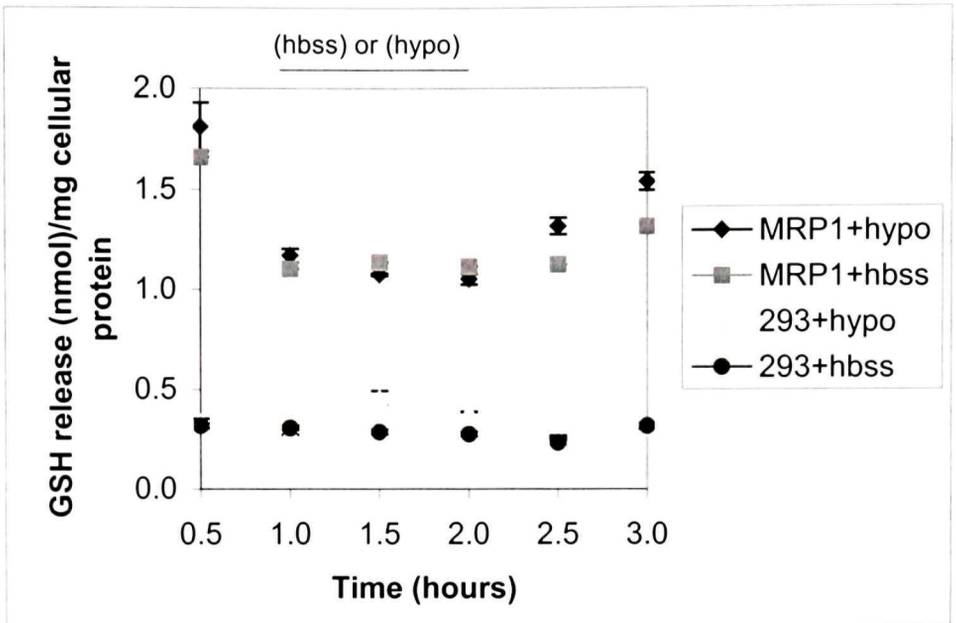
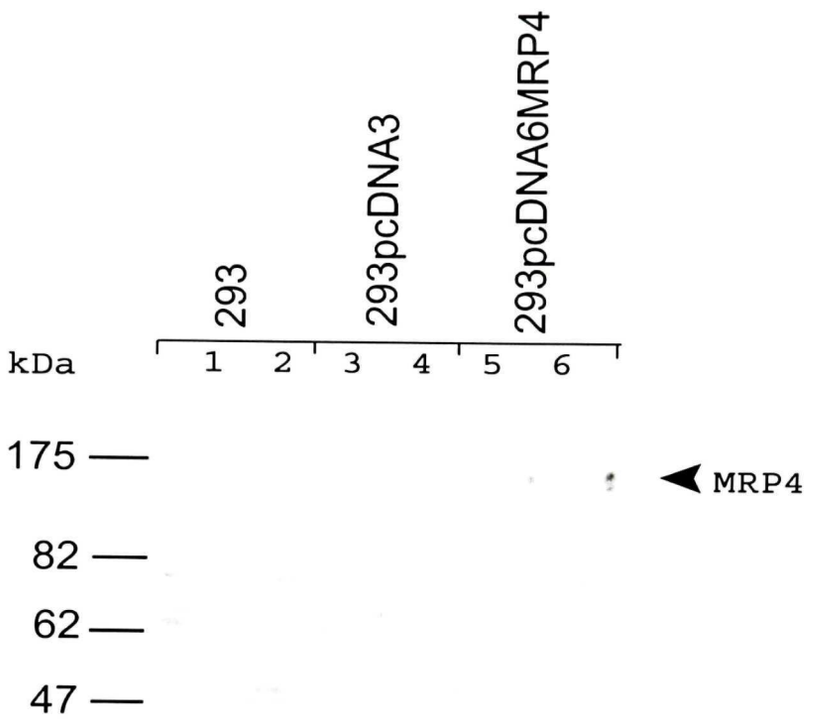


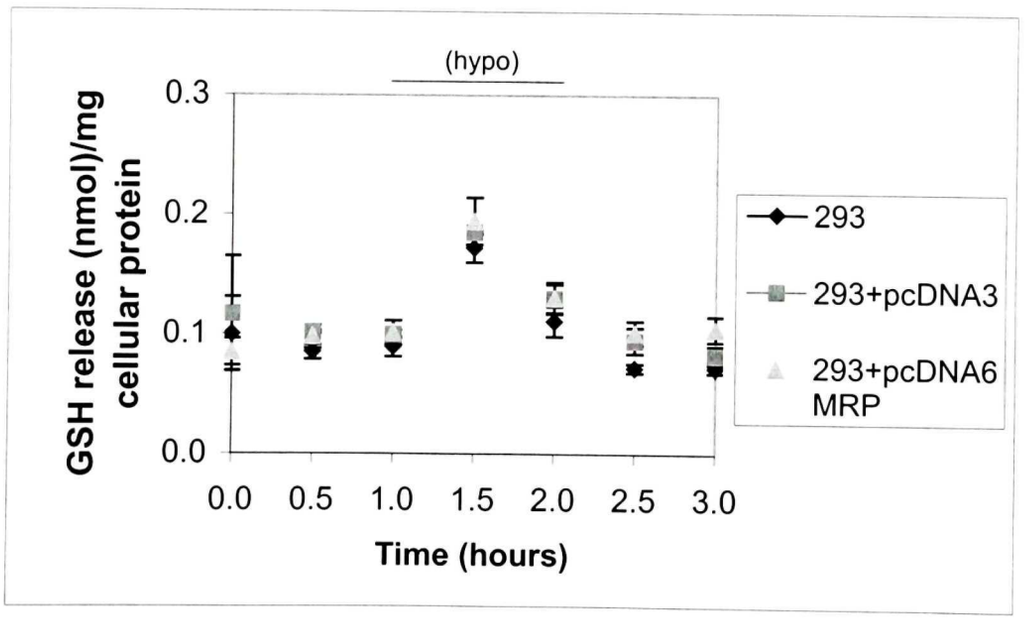
Figure 20: Response of hypotonic GSH efflux in cells with and without MRP1. Representative experiment in which monolayers of HEK cells expressing MRP1 (HEK-MRP1) and lacking MRP1 (HEK-293) were cultured, and baseline GSH efflux was established prior to adding 70%HBSS. Bar represents duration of hypotonic treatment (n=3 for each data set).

Figure 21: Response of hypotonic GSH efflux in cells with and without MRP4. Monolayers of FuGENE 6 transiently transfected MRP4-expressing HEK cells (HEK-MRP4) and cells lacking MRP1 (HEK-293) were cultured, and baseline GSH efflux was established prior to adding 70%HBSS. Bar represents duration of hypotonic treatment. **A.** MRP4 expression in FuGENE 6 transiently transfected HEK-293 cells. Total protein was harvested and a Western blot was performed using an anti-V5 antibody against MRP4 . HEK-293 cells and HEK-293 cells transiently transfected with empty vector pcDNA3 were used as negative controls, and 100μg of protein was loaded in each well. **B.** Comparison of hypotonically-induced GSH effluxes in MRP-expressing and nonexpressing cells (n=3 for each data set).

A


293
293pcDNA3
293pcDNA6MRP4
kDa
1 2 3 4 5 6
175
82
62
47
MRP4

B


(hypo)
GSH release (nmol)/mg cellular protein
0.3
0.2
0.1
0.0
0.0 0.5 1.0 1.5 2.0 2.5 3.0
Time (hours)
293
293+pcDNA3
293+pcDNA6 MRP

CHAPTER 4: DISCUSSION

4.1 Role of CFTR in GSH release

The elevation of GSH efflux by CFTR agonists (cAMP/IBMX/forskolin) in cells irrespective of CFTR expression is a new finding and implies that CFTR is not a significant transporter of GSH. The presence of CFTR did elevate basal GSH release, however, by comparison of doxycycline-induced CFTR-expressing cells to vector-only control cells exposed to doxycycline. This is consistent with a previous study in which apical fluid GSH levels were higher in CFTR-expressing cells (Gao *et al.*, 1999) but not with the patch clamp data of Linsdell *et al.* (1998) that indicated CFTR permeability to GSH from the cytoplasmic side. While GSH conductance was detected using excised patches, it seems unlikely that CFTR mediates significant GSH efflux under physiological conditions. Nevertheless, CFTR expression does upregulate GSH release through some mechanism that is independent of channel stimulation by PKA.

A recent paper by Kogan *et al.* (2003) demonstrated that CFTR can mediate glutathione flux in crude membrane vesicles and reconstituted proteoliposomes. This would appear to contradict the conclusion of this study, however, there were several differences in methodology that might explain the apparent discrepancy. Kogan *et al.* used radiolabelled GSH to assay transport, which may detect smaller fluxes than the spectrophotometric assay used here. More importantly, Kogan *et al.* found no CFTR-associated GSH flux in native membranes when assays were performed with ATP present. CFTR-dependent GSH transport only became significant when AMP-PNP replaced ATP. This non-hydrolyzable analog could not be employed under the

physiological conditions used in the present study. Finally, it is possible that CFTR-mediated GSH flux was present in both studies, but was only detectable after eliminating other membrane proteins by purification and reconstitution of CFTR in proteoliposomes. If those other proteins contributed most of the physiologically-relevant GSH transport, this would explain why the CFTR-mediated component was not detectable. The issue of CFTR-mediated GSH flux remains somewhat controversial, as Jensen *et al.* (2001) found no ATP-dependent transport of radioactive GSH in CFTR-containing membrane vesicles from Sf9 cells or BHK cells using biochemical methods similar to those used by Kogan *et al.*.

The advantage of using an inducible system is that it allows comparison of cells with or without expression of CFTR, however experiments revealed that uninduced (-DOX) IB3-837 cells (which should not express significant CFTR) had basal GSH effluxes that were comparable to those of DOX-induced IB3-837 cells (which should express CFTR). The simplest explanation for this result is that very low levels of CFTR are sufficient to upregulate GSH release, and these levels of CFTR were achieved even in uninduced cells because the tetracycline-responsive element (TRE) promoter is slightly leaky. This is consistent with the results of Ye *et al.* (2001), who found low levels of CFTR mRNA expression in this line even in the absence of doxycycline. For this reason, cells transfected with empty vector were used as controls in addition to +/- doxycycline.

Standard curves were obtained prior to each experiment, and served as internal controls for the assay and for any slight discrepancies in experimental conditions from day to day. Care was taken to maintain the same experimental conditions throughout,

nevertheless subtle differences in cell confluency, passage number, and the activity of glutathione reductase may account for the variability observed.

4.2 Hypotonically-stimulated GSH efflux

Hypotonicity stimulates the efflux of many solutes such as Na^+ (Venosa *et al.*, 2003), K^+ and Cl^- (De Smet *et al.*, 1998; Roman *et al.*, 1996; Hug *et al.*, 1995; Mastrocola *et al.*, 1991), HCO_3^- (Nicholl *et al.*, 2002; Wojnowski *et al.*, 1992), ATP (Boudreault *et al.*, 2002; Feranchak *et al.*, 1998; Wang *et al.*, 1996), and organic osmolytes such as the amino acid taurine (Boese *et al.*, 1996; Galietta *et al.*, 1996) and to a lesser extent glutamate, aspartate, glycine, phosphoethanolamine and GABA (Estevez *et al.*, 1999; Manolopoulos *et al.*, 1997; Kimelberg *et al.*, 1990). The present study revealed that hypotonic stress increased GSH efflux, which has not been reported previously. Hypotonically-induced GSH efflux was not dependent on the expression of CFTR and was observed in HEK cells, therefore it is not airway cell-specific. Nevertheless, it may be relevant to the hypotonic/defensin hypothesis for pathogenesis of CF airway infection, which proposes that defective chloride transport results in an abnormally high salt concentration (i.e. hypertonic) in the epithelial lining fluid (ELF) that in turn inhibits its bactericidal activity (Smith *et al.*, 1996). For example, if ELF is normally hypotonic and this promotes GSH release, hypertonicity in cystic fibrosis might be expected to reduce GSH release and contribute to low luminal GSH levels in CF. However, most recent evidence suggests that the ionic composition and osmolality of ELF are not affected in CF (Jayaraman *et al.*, 2001; Knowles *et al.*, 1997), therefore loss of the small upregulation of basal GSH release by mutant CFTR expression may be more relevant to the disease. As

expected, hypotonic stress also stimulated anion efflux, consistent with a regulatory volume decrease after hypotonic swelling.

In this study, no evidence for significant GSH efflux through CFTR was obtained, therefore alternative pathways were explored. In HEK cells, MRP1 overexpression did not lead to larger GSH efflux responses to hypotonic stress, although basal release was elevated. This suggests that MRP1 could still be involved, perhaps indirectly, in mediating GSH efflux. Using HEK-293 cells transiently transfected with MRP4, we also observed a hypotonically-mediated GSH efflux, but MRP4 did not appear to have an effect on GSH transport. This contrasts with the results of Lai *et al.* (2002), however there were several differences between the experimental protocols. Different cell types (MDCKII/HepG2 vs. HEK cells) and GSH collection methodologies were used (cell suspension vs. cell monolayer supernatant, 100 minutes vs. 30 minutes). Further studies are needed to assess the role of MRP in GSH transport in HEK-293 cells, as a recent study has shown that MRP4 can apparently transport cyclic nucleotides such as cAMP (Chen *et al.*, 2001).

4.3 Signaling pathways

PI3-kinase activation occurs in response to hypotonic stress in hepatocytes (Krause *et al.*, 1996) and may mediate ATP efflux under these conditions in HTC hepatoma cells (Feranchak *et al.*, 1998). Our results suggest that GSH efflux is not regulated by PI3-kinase because the inactive analogue LY303511 also elevated GSH efflux and another PI3-kinase inhibitor (wortmannin) had no effect on GSH transport. In addition to PI3-kinase, LY294002 is known to inhibit casein kinase 2 (Davies *et al.*,

2000) as well as the PI3-kinase related protein kinases (Stein, 2001), and so it is possible that its effects on GSH release may be due to inhibition of these other kinases.

It has been reported that swelling-induced efflux of ions (Tilly *et al.*, 1993) and amino acids (Song *et al.*, 1998) can be reduced by inhibiting protein tyrosine kinases. Our results differ from those previous studies, in that genistein application caused an elevation of GSH efflux. It is possible that the cell type is an important determinant in tyrosine kinase-regulated GSH efflux, as previous studies were done using intestinal and cardiac cells rather than airway cells. Tyrosine kinases may make some contribution to the regulation of GSH efflux, since genistein had a much larger effect than its inactive analogue daidzein..

While Song *et al.* (1998) found that PKC inhibition reduced the osmotically-induced release of amino acids from rat cardiac cells, our results suggest a different role of PKC in efflux pathways, as chelerythrine seemed to potentiate the hypotonic efflux of glutathione. PKC is known to be activated during cell swelling in hepatocytes (Roman *et al.*, 1998), which may be part of the cellular response to restore homeostasis. Therefore, when PKC is inhibited, the decrease in swelling compensation would effectively result in a greater net level of cellular stress, resulting indirectly in a higher GSH efflux as a stress response. Another possibility is that PKC plays a more direct role in GSH efflux, whereby PKC is inhibited during normal conditions, and release of this inhibition upon exposure to hypotonic stress causes elevated GSH efflux.

It has been shown that ATP is released from cells in response to hypotonic stress (Guyot *et al.*, 2002; Wang *et al.*, 1996). This raises the possibility that ATP acts as an autocrine signaling molecule, binding to purinergic receptors on the cell surface to initiate GSH efflux. However, based on our results, ATP and purinergic receptors are not involved in hypotonic GSH efflux since the addition or depletion of extracellular ATP did not alter GSH release. The physiological role of intracellular ATP levels in controlling GSH release is unclear, since exogenous ATP might be redundant if intracellularly derived ATP is already high, or if exogenous apyrase does not significantly reduce physiological ATP.

Our data suggests that p38 MAP kinases and ERKs could be involved in modulating GSH efflux, since both SB203580 and PD98059 increased basal GSH efflux but little or no alteration of the hypotonically-induced release. Since these inhibitors elevated basal GSH efflux, MAP kinases may normally exert an inhibitory effect on release. The lack of response during exposure to hypotonic solution was surprising however, since these pathways are usually activated by stress-related signals, including hypoosmotic stress in rat hepatocytes (Kim *et al.*, 2000; vom Dahl *et al.*, 2001).

Evidence that GSH release is tonically inhibited in the basal state comes from the relative potencies of various activators and inhibitors. Deactivating downstream enzymes (e.g. PKC) that are presumably closer to the GSH release mechanism elicits the greatest GSH release, whereas less vigorous responses are observed after inhibiting more proximal enzymes.

All of the drugs used in this work have been used previously on intact cells in other studies. However, the brefeldin A experiments should be interpreted cautiously since BFA disrupts the endoplasmic reticulum and vesicle trafficking, which is probably stressful for cells and could have diverse effects. Specificity is also a consideration for PI3-kinase and PKC studies; LY294002 and its inactive analogue elicited a large GSH efflux, while wortmannin did not. Although widely used as a PKC inhibitor, the potency and specificity of chelerythrine has been questioned (Lee *et al.*, 1998). Recently, p38 MAPK was found to be activated by chelerythrine via a PKC-independent mechanism (Yu *et al.*, 2000), raising the possibility that chelerythrine affects other signaling pathways.

4.4 Possible mechanisms of GSH efflux in airway epithelial cells

GSH release induced by LY294002 or wortmannin does not seem to be associated with the increased halide permeability that occurs during hypotonic stress. Also, CFTR does not appear to mediate GSH release in IB3 and BHK cells, since exposure to cAMP/IBMX/forskolin or hypotonic solution stimulated GSH release independently of CFTR expression.

The role of exocytosis was explored using brefeldin A, but it caused only a modest decrease in hypotonic-stimulated GSH release. Nevertheless, exocytotic membrane insertion and release of vesicular GSH cannot be excluded; indeed, GSH may be in the same vesicles that contain ATP (Maroto *et al.*, 2001). The GSH concentration in vesicles is not known, but may be comparable to the cytoplasm (1-10mM; Kogan *et al.*, 2001).

cAMP stimulates efflux of the protein α_1-antitrypsin in colonic epithelial cells (Jilling *et al.*, 1996) and exocytosis of surfactant from alveolar type II cells (Rooney, 2001), therefore it is conceivable that cAMP stimulates exocytosis of GSH-containing vesicles. Low antioxidant levels in the ASF causes abnormalities in the surfactant system (Morris *et al.*, 1994), thus coordinated release with glutathione would help maintain an optimal environment for surfactant secretion and signal transduction. Using the same cellular signal (cAMP) to secrete surfactant and a protective molecule (i.e. glutathione) would be an elegant solution to this physiological problem. Our results indicate that cAMP by itself can stimulate exocytotic release of GSH, as cAMP caused elevated GSH efflux from both IB3 and BHK cells, independently of CFTR.

If indeed glutathione efflux occurs via a vesicular transport method, GSH transport could occur through two types of pathways, constitutive and regulated secretion. The constitutive secretory pathway allows for continual secretion of proteins needed outside the cell and could mediate the low basal rate of GSH efflux seen under control conditions. The regulated secretory pathway, by contrast, may require an external stimulus such as hypotonic stress or cAMP.

Although CFTR does not appear to mediate GSH transport, CFTR-expressing cells (IB3-837) had higher basal levels of GSH efflux compared to those lacking CFTR (IB3-TA8). Its presence may be sufficient to promote membrane turnover. Alternatively, GSH may be released through a specific transporter that remains to be identified.

SUMMARY

The results in this study demonstrate that cAMP and hypotonic stress stimulate glutathione release. GSH does not appear to be directly transported across the plasma membrane via a CFTR-mediated pathway in airway and other cells, although release is higher when CFTR is expressed. The signaling pathways involved in the hypotonic-mediated efflux of glutathione in airway epithelial cells were explored using pharmacological methods. Inhibition of some signaling pathways elevated GSH efflux, suggesting that GSH is tonically inhibited under basal conditions. Although some evidence exists to support exocytotic GSH release, further studies are needed to establish the mechanism of release.

REFERENCES

AKABAS MH (2000). Cystic fibrosis transmembrane conductance regulator: structure and function of an epithelial chloride channel. *Journal of Biological Chemistry* **275,** 3729-3732.

AKIYAMA T, ISHIDA J, NAKAGAWA S, OGAWARA H, WATANABE S, ITOH N, SHIBUYA N & FUKAMI Y (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry* **262(12)**; 5592-5595.

ALESSI DR, CUENDA A, COHEN P, DUDLEY DT & SALTIEL AR (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo. Journal of Biological Chemistry* **270**, 27489-27494.

ANDERSON MP, BERGER HA, RICH DP, GREGORY RJ, SMITH AE, AND WELSH MJ (1991). Nucleoside triphosphates are required to open the CFTR chloride channel. *Cell* **67**: 775-784.

ARCARO A, ZVELEBILMJ, WALLASCH C ULLRICH A, WATERFIELD MD, & DOMIN J (2000). Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *Molecular and Cellular Biology* **20(11)**: 3817-3830.

BLAU H. "Cystic Fibrosis", [online], [cited May 8, 2003], available from World Wide Web: http://www.utoronto.ca/kids/cystic.htm.

BORGES R, MACHADO JD, BETANCOR G & CAMACHO M (2002). Pharmacological regulation of the late steps of exocytosis. *Annals of the New York Academy of Sciences* **971**: 184-192.

BOESE SH, WEHNER F & KINNE RK (1996). Taurine permeation through swelling-activated anion conductance in rat IMCD cells in primary culture. *American Journal of Physiology* **271(3 Pt 2)**: F498-F507.

BOUDREAULT F & GRYGORCZYK R (2002). Cell swelling-induced ATP release and gadolinium-sensitive channels. *American Journal of Physiology - Cell Physiology* **282(1)** : C219-C226.

BÜCHLER M, KONIG J, BROM M, KARTENBECK J, SPRING H, HORIE T & KEPPLER D (1996). cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *Journal of Biological Chemistry* **271(25)**: 15091-15098.

CADENA DL & GILL GN (1992). Receptor protein tyrosine kinases. In: *Protein Phosphorylation*, edited by Marks F. New York: VCH Publishers, pp. 265-284.

CANTIN AM & BEGIN R (1991). Glutathione and inflammatory disorders of the lung. *Lung* **169(3)**: 123-138.

CANTIN AM, NORTH SL, HUBBARD RC & CRYSTAL RG (1987). Normal alveolar epithelial lining fluid contains high levels of glutathione. *Journal of Applied Physiology* **63(1)**: 152-157.

CHEN ZS, LEE K & KRUH GD (2001). Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. *Journal of Biological Chemistry* **276(36)**: 33747-33754.

CHENG J, MOYER BD, MILEWSKI M, LOFFING J, IKEDA M, MICKLE JE, CUTTING GR, LI M, STANTON BA & GUGGINO WB (2002). A Golgi-associated PDZ domain protein modulates cystic fibrosis transmembrane regulator plasma membrane expression. *Journal of Biological Chemistry* **277(5)**: 3520-3529.

COMHAIR SAA & ERZURUM SC (2002). Antioxidant responses to oxidant-mediated lung diseases. *American Journal of Physiology: Lung Cellular and Molecular Physiology* **283**: L246-L255.

CUENDA A, ROUSE J, DOZA YN, MEIER R, COHEN P, GALLAGHER TF, YOUNG PR & LEE JC (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Letters* **364**, 229-233.

DAVIES SP, REDDY H, CAIVANO M & COHEN P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* **351**: 95-105.

DEAN M, HAMON Y & CHIMINI G (2001). The human ATP-binding cassette (ABC) transporter superfamily. *Journal of Lipid Research* **42**: 1007-1017.

DE SMET P, LI J & VAN DRIESSCHE W (1998). Hypotonicity activates a lanthanide-sensitive pathway for K^+ release in A6 epithelia. *American Journal of Physiology* **275(1 Pt 1)** : C189-C199.

DICKINSON DA & FORMAN HJ (2002). Cellular glutathione and thiols metabolism. *Biochemical Pharmacology* **64(5-6)**: 1019-1026.

DIEDERICHS K, DIEZ J, GRELLER G, MÜLLER C, BREED J, SCHNELL C, VONRHEIN C, BOOS W & WELTE W (2000). Crystal structure of MalK, the ATPase subunit of the trehalose/maltose ABC transporter of the archaeon *Thermococcus litoralis*. *EMBO Journal* **19(22)**: 5951-5961.

DIMMELER S, FLEMING I, FISSLTHALER B, HERMANN C, BUSSE R & ZEIHER AM (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**: 601-605.

DONALDSON JG, LIPPINCOTT-SCHWARTZ J, BLOOM GS, KREIS TE & KLAUSNER RD (1990). Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. *Journal of Cell Biology* **111**: 2295-2306.

DRÖGE W, SCHULZE-OSTHOFF K, MIHM S, GALTER D, SCHENK H, ECK H-P, ROTH S & GMÜNDER H (1994). Functions of glutathione and glutathione disulfide in immunology and immunopathology. *FASEB J.* **13**: 1131-1138.

ECKLY-MICHEL AE, LE BEC A & LUGNIER C (1997). Chelerythrine, a protein kinase C inhibitor, interacts with cyclic nucleotide phosphodiesterases. *European Journal of Pharmacology* **324**: 85-88.

ESTEVEZ AY, O'REGAN MH, SONG D & PHILLIS JW (1999). Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. *Neurochemical Research* **24(3)**: 447-452.

FERANCHAK AP, ROMAN RM, SCHWIEBERT EM & FITZ JG (1998). Phosphatidylinositol 3-kinase contributes to cell volume regulation through effects on ATP release. *Journal of Biological Chemistry* **273(24)**: 14906-14911.

FREDHOLM BB, ARSLAN G, HALLDNER L, KULL B, SCHULTE G & WASSERMAN W (2000). Structure and function of adenosine receptors and their genes. *Naunyn Schmiedebergs Arch Pharmacol* **362(4-5)**: 364-374.

GALIETTA LJ, ROMEO G & ZEGARRA-MORAN O (1996). Volume regulatory taurine release in human tracheal 9HTEo$^-$ and multidrug resistant 9HTEo$^-$/Dx cells. *American Journal of Physiology* **271(3 Pt 1)** : C728-C735.

GAO L, BROUGHMAN JR, IWAMOTO T, TOMICH JM, VENGLARIK CJ & FORMAN HJ (2001). Synthetic chloride channel restores glutathione secretion in cystic fibrosis airway epithelia. *American Journal of Physiology (Lung Cellular and Molecular Physiology)* **281(1)**: L24-L30.

GAO L, KIM KJ, YANKASKAS JR & FORMAN HJ (1999). Abnormal glutathione transport in cystic fibrosis airway epithelia. *American Journal of Physiology* **277(1 Pt 1)**: L113-L118.

GATSIOS P, TERSTEGEN L, SCHLIESS F, HAUSSINGER D, KERR IM, HEINRICH PC & GRAEVE L (1998). Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock. *Journal of Biological Chemistry* **273(36)**: 22962-22968.

GRYGORCZYK R & HANRAHAN JW (1997). CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. *American Journal of Physiology* **272** (*Cell Physiology* **41**): C1058-C1066.

GUYOT A & HANRAHAN JW (2002). ATP release from human airway epithelial cells studied using a capillary cell culture system. *Journal of Physiology* **545(Pt 1)**: 199-206.

HANRAHAN JW, GENTZSCH M & RIORDAN JR (2002). Chapter 29: The Cystic Fibrosis Transmembrane Conductance Regulator (ABCC7). In: *ABC Proteins: From Bacteria to Man*, edited by Holland IB, Cole SPC, Kuchler K & Higgins CF. London: Academic Press, Ltd., pp. 589-618.

HEIJN M, OUDE ELFERINK RP & JANSEN PL (1992). ATP-dependent multispecific organic anion transport system in rat erythrocyte membrane vesicles. *American Journal of Physiology* **262 (1 Pt 1)**: C104-C110.

HOPPER E, BELINSKY MG, ZENG H, TOSOLINI A, TESTA JR & KRUH GD (2001). Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. *Cancer Letters* **162(2)**: 181-191.

HUG T, KOSLOWSKY T, ECKE D, GREGER R & KUNZELMANN K (1995). Actin-dependent activation of ion conductances in bronchial epithelial cells. *Pflügers Arch.* **429(5)** : 682-690.

HUNG LW, WANG IX, NIKAIDO K, LIU PQ, AMES GF & KIM SH (1998). Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature* **396(6712)**: 703-707.

INSEL PA, OSTROM RS, ZAMBON AC, HUGHES RJ, BALBOA MA, SHEHNAZ D, GREGORIAN C, TORRES B, FIRESTEIN BL XING M & POST SR (2001). P2Y receptors of MDCK cells: epithelial cell regulation by extracellular nucleotides. *Clinical and Experimental Pharmacology and Physiology* **28**: 351-354.

IRVINE T (2002). Studies of split R domain deleted CFTR channels expressed in mammalian cells. M.Sc. thesis, Department of Physiology, McGill University.

ISHIDA-TAKAHASHI A OTANI H, TAKAHASHI C, WASHIZUKA T, TSUJI K, NODA M, HORIE M & SASAYAMA S (1998). Cystic fibrosis transmembrane conductance regulator mediates sulphonylurea block of the inwardly rectifying K^+ channel Kir6.1. *Journal of Physiology* **508.1**: 23-30.

ISHIKAWA T, MULLER M, KLUNEMANN C, SCHAUB T & KEPPLER D (1990). ATP-dependent primary active transport of cysteinyl leukotrienes across liver canalicular membrane. Role of the ATP-dependent transport system for glutathione S-conjugates. *Journal of Biological Chemistry* **265(31)**: 19279-19286.

ISHIKAWA T (1989). ATP/Mg^{2+}-dependent cardiac transport system for glutathione S-conjugates: a study using rat heart sarcolemma vesicles. *Journal of Biological Chemistry* **264**: 17343-17348.

JAYARAMAN S, JOO NS, REITZ B, WINE JJ & VERKMAN AS (2001). Submucosal gland secretions in airways from cystic fibrosis patients have normal [Na^+] and pH but elevated viscosity. *Proceedings of the National Academy of Sciences U.S.A.* **98(14)**: 8119-8123.

JEDLITSCHKY G, LEIER I, BUCHHOLZ U, HUMMEL-EISENBEISS J, BURCHELL B & KEPPLER D (1997). ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. *Biochemical Journal* **327 (Pt 1)**: 305-310.

JEDLITSCHKY G, LEIER I, BUCHHOLZ U, CENTER M & KEPPLER D (1994). ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Research* **54(18)**: 4833-4836.

JENSEN TJ, CHANG X-B, RIORDAN JR (2001). Evidence that CFTR does not transport MRP substrates. *The Bulletin: Mount Desert Island Biological Laboratory* **40**: 6-7.

JILLING T & KIRK KL (1996). Cyclic AMP and chloride-dependent regulation of the apical constitutive secretory pathway in colonic epithelial cells. *Journal of Biological Chemistry* **271(8)**: 4381-4387.

KAI H, YOSHITAKE K, ISOHAMA Y, HAMAMURA I, TAKAHAMA K & MIYATA T (1994). Involvement of protein kinase C in mucus secretion by hamster tracheal epithelial cells in culture. *American Journal of Physiology* **267(5 Pt 1)**: L526-L530.

KARTNER N & RIORDAN JR (1998). Characterization of polyclonal and monoclonal antibodies to cystic fibrosis transmembrane conductance regulator. *Methods Enzymology* **292**: 629-652.

KEPPLER D (1999). Export pumps for glutathione s-conjugates. *Free Radical Biology & Medicine* **27**: 985-991.

KEPPLER D & KONIG J (1997). Hepatic canalicular membrane 5: expression and localization of the conjugate export pump encoded by the MRP2 (cMRP/cMOAT) gene in liver. *FASEB J* **11(7)**: 509-516.

KEPPLER D, LEIER I & JEDLITSCHKY G (1997). Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. *Biological Chemistry* **378(8)**: 787-791.

KHAKH BS, BURNSTOCK G, KENNEDY C, KING BF, NORTH RA, SEGUELA P, VOIGT M & HUMPHREY PP (2001). International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacological Reviews* **53(1)**: 107-118.

KIM RD, DARLING CE, CERWENKA H & CHARI RS (2000). Hypoosmotic stress activates p38, ERK 1 and 2 and SAPK/JNK in rat hepatocytes. *Journal of Surgical Research* **90**: 58-66.

KIMELBERG HK, GODERIE SK, HIGMAN S, PANG S & WANIEWSKI RA (1990). Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *Journal of Neuroscience* **10(5)**: 1583-1591.

KIUCHI Y, SUZUKI H, HIROHASHI T, TYSON CA & SUGIYAMA Y (1998). cDNA cloning and inducible expression of human multidrug resistance associated protein 3 (MRP3). *FEBS Letters* **433(1-2)**: 149-152.

KLEIZEN B, BRAAKMAN I & DE JONGE HR (2000). Regulated trafficking of the CFTR chloride channel. *European Journal of Cell Biology* **79**: 544-556.

KLINGER M, FREISSMUTH M & NANOFF C (2002). Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell Signaling* **14(2)**: 99-108.

KNOWLES MR, ROBINSON JM, WOOD RE, PUE CA, MENTZ WM, WAGER GC, GATZY JT & BOUCHER RC (1997). Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *Journal of Clinical Investigation* **100(10)**: 2588-2595.

KOGAN I, RAMJEESINGH M, LI C, KIDD JF, WANG Y, LESLIE EM, COLE SP & BEAR CE (2003). CFTR directly mediates nucleotide-regulated glutathione flux. *EMBO Journal* **22(9)**: 1981-9.

KOGAN I, RAMJEESINGH M, HUAN LJ, WANG Y & BEAR CE (2001). Perturbation of the pore of the cystic fibrosis transmembrane conductance regulator (CFTR) inhibits its ATPase activity. *Journal of Biological Chemistry* **276(15)**: 11575-11581.

KOOL M, VAN DER LINDEN M, DE HAAS M, BAAS F & BORST P (1999). Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. *Cancer Research* **59(1)**: 175-182.

KOOL M, DE HAAS M, SCHEFFER GL, SCHEPER RJ, VAN EIJK MJ, JUIJN JA, BAAS F & BORST P (1997). Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Research* **57(16)**: 3537-3547.

KRAUSE U, RIDER MH & HUE L (1996). Protein kinase signaling pathway triggered by cell swelling and involved in the activation of glycogen synthase and acetyl-CoA carboxylase in isolated rat hepatocytes. *Journal of Biological Chemistry* **271(28)**: 16668-16673.

KULCZYCKI LL, KOSTUCH M & BELLANTI JA (2003). A clinical perspective of cystic fibrosis and new genetic findings: relationship of CFTR mutations to genotype–phenotype manifestations. *American Journal of Medical Genetics* **116A**: 262–267.

LAI L & TAN TM (2002). Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues. *Biochemical Journal* **361(Pt 3)**: 497-503.

LAVIN MF, WATTERS D & SONG Q (1996). Role of protein kinase activity in apoptosis. *Experientia* **52(10-11)**: 979-994.

LAZAROWSKI ER, BOUCHER RC & HARDEN TK (2000). Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. *Journal of Biological Chemistry* **275(40)**: 31061-31068.

LEE K, BELINSKY MG, BELL DW, TESTA JR & KRUH GD (1998). Isolation of MOAT-B, a widely expressed multidrug resistance-associated protein/canalicular multispecific organic anion transporter-related transporter. *Cancer Research* **58(13)** : 2741-2747.

LEE MG, WIGLEY WC, ZENG W, NOEL LE, MARINO CR, THOMAS PJ & MUALLEM S (1999). Regulation of Cl^-/HCO_3^- exchange by cystic fibrosis transmembrane conductance regulator expressed in NIH 3T3 and HEK 293 cells. *Journal of Biological Chemistry* **274(6)**: 3414-3421.

LEE SK, QING WG, MAR W, LUYENGI L, MEHTA RG, KAWANISHI K, FONG HH, BEECHER CW, KINGHORN AD & PEZZUTO JM (1998). Angoline and chelerythrine, benzophenanthridine alkaloids that do not inhibit protein kinase C. *Journal of Biological Chemistry* **273(31)**: 19829-19833.

LEIER I, JEDLITSCHKY G, BUCHHOLZ U, CENTER M, COLE SP, DEELEY RG & KEPPLER D (1996). ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump. *Biochemical Journal* **314 (Pt 2)**: 433-437.

LEIER I, JEDLITSCHKY G, BUCHHOLZ U, COLE SP, DEELEY RG & KEPPLER D (1994). The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *Journal of Biological Chemistry* **269(45)**: 27807-27810.

LINSDELL P & HANRAHAN JW (1998). Adenosine triphosphate-dependent asymmetry of anion permeation in the cystic fibrosis transmembrane conductance regulator chloride channel. *Journal of General Physiology* **111**: 601-614.

LINSDELL P & HANRAHAN JW (1998). Glutathione permeability of CFTR. *American Journal of Physiology* **275(1 Pt 1)**: C323-C326.

LINSDELL P, TABCHARANI JA & HANRAHAN JW (1997). Multi-ion mechanism for ion permeation and block in the cystic fibrosis transmembrane conductance regulator chloride channel. *Journal of General Physiology* **110**: 365-377.

LINSDELL P, TABCHARANI JA, ROMMENS JM, HOU Y-X, CHANGE X-B, TSUI L-C, RIORDAN JR & HANRAHAN JW (1997). Permeability of wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride channels to polyatomic anions. *Journal of General Physiology* **110**: 355-364.

LIPPINCOTT-SCHWARTZ J, COLE NB & DONALDSON JG (1998). Building a secretory apparatus: role of ARF1/COPI in Golgi biogenesis and maintenance. *Histochemistry and Cell Biology* **109(5-6)**: 449-462.

LIVNEH E & FISHMAN DD (1997). Linking protein kinase C to cell-cycle control. *European Journal of Biochemistry* **248(1)**: 1-9.

LYCZAK JB, CANNON CL & PIER GB (2002). Lung infections associated with cystic fibrosis. *Clinical Microbiology Reviews* **15(2)**: 194–222.

MANOLOPOULOS VG, VOETS T, DECLERCQ PE, DROOGMANS G & NILIUS B (1997). Swelling-activated efflux of taurine and other organic osmolytes in endothelial cells. *American Journal of Physiology* **273(1 Pt 1)** : C214-C222.

MAROTO R & HAMILL OP (2001). Brefeldin A block of integrin-dependent mechanosensitive ATP release from *Xenopus* oocytes reveals a novel mechanism of mechanotransduction. *Journal of Biological Chemistry* **276(26)**: 23867-23872.

MASTROCOLA T, FLAMIGNI A & RUGOLO M (1991). Hypotonic shock activated Cl^- and K^+ pathways in human fibroblasts. *Biochim et Biophys Acta.* **1069(2)**: 201-208.

McNICHOLAS CM, GUGGINO WB, SCHWIEBERT EM, HEBERT SC, GIEBISCH G & EGAN ME (1996). Sensitivity of a renal K^+ channel (ROMK2) to the inhibitory sulfonylurea compound glibenclamide is enhanced by coexpression with the ATP-binding cassette transporter cystic fibrosis transmembrane regulator. *Proceedings of the National Academy of Sciences USA* **93**: 8083-8088.

MEISTER A (1988). Glutathione metabolism and its selective modification. *Journal of Biological Chemistry* **263(33)**: 17205-17208.

MORRIS PE & BERNARD GR (1994). Significance of glutathione in lung disease and implications for therapy. *American Journal of the Medical Sciences* **307(2)** : 119-127.

MOYER BD, DENTON J, KARLSON KH, REYNOLDS D, WANG S, MICKLE JE, MILEWSKI M, CUTTING GR, GUGGINO WB, LI M & STANTON BA (1999). A PDZ-interacting domain in CFTR is an apical membrane polarization signal. *Journal of Clinical Investigation* **104**: 1353-1361.

NAREN AP, DI A, CORMET-BOYAKA E, BOYAKA PN, McGHEE JR, ZHOU W, AKAGAWA K, FUJIWARA T, THOME U, ENGELHARDT JF, NELSON DJ & KIRK KL (1999). Syntaxin 1A is expressed in airway epithelial cells, where it modulates CFTR Cl- channels. *Journal of Clinical Investigation* **105**: 377-386.

NAREN AP, QUICK MW, COLLAWN JF, NELSON DJ & KIRK KL (1998). Syntaxin 1A inhibits CFTR chloride channels by means of domain-specific protein-protein interactions. *Proceedings of the National Academy of Sciences USA* **95**: 10972-10977.

NECHUSHTAN H & RAZIN E (2001). Studies of different aspects of the role of protein kinase C in mast cells. *International Archives of Allergy and Immunology* **124(1-3)**: 130-132.

NEDELJKOVIĆ A, RADULOVIĆ S & BJELOGRLIĆ S (2001). Pleiotropic effect of genistein makes it a promising cancer protective compound. *Archive of Oncology* **9(3)**: 171-174.

NICHOLL AJ, KILLEY J, LEONARD MN & GARNER C (2002). The role of bicarbonate in regulatory volume decrease (RVD) in the epithelial-derived human breast cancer cell line ZR-75-1. *Pflügers Arch.* **443(5-6)** : 875-881.

NIES AT, KONIG J, PFANNSCHMIDT M, KLAR E, HOFMANN WJ & KEPPLER D (2001). Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. *International Journal of Cancer* **94(4)**: 492-499.

NIISATO N & MARUNAKA Y (1999). Activation of the Na^+-K^+ pump by hyposmolality through tyrosine kinase-dependent Cl^- conductance in Xenopus renal epithelial A6 cells. *Journal of Physiology* **518 (Pt 2)**: 417-432.

NILIUS B & DROOGMANS G (2003). Amazing chloride channels: an overview. *Acta Physiologica Scandinavica* **177**: 119-147.

NISHIZUKA Y (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**: 607-614.

NORTH RA (2002). Molecular physiology of P2X receptors. *Physiological Reviews* **82(4)**: 1013-1067.

PARK CS, LEEM CH, JANG YJ & SHIM YH (2000). Vesicular transport as a new paradigm in short-term regulation of transepithelial transport. *Journal of Korean Medical Science* **15**: 123-132.

PASYK E & FOSKETT K (1995). Mutant (ΔF508) cystic fibrosis transmembrane conductance regulator Cl^- channel is functional when retained in the endoplasmic reticulum of mammalian cells. *Journal of Biological Chemistry* **270**: 12347-12350.

PAULUSMA CC, BOSMA PJ, ZAMAN GJ, BAKKER CT, OTTER M, SCHEFFER GL, SCHEPER RJ, BORST P & OUDE ELFERINK PR (1996). Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein. *Science* **271(5252)**: 1126-1128.

PETERSON JD, HERZENBERG LA, VASQUEZ K & WALTENBAUGH C (1998). Glutathione levels in antigen-presenting cells modulate T_H1 versus T_H2 response patterns. *Proceedings of the National Academy of Sciences USA* **95**: 3071-3076.

POWIS G, BONJOUKLIAN R, BERGGREN MM, GALLEGOS A ABRAHAM R, ASHENDEL C, ZALKOW L, MATTER WF, DODGE J GRINDEY G, *et al.* (1994). Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Research* **54(9)**: 2419-2423.

RATJEN F & DÖRING G (2003). Cystic fibrosis. *Lancet* **361**: 681–689.

REID JM & O'NEIL RG (2000). Osmomechanical regulation of membrane trafficking in polarized cells. *Biochemical and Biophysical Research Communications* **271**: 429-434.

RIORDAN JR (1999). Therapeutic strategies for treatment of CF based on knowledge of CFTR. *Pediatric Pulmonology Supplement* **18**: 83-87.

ROMAN RM, BODILY KO, WANG Y, RAYMOND JR & FITZ JG (1998). Activation of protein kinase Cα couples cell volume to membrane Cl^- permeability in HTC hepatoma and Mz-ChA-1 cholangiocarcinoma cells. *Hepatology* **28**: 1073-1080.

ROMAN RM, WANG Y & FITZ JG (1996). Regulation of cell volume in a human biliary cell line: activation of K^+ and Cl^- currents. *American Journal of Physiology* **271(2 Pt 1)** : G239-G248.

ROONEY SA (2001). Regulation of surfactant secretion. *Comparative Biochemistry and Physiology* **129(1)**: 233-243.

ROUM JH, BOROK Z, MCELVANEY NG, GRIMES GJ, BOKSER AD, BUHL R & CRYSTAL RG (1999). Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. *Journal of Applied Physiology* **87(1)**: 438-443.

ROUM JH, BUHL R, MCELVANEY NG, BOROK Z & CRYSTAL RG (1993). Systemic deficiency of glutathione in cystic fibrosis. *Journal of Applied Physiology* **75(6)**: 2419-2424.

ROYLE SJ & MURRELL-LAGNADO RD (2002). Constitutive cycling: a general mechanism to regulate cell surface proteins. *Bioessays* **25**: 39-46.

SAMBROOK J & RUSSELL DW (2001). *Molecular Cloning, A Laboratory Manual, 3rd Edition*. Cold Spring Harbor, New York: Cold Spring Harbour Laboratory Press.

SAMUELSSON B (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220(4597)**: 568-575.

SAXENA R, SAKSA BA, FIELDS AP & GANZ MB (1993). Activation of Na/H exchanger in mesangial cells is associated with translocation of PKC isoforms. *American Journal of Physiology* **265(1 Pt 2)**: F53-60.

SCHENK PW & SNAAR-JAGALSKA BE (1999). Signal perception and transduction: the role of protein kinases. *Biochim et Biophys Acta* **1449(1)**: 1-24.

SCHMITT L (2002). The first view of an ABC transporter: the X-ray crystal structure of MsbA from *E.coli*. *ChemBioChem* **3**: 161-165.

SCHRAMEK H (2002). MAP kinases: from intracellular signals to physiology and disease. *News in Physiological Sciences* **17**: 62-67.

SCHREIBER R, NITSCHKE R, GREGER R & KUNZELMANN K (1999). The cystic fibrosis transmembrane conductance regulator activates aquaporin 3 in airway epithelial cells. *Journal of Biological Chemistry* **274(17)**: 11811-11816.

SCHWIEBERT EM (2001). ATP release mechanisms, ATP receptors and purinergic signaling along the nephron. *Clinical and Experimental Pharmacology and Physiology* **28**: 340-350.

SCHWIEBERT EM, EGAN ME, HWANG TH, FULMER SB, ALLEN SS, CUTTING GR & GUGGINO WB (1995). CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* **81(7)**: 1063-1073.

SCHWIEBERT EM, GASEK F, ERCOLANI L, WJASOW C, GRENERT DC, KARLSON K & STANTON BA (1994). Heterotrimeric G proteins, vesicle trafficking, and CFTR Cl^- channels. *American Journal of Physiology* **267** (*Cell Physiology* **36**): C272-C281.

SHEPPARD DN & WELSH MJ (1999). Structure and function of the CFTR chloride channel. *Physiological Reviews* **79**: S23-S45.

SMITH JJ, TRAVIS SM, GREENBERG EP & WELSH MJ (1996). Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* **85(2)**: 229-236.

SONG D, O'REGAN MH & PHILLIS JW (1998). Protein kinase inhibitors attenuate cardiac swelling-induced amino acid release in the rat. *Journal of Pharmacy and Pharmacology* **50**: 1280-1286.

STEIN RC (2001). Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment. *Endocrine-Related Cancer* **8**: 237-248.

STUTTS MJ, ROSSIER BC & BOUCHER RC (1997). Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics. *Journal of Biological Chemistry* **272(22)**: 14037-14040.

SURAPISITCHAT J, HOEFEN RJ, PI X YOSHIZUMI M, YAN C & BERK BC (2001). Fluid shear stress inhibits TNF-α activation of JNK but not ERK1/2 or p38 inhuman umbilical vein endothelial cells: inhibitory crosstalk among MAPK family members. *Proceedings of the National Academy of Sciences USA* **98(11)**: 6476-6481.

SUTHANTHIRAN M, ANDERSON ME, , SHARMA VK & MEISTER A (1990). Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigens. *Proceedings of the National Academy of Sciences USA* **87**: 3343-3347.

SWIATECKA-URBAN A, DUHAIME M, COUTERMARSH B, KARLSON KH, COLLAWN J, MILEWSKI M, CUTTING GR, GUGGINO WB, LANGFORD G & STANTON BA (2002). PDZ domain interaction controls the endocytic recycling of the cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry* **277(42)**: 40099-40105.

TABCHARANI JA, LINSDELL P & HANRAHAN JW (1997). Halide permeation in wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride channels. *Journal of General Physiology* **110**: 341-354.

TAMMUR J, PRADES C, ARNOULD I, RZHETSKY A, HUTCHINSON A, ADACHI M, SCHUETZ JD, SWOBODA KJ, PTACEK LJ, ROSIER M, DEAN M & ALLIKMETS R (2001). Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. *Gene* **273(1)**: 89-96.

TIETZE F (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Analytical Biochemistry* **27**: 502-522.

TILLY BC, VAN DEN BERGHE N, TERTOOLEN LGJ, EDIXHOVEN MJ & DE JONGE HR (1993). Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. *Journal of Biological Chemistry* **268(27)**: 19919-19922.

TWAMLEY GM & COURTNEIDGE SA (1996). Non-receptor protein tyrosine kinases. In: *Protein Phosphorylation*, edited by Marks F. New York: VCH Publishers, pp. 237-264.

VAN DER WIJK T, TOMASSEN SF, DE JONGE HR & TILLY BC (2000). Signalling mechanisms involved in volume regulation of intestinal epithelial cells. *Cellular Physiology and Biochemistry* **10(5-6)**: 289-296.

VENOSA RA (2003). Hypotonic stimulation of the Na^+ active transport in frog skeletal muscle: role of the cytoskeleton. *Journal of Physiology* **548(Pt 2)**: 451-459.

VLAHOS CJ, MATTER WF, HUI KY & BROWN RF (1994). A specific inhibitor of phosphatidylinositol 3-kinase 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *Journal of Biological Chemistry* **269(7)**; 5241-5248.

VOM DAHL S, SCHLIESS F, GRAF D & HÄUSSINGER D (2001). Role of p38 MAPK in cell volume regulation of perfused rat liver. *Cell Physiology and Biochemistry* **11**: 285-294.

WALSH DE, HARVEY BJ & URBACH V (2000). CFTR regulation of intracellular calcium in normal and cystic fibrosis human airway epithelia. *Journal of Membrane Biology* **177**: 209-219.

WANG S, YUE H, DERIN RB, GUGGINO WB & LI M (2000). Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. *Cell* **103(1)**: 169-179.

WANG Y, ROMAN R, LIDOFSKY SD & FITZ JG (1996). Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. *Proceedings of the National Academy of Sciences USA* **93(21)**: 12020-12025.

WEI L, VANKEERBERGHEN A, CUPPENS H, EGGERMONT J, CASSIMAN J-J, DROOGMANS G & NILIUS B (1999). Interaction between calcium-activated chloride channels and the cystic fibrosis transmembrane conductance regulator. *Pflügers Arch* **438**: 635-641.

WELSH MJ & SMITH AE (1993). Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell* **73**: 1251-1254.

WILKINSON DJ, STRONG TV, MANSOURA MK, WOOD DL, SMITH SS, COLLINS FS & DAWSON DC (1997). CFTR activation: additive effects of stimulatory and inhibitory phosphorylation sites in the R domain. *American Journal of Physiology: Lung Cellular and Molecular Physiology* **273**: L127-L133.

WOJNOWSKI L, KERSTING U & OBERLEITHNER H (1992). Renal potassium bicarbonate release in humans exposed to an acute volume load. *Clinical Investigator* **70(8)**: 692-697.

WRIGHT EM, HIRSCH JR, LOO DD & ZAMPIGHI GA (1997). Regulation of Na^+/glucose cotransporters. *Journal of Experimental Biology* **200 (Pt 2)**: 287-293.

WYMANN MP & PIROLA L (1998). Structure and function of phosphoinositide 3-kinases. *Biochimica et Biophysica Acta* **1436**: 127-150.

YE L, CHAN S, CHOW YH, TSUI LC & HU J (2001). Regulated expression of the human CFTR gene in epithelial cells. *Molecular Therapy* **3(5 Pt 1)**: 723-733.

YU R, MANDLEKAR S, TAN T-H & KONG A-N (2000). Activation of p38 and c-Jun N-terminal kinase pathways and induction of apoptosis by chelerythrine do not require inhibition of protein kinase C. *Journal of Biological Chemistry* **275(13)**: 9612-9619.

ZENG H, LIU G, REA PA & KRUH GD (2000). Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Research* **60(17)**: 4779-4784.

ZIELENSKI J (2000). Genotype and phenotype in cystic fibrosis. *Respiration* **67**: 117–133.