Osmoregulation of chloride channels in epithelial cells

Osmoregulatie van chloride kanalen in epitheelcellen

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in epitheelcellen

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獻給我的父母和兩個弟弟

並以此紀念

Arnoldus Christiaan Breedveld
(1950-2008)

This thesis is dedicated to

My parents and my brothers, for always being there for me
&
Arnoldus Christiaan Breedveld (1950-2008), for believing in me.

Contents

Abbreviations

Abbreviations

AA	arachidonic acids
ABA	hormonal abscisic acid
ABCA1	ATP-binding cassette protein A1
ABP	actin-binding protein
AP-2	associated adaptin
AQP	aquaporins
ATP	adenosine 5'-triphosphate
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester
cAMP	cyclic adenosine 3':5'-monophosphoric acid
CD	2-hydroxypropyl-β-cyclodextrin
cDNA	complementary DNA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CPAE cells	calf pulmonary artery endothelial cells
DAG	1,2 diacylglycerol
DIDS	4,4'-di-isothiocyanatostilbene-2,2'-disulfonic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfonyloxide
DN	dominant negative
DPC	diphenylamine-2-carboxylate
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
ENaC	Epithelial Na^+ channel
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
F-actin	filamentous actin
FAK	focal adhesion kinase
F508delCFTR	deletion of phenylalanine at amino acid position 508 of CFTR
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor

GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
G protein	guanine nucleotide-binding regulatory protein
GTP	guanosine 5'-triphosphate
^{3}H	hydrogen isotope 3/ tritium
HDL	high-density lipoproteins
hepes	*N*-2-hydroxy-ethylpiperazine-*N*'-2-ethane-sulfonic acid
Hog1p	high osmolarity glycerol response protein
HRP	horseradish peroxidase
^{125}I	iodium isotope 125
IP_3	inositol 1,4,5-triphosphate
K^+_{swell}	stretch-activated K^+ channels and swelling activated K^+ channels
Kv	voltage-gated K^+ channels
LDL	low density lipoproteins
MAP kinase	mitogen-activated protein kinase
Maxi-K^+	Ca^{2+}-dependent intermediate K^+ channel
MDCK cells	Madin-Darby canine kidney cells
MEK	mitogen-activated/ERK-activating kinase
MLCK	myosin light chain kinase
MMTS	methylmethanethio-sulphonate
MscL	mechanosensitive channel
NEM	N-ethylmaleimide
NPC	Niemann Pick C1 protein
NPPB	5-nitro-2-(3-phenyl-propylamino)-benzoate
NSF	N-ethylmaleimide-sensitive factor
PAGE	polyacrylamide gel electrophorese
PBS	phosphate-buffered saline
PC-LUVs	phosphatidylcholine large unilaminar vesicles
PI-PLCβ	phosphatidylinositol-specific phospholipase Cβ
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLA	phospholipase A
PtdIns-3K	phosphatidyl inositol 3 kinase
^{86}Rb	rubidium isotope 86
RT-PCR	reverse transcriptase polymerase chain reaction

RVD	regulatory volume decrease
RVI	regulatory volume increase
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SFK	SRC family kinases SFK
SiRNA	small interfering RNA
SITS	4-acetamido-4'-isothiocyanostilbene
SNAP	soluble NSF attachment protein
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TMAO	trimethylamine N-oxide
TKR	tyrosine kinase receptor
TRPC	transient receptor potential channels
t-SNARE	target membrane SNARE
TTBS	Tris-Tween-buffered saline
VAMP2	vesicle-associated protein on the v-SNARE
VRAC	volume regulated anion channels
v-SNARE	vesicle SNARE
wt	wild-type

List of chemical compounds and their action

ABA	Increases the $[Ca^{2+}]_i$ and activates the K^+ channels
Apyrase	Nonspecific adenosine tri/biphosphatase (ATPase) that converts ATP into AMP and Pi
BAPTA-AM	A cell permeable calcium chelator
Brefeldin A	An inhibitor of vesicle transport between the ER and the Golgi. It disturbes intracellular membrane flow and Golgi function
C. botulinium neurotoxin F	Zinc endopeptidase causing specific cleavage of vesicle associated SNARE protein VAMP/synaptobrevin
C. botulinium exoenzyme C3	Enzyme that inactivates Rho A, B and C (not Rho D) through ADP-ribosylation
cytochalasin B	Disrupts F-actin microfilaments
DIDS	Cl^--channel blocker
DPC	Cl^--channel blocker
Herbimycin A	Tyrosine kinases inhibitor
KT5926	A potent and selective inhibitor of MLCK
MMTS	A sulfhydryl reagent methylmethanethio-sulphonate that inhibit AQP
NEM	Binds to the SNARE protein SNAP-25 and prevents vesicle fusion
NPPB	Cl^--channel blocker
SITS	Cl^--channel blocker
Tamoxifen	P-glycoprotein inhibitor and anion channel (VRAC) blocker
TMAO	Counteracts the protein denaturing effect of urea
Vanadate	Phosphotyrosine phosphatase inhibitor
Wortmannin	A specific inhibitor of phosphatidylinositol-3-kinase

Chapter 1

Introduction

Introduction

Water movement across the plasma membrane

The plasma membrane of mammalian cells is formed by two layers of lipids (lipid bilayer), primarily phospholipids, glycolipids and cholesterol, in which many different proteins are embedded. Phospholipid consists of a glycerol backbone esterified to fatty acids (the "lipid tail") and, via a phosphate group, to either choline, serine, inositol or ethanolamine (the "head group"). Whereas the head group is hydrophilic and oriented towards the outer surface of the membrane, the lipid tail is hydrophobic and pointed towards the inner part.

The plasma membrane is impermeable to large molecules like carbohydrates and proteins but readily permeable to small uncharged molecules like oxygen, water, and carbon dioxide. Molecules can move through the membrane either by direct diffusion or through specialized channels or transport proteins (facilitated diffusion). In an isolated system, as stated by the Second Law of Thermodynamics, all events move spontaneously from a higher energy state to a lower energy state and are driven by the tendency to increase the entropy (degree for randomness/ disorder) [1]. When molecules are evenly distributed throughout the available space, the entropy is at its maximum. Therefore, free moving molecules and atoms (i.e. not part of a crystal structure and not restrained by additional forces) tend to distribute themselves over an as large as possible area. As a consequence of the Second Law of Thermodynamics, differences in the concentrations of non-permeable solutes between the cell and its surrounding medium will result in a redistribution of the solvent (e.g. water) to maintain the lowest energy possible, a phenomenon known as osmosis. Therefore, in response to an osmotic imbalance, water will move across a semi-permeable membrane until the water molecules are equally distributed, resulting in swelling or shrinkage [2].

The Gibbs-Donnan effect refers to the observation that under certain conditions charged molecules or ions fail to distribute evenly across a semi-permeable membrane. At the Gibbs-Donnan equilibrium, the total number of positive charges balanced the total number of negative charges (bulk electroneutrality). Due to the presence of charged membrane-impermeable macromolecules and the electrogenic Na^+/K^+ pump, however, the Gibbs Donnan equilibrium will never be obtained in an intact cell, leading to an asymmetric distribution of permeable ions and the generation of the membrane potential [3].

A consequence of the presence of negatively charged membrane-impermeable proteins is the constant tendency of cells to accumulate water. To counteract a potential increase in volume, sodium is removed from the cells by the Na^+/K^+ pump on the expenditure of metabolic energy. Because alterations in cell volume may affect many biochemical and physiological processes, almost all cell types have additional mechanisms to regulate their volume and to limit or prevent swelling and shrinkage [3].

Physiological and pathological disturbances in osmolarity

Under physiological conditions, the osmolarity of the interstitial fluid is tightly controlled to protect the cells in the body against major disturbances in the extracellular osmolarity. Therefore, osmotic stress is generally due to intracellular changes caused by the uptake or release of osmotically active substances (amino acids, sugars etc.), the formation or degradation of macromolecules (proteins, glycogen) or hormone-induced alterations in cellular metabolism.

Pathophysiological disturbances in osmolarity can be caused by alterations in both the extra- and intracellular environment. A change in the extracellular environment occurs during hypernatremia. Hypernatremia is caused by elevated sodium levels in the blood as a result of dehydration due to for instance diarrhoea or diabetes insipidus. Alterations in the intracellular enviroment are mainly caused by a change in the cellular metabolic activity. For example during ischaemia, anaerobic metabolic processes result in an intracellular accumulation of metabolites, including lactate and other glycolytic products [4]. Furthermore, ATP depletion inhibits the Na^+/K^+–pump, leading to dissipation of the Na^+ and K^+ gradients and membrane depolarization. Now that the intracellular compartment has become less negative, Cl^- has a tendency to accumulate intracellularly, leading to osmotic cell swelling [5,6,7].

Changes in ion concentrations may play a role in apoptosis or necrosis. During apoptosis, inhibition of the Na^+/ K^+–ATPase results in decreased intracellular potassium and increased intracellular sodium concentrations [8]. The enhanced efflux of potassium was found to augment apoptosis [8]. In addition, an increased efflux of chloride resulted in apoptotic volume decrease (RVD) [8]. Apoptosis can be prevented by a high extracellular concentration of potassium and by potassium or chloride channel blockers [9,10].

Mechanisms of osmoregulation

Bacteria, fungi, algae and plants have evolved a rigid cell wall and are therefore less susceptible to changes in the osmolarity of their environment. However, a cell wall can only protect bacteria and cells from excessive swelling but not from shrinkage. Organic osmolytes such as amino acids, polyols and methylamines are used to increase the intracellular osmolarity and to facilitate the uptake of water. Bacteria like *Halobacterium salanarium* are able to survive in a high salt enviroment (halophilic) by accumulating amino acids to prevent excessive water loss [11]. Organisms living in a very dry environment like xerophilic yeasts and fungi produce glycerol to increase their internal osmolarity to promote the uptake of water. Other yeasts and fungi either produce or accumulate polyols such as sorbitol, xylitol and mannitol to maintain volume [11].

Subject to salt stress, plants generally accumulate organic osmolytes such as proline, valine, isoleucine, betaine, aspartic acid, glucose, fructose, sucrose, mannitol and/or inositol by increasing their synthesis. Plant cells are generally hypertonic compared to their environment. As a result, water will enter the cells and develop an internal pressure known as turgor. High turgor pressure opens the stomata pores that allow water and gas exchange between the plant and the air. Under high temperature and low humidity, abscisic acid (ABA) is released, leading to an increase in $[Ca^{2+}]_i$ and an activation of the K^+ channels. As a consequence stomata will close to prevent a further loss of water [11].

Marine animals use both organic and inorganic osmolytes to maintain osmotic balance. The salt concentration of the blood and other body fluids of elasmobranchs, sharks and rays, is only one-third of the concentration of seawater. This low salt concentration is maintained by sodium and chloride excretion through rectal gland and kidney. The shark kidney however, actively reabsorbs urea. As a result, the total osmotic concentration of the blood equals or slightly exceeds that of the seawater [12].

Saltwater bony fish are osmotically hypotonic as compared to their environment. Therefore, saltwater fish are at a constant risk of dehydration. To prevent water loss, these fish drink sea water and expell the ingested salts via the gills (Na^+, Cl^-) or urine (Mg^{2+}, SO_4^{2-}) by active transport. In contrast, the body fluids of freshwater fish have a higher osmolarity as compared to their environment and have a tendency to accumulate water. Excess water is removed by producing large volumes of diluted urine. The subsequent loss of ions is compensated for by active uptake via the gills [11]. Water regulation of amphibians is very similar to

that of freshwater fish. However, the skin serves as the main organ for the active uptake of ions [12].

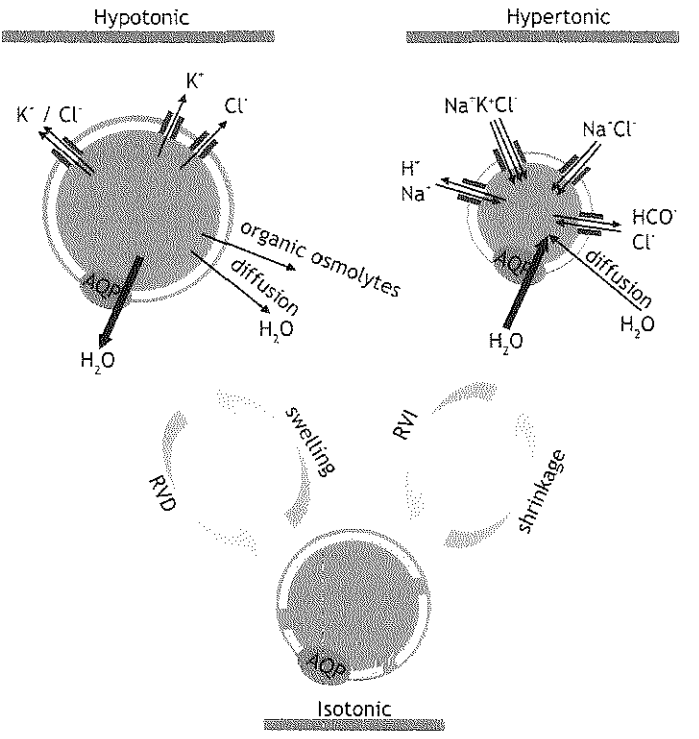

Figure 1: **Schematic illustration of cell volume regulation in mammalian cells.** Alteration in extracellular osmolarity will result in redistribution of water, which rapidly leads to cell volume changes. Upon cell shrinkage, ion transporters are activated for the uptake of NaCl, facilitating water influx. This mechanism is commenly referred to as regulatory volume increase (RVI). Upon cell swelling, KCl and organic osmolytes are respectively released through ion channels and organic osmolytes release pathways, which will leads to water efflux, a phenomenon known as regulatory volume decrease (RVD). (see page 172 for color figure)

In mammals, the osmolarity of the interstitial fluid is tightly regulated by adaptive behaviour (thirst sensation) and by varying the salt concentration and volume of the urine, which protect the cells against major disturbances in extracellular osmolarity. Therefore, osmotic stress generally originates from changes in the intracellular osmolarity. Because both swelling and shrinkage are potentially

deleterious for the cells, compensatory mechanisms have been developed. In mammalian cells, shrinkage activates a regulatory volume increase (RVI) by the uptake of NaCl through activation of Na^+/H^+ and Cl^-/HCO_3^- exchangers or by activation of Na^+/ Cl^- or $Na^+/ K^+/ 2Cl^-$ symporters [13,14]. Cell swelling activates a regulatory volume decrease (RVD). The RVD is mediated by the concomittant release of KCl through K^+ - and Cl^- - selective ion channels or K^+/Cl^- - symporters [13-15] and by the efflux of small molecules through an organic osmolytes release pathway (Figure 1) [16-18].

Characteristics and molecular identity of VRAC

Upon osmotic swelling, K^+- and Cl^--selective ion channels are rapidly activated. Different K^+ channels participate in cell volume regulation including voltage-gated K^+ channels (Kv), Ca^{2+}-dependent intermediate K^+ channels (maxi-K^+), stretch-activated K^+ channels and swelling-activated K^+ channels (K^+_{swell}) [19]. The molecular identity of the ubiquitously expressed Volume-Regulated Anion Channel (VRAC), however, despite its marked biophysical characteristics, has not yet been resolved. Several candidates (CLC-2, CLC-3, ICln, P-glycoprotein and CaCCs) have been proposed, but none of them full fill all the electrical and pharmacological criteria (table 1) [20-22]. Potential novel VRAC candidates will be discussed more extensively in Chapter 7.

Osmosensing

A direct activation of the mechanosensitive channel MscL by osmotic swelling-induced membrane stretch has been found in the bacterium *Escherichia coli* [1, 23]. In many other organisms, however, the mechanism of osmosensing has been found to be much more complicated or still remains an enigma.

In the yeast *Saccharomyces cerevisiae*, cell shrinkage activates the mitogen-activated protein (MAP) kinase Hog1p (high osmolarity glycerol response protein) leading to an increase in glycerol synthesis and the restoration of the osmotic gradient [11,24]. Hog1p is highly similar to the mammalian p38/JNK MAP kinases. The Hog pathway is activated by the osmo-sensor Sho1p and by a 'two-component system' composed of the Sln1p histidine kinase and the response regulator Ssk1p [11,24]. Sln1p is stimulated by a low extracellular osmolarity and inactivates Ssk1p by phosphorylation. Eventually, this results in the inhibition of Pbs2p and finally Hog1p [11,24]. In yeast, the mucin-like proteins Msb2 and Hkr1were also recognized as stress sensors which, together with Sho1, stimulate the Hog

pathway during hyperosmotic shrinkage. Mucins are transmembrane proteins with heavily glycosylated extracellular domains and characterized by a Ser- and Thr - enriched stretch of amino acids (STR domain) in the extracellular region. Glycosylated polymers change their properties depending on the degree of water accessibility. Therefore, high osmolarity might produce significant changes in the STR region that are then transmitted to the rest of the protein [24]. Similar osmosensing systems have been reported in bacteria, fungi and plants. In plants, ATHK1, a transmembrane histidine kinase structurally related to Sln1p has been found [25].

Table 1: Characteristics of VRAC current compared to that of several VRAC candidate.

	VRAC	ClC-2	ClC-3	IcIn	CaCC
Strong outward rectification	Yes	No	Yes	Yes	Yes
Anion selectivity Eisenman's sequence I $^{\Psi}$	Yes	No	Yes	Yes	Yes
Blocked by SITS, DPC, DIDS, NPPB Niflumic acids	Yes	Yes	Yes*	Yes	Yes
Blocked by extracellular nucleotides	Yes	?	Yes	Yes	No
Blocked by cyclamate AZT	No	?	?	Yes	?
Inactivation at positive potential >60 mV	Yes	Yes	Disputed	Yes	No

* Not by DPC

$^{\Psi}$ $SNC^- > I^- > NO_3^- > Br^- > Cl^- > F^-$

? Not known

To date, little is known about the mechanisms mammalian cells utilize to detect changes in their volume. Several mechanisms have been proposed, including activation of mechano-sensitive ion channels, macromolecular crowding as well as signalling via the extracellular matrix and integrin-receptors. Stretching or compression of the membrane can activate mechano-sensitive ion channels. This type of ion channel is expressed in many cell types [1,26]. Transient receptor potential vanilloid-4 (TRPV4), a non-selective cation channel, may directly sense volume changes [19]. This channel is activated by hypo-osmolarity and membrane stretch. A change in the concentration of cytosolic proteins due to a change in cell volume may also function as an osmo-sensor or osmo-transducer. Alterations in the protein concentration can markedly influence thermodynamic

and catalytic activities of enzymes regulating membrane transport, through a phenomenon known as macromolecular crowding [27]. A most interesting mechanism for osmo-sensing and -transduction, especially for epithelial cells, is signalling via the extracellular matrix (ECM) and integrin receptors. The ECM is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produce them [28]. Among the matrix proteins that are potentially involved in stretch-sensing are the proteoglycans. Proteoglycans are major protein-glycosaminoglycan (polysaccharide chains) complexes that can bind signalling molecules and subsequently stimulate or inhibit their signalling activity. Other ECM proteins are collagens, fibronectin and laminin. Binding to these ECM proteins are the integrins, transmembrane heterodymeric receptors composed of α- and ß- subunits. The extracellular domain of integrins are bound to the ECM, whereas the cytoplasmic domain is associated with the actin cytoskeleton. Integrins can establish links between the inside and outside of the cells. Upon stimulation, integrins aggregate and cluster in focal adhesions and trigger phosphorylation of a number of intracellular proteins. Because integrins are connected to bundles of actin filaments, changes in the matrix can lead to a remodelling of cytoskeleton [29,30]. In addition, tyrosine kinase receptors (TKRs), which also consist of an extracellular domain, a transmembrane element and an intracellular domain with intrinsic sites for protein tyrosine kinase and phosphorylation, have been proposed as volume sensors [19].

Intracellular signalling

Lipid-derived second messengers

Activated phosphatidylinositol-specific phospholipase Cß (PI-PLCß) catalyses the splicing of PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), leading to protein kinase C (PKC) activation and IP_3 receptor-mediated intracellular Ca^{2+} mobilization. In Ehrlich ascites tumor cells, PIP_2 levels were found to be decreased upon cell shrinkage and increased upon cell swelling [31]. DAG- and Ca^{2+}- dependent PKC were found to contribute to the RVI in NIH/3T3 mouse fibroblasts [32] and Ehrlich ascites tumor cells [33]. In Ehrlich ascites tumor cells, PKC was found to be activated in the late phase of RVD response, leading to VRAC inhibition and termination of the RVD [34].

Calcium

In several cell models, hypo-osmotic swelling results in an increase in the intracellular free calcium concentration. In many cell models, Ca^{2+} has been found to be directly involved in volume regulation and in the activation of compensatory conductances [35-39]. Other cell types, however, including Intestine 407 cells, do not depend on a rise in $[Ca^{2+}]_i$ to elicit the RVD response [19]. Although Ca^{2+} is not directly involved in the activation of VRAC in these cells, Ca^{2+}-mobilizing hormones like bradykinin or histamine are able to potentiate the hypotonicity-provoked anion conductance [40,41].

Tyrosine kinases

Tyrosine kinases such as MAP kinases (ERK-1/2, p38), FAK and SRC are known to be activated upon hyposmotic swelling. In Intestine 407 cells protein tyrosine phosphorylation was found to be required for VRAC activation and for eliciting the RVD response [42]. This dependency on tyrosine kinases has since been established for many other cell types, including cardiomyocytes [43], endothelial cells [44] and lymphocytes [45]. In a number of cell lines, however, including ROS17/2.8 osteoblasts [46] and in CPAE cells, tyrosine kinase inhibitors were found to be ineffective [47].

G proteins

Anion-selective currents in cell types such as human HT29cl19A colonocytes are activated by intracellular administration of GTPγS (to activation guanine nucleotide-binding regulatory proteins, known as G proteins) and inactivated by GDPβS [48]. The involvement of $p21^{rho}$ (a small G protein) in the activation of osmosensitive efflux in Intestine 407 cells was demonstrated by intracellular delivery of *Clostridium botulinum* exoenzyme C3. Similar results were obtained with bovine endothelial cells [49].

Arachidonic acid

Increased release of arachidonic acid upon osmotic swelling is reported for many cell types, including human neuroblastoma cells [50], Ehrlich ascites tumor cells [51] and human platelets [52]. The increase is mainly caused by the activation of phospholipase A2 (PLA_2). Inhibition of PLA_2 was found to diminish the volume-sensitive anion conductance in bovine pigmented ciliary epithelial cells [53], rat brain endothelial cells [54] and human platelets [52]. Arachidonic acid can

be metabolized in many ways. Leukotriene D_4, one of its metabolites, has been reported to be involved in the development of RVD in multiple cell types [18].

Vesicular transport

Emerging evidence demonstrates the involvement of vesicular transport in ion channel regulation [55-58]. Intracellular vesicular transport can be divided into a secretory and an endocytic pathway. The constitutive secretory pathway begins with the synthesis of proteins within the endoplasmic reticulum (ER). Subsequently, the proteins are transported in vesicles to the Golgi apparatus, where glycosylation can take place, to be finally delivered to the cell surface by means of exocytosis. Exocytosis is essential for secreting ECM components, for incorporating components of the plasma membrane (lipids), as well as for membrane insertion of transmembrane proteins (receptors, ion channels). The endocytic pathway starts with the formation of a membrane bud, followed by the formation of a vesicle that ends up in the lysosome or cycles back to the plasma membrane. Bud formation is facilitated by assembly of a protein coat, leading to a distortion of membrane conformation, and the formation of a coated pit. Coated vesicles can be divided into COP (coat protein)-coated vesicles and clathrin-coated vesicles [59]. COPI-coated vesicles bud from the Golgi and carry their cargo from the Golgi to the ER. In contrast, COPII-coated vesicles bud from the ER and are responsible for the transport from the ER to the Golgi. Clathrin-coated vesicles are responsible for the uptake of extracellular molecules from the plasma membrane by endocytosis as well as for transport of molecules from the *trans* Golgi network to the lysosomes. Clathrin-mediated internalization involves the assembly of clathrin and its associated adaptin (AP-2) complex on the plasma membrane, concentration of membrane proteins in clathrin-coated pits, budding of the coated pits from the membrane mediated by dynamin, release of clathrin and AP-2 from the vesicles, and reformation of new coated pits [60] (Figure 2).

Docking and fusion of vesicles

Vesicle docking and fusion is mediated by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. An interaction between the vesicle-associated protein VAMP2 on the vesicle (a "v-SNARE"), syntaxin on the plasma membrane (a "t-SNARE") and SNAP25, an N-ethylmaleimide-sensitive factor recruited from the cytosol, results in the formation of a complex that directs membrane docking and fusion [61].

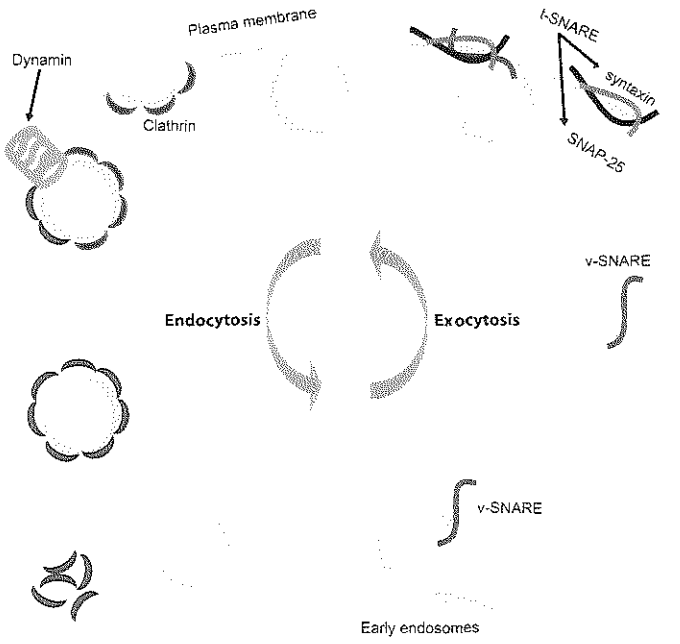

Figure 2: **Scheme of endo- and exocytosis.** Endocytosis begins with the assembly of clathrin coat, which leads to the formation of a vesicle. Dynamin mediates the release of vesicle from the membrane, allowing it to travel towards the early endosomes. Exocytosis is facilitated by the binding of v-SNARE to the t-SNARE. (see page 170 for color figure)

Directing vesicular transport: the Rab proteins

Coordination of vesicle trafficking requires Rab proteins. The Rab proteins are a family of small GTP-binding proteins that function in specific vesicle transport processes. Loss of function mutations of Rab GTPases result in many human disease such as bleeding and pigmenting disorders (Griscelli syndrome), kidney disease (tuberous sclerosis) and blindness (choroideremia). Overexpression of Rab GTPases has been observed in a number of human cancers (prostate, liver and breast cancer) [62]. A guanine nucleotide exchange factor (GEF) activates Rab by exchanging GTP for GDP which leads to its association to the transport vesicle. After vesicle fusion, a Rab GTPase hydrolyzes the GTP to GDP resulting in the release from the vesicle [63] (Figure 3). Approximately 60 Rab proteins are encoded by the human genome [64,65], but only a few have been characterized

extensively. Rab5 is involved in the clathrin-coated endocytosis pathway and regulates transport from the plasma membrane to the early endosomes [66]. The cycling back of early endosomes back to the plasma membrane requires Rab4 and Rab11 [67,68] (Figure 3). In addition, Rab7 mediates the transition from early to late endosomes. Rab proteins have been reported to modulate cell surface expression and function of epithelial ion channels such as the cystic fibrosis transmembrane conductance regulator (CFTR), the epithelial sodium channel (ENaC) and calcium channels [69].

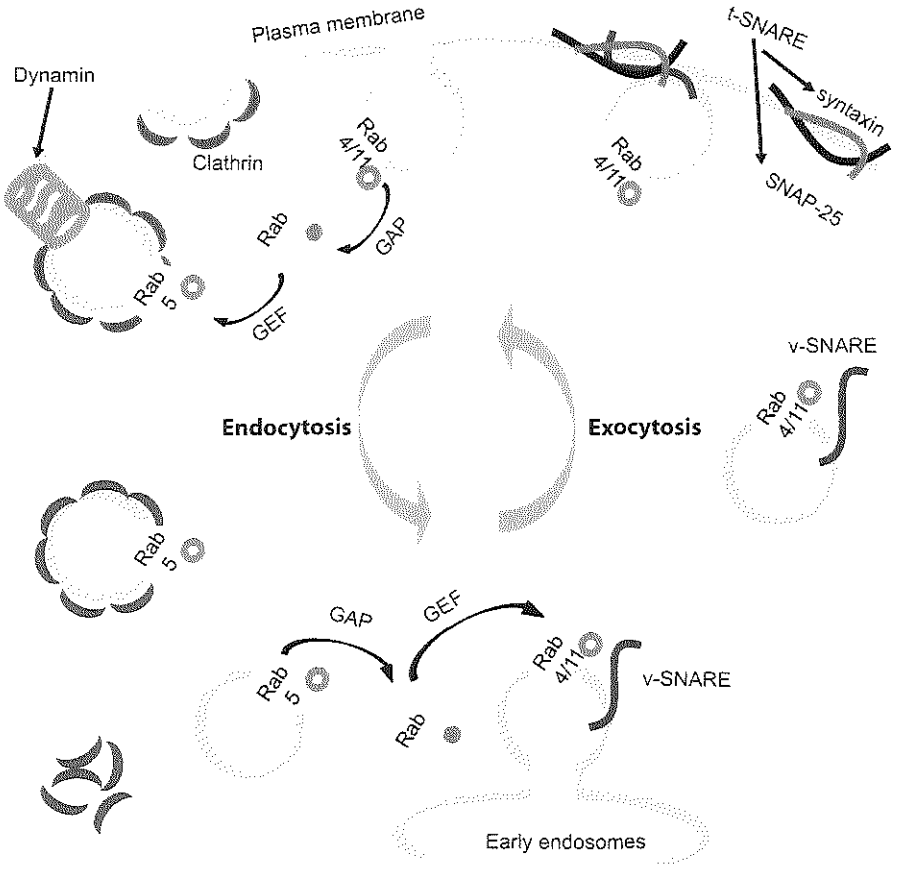

Figure 3: **Rab proteins and their functions.** Rab5 is involved in the clathrin-coated endocytosis pathway. Exocytosis requires Rab4 and/or Rab11. Guanine nucleotide exchange factor (GEF) activates Rab by exchanging GDP (closed circles) for GTP (open circles). After vesicle fusion, a Rab GTPase hydrolyzes the GTP (open circles) to GDP (closed circles) resulting in a release of Rab proteins from the vesicle. (see page 171 for color figure)

Cholesterol and vesicular transport

Besides phospholipids, the plasma membrane also consist of considerable amounts of sphingolipids, glycolipids and cholesterol. More than 90% of cellular free cholesterol is located at the plasma membrane [70]. Cholesterol intercalates between the hydrophobic tails of the membrane lipids and interferes with the tight packing of phospholipids [71]. Cholesterol therefore, is an important determinant for the membrane fluidity and permeability. The cholesterol level of the ER pool depends on the circulation of cholesterol from and to the plasma membrane [72]. The relatively small pool of cholesterol in the endoplasmic reticulum responds rapidly to changes in plasma membrane cholesterol and affects intracellular vesicular transport. Depletion of plasma membrane cholesterol promotes exocytosis and *vice versa*, an increase stimulates endocytosis [73,74]. In addition, intracellular accumulation of cholesterol was found to block exocytosis through a mechanism involving Rab4 [75].

Aim and scope of this thesis

The major aims of this study were to further explore the regulatory mechanisms of Volume-Regulated Anion Channels (VRACs) and to search for molecular candidates for VRAC. Intestine 407 cells are extremely suitable for this purpose because they lack the expression of chloride conductances other than the cell swelling-activated anion conductance. **Chapter 2** provides an overview of the different model systems and methods that can be used to investigate cell volume regulation in mammalian intestinal epithelial cells. In **Chapter 3**, we demonstrate that exocytosis contributes to the development of the compensatory anion conductance by recruitment of additional anion channels from intracellular compartments to the plasma membrane. Furthermore, impaired intracellular cholesterol transport as result of defective NPC1 transport protein was found to reduce hypotonicity-provoked anion efflux. Intracellular accumulation of cholesterol has also been observed in cultured cell models of CF cells. Using our experience with Intestine 407 and NPC cells, we tested the hypothesis that cholesterol depletion would promote recruitment and plasma membrane retention of F508delCFTR (**Chapter 4**). The role of Rab proteins and vesicular traffic in the regulation of VRAC is described in **Chapter 5**. The data suggest that a significant fraction of the volume-sensitive Cl^- channels is localized intracellularly and is recruited to the plasma membrane upon osmotic cell swelling. As a consequence, anion channels localized primarily in intracellular compartments can no longer be excluded as VRAC candidates. **Chapter 6** shows an

inventory of the putative intracellular Cl^-- channels. Finally, the role of aquaporin (AQP) water channels in cell volume regulation was studied. Using AQP knockdown cells, we found that the hypotonicity-evoked fast increase in cell volume is a main trigger for the activation of VRAC (**Chapter 7**).

Taken together, the studies described in this thesis indicate a novel mechanism of VRAC activation, i.e. cell swelling-induced channel recruitment, and suggest a role for intracellularly localized chloride channels in cell volume regulation.

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Chapter 2

Osmosignalling and Volume Regulation in Intestinal Epithelial Cells

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Abstract

Most cells have to perform their physiological functions under a variable osmotic stress which, because of the relatively high permeability of the plasma membrane for water, may result in frequent alterations in cell size. Intestinal epithelial cells are especially prone to changes in cell volume due to their high capacity of salt and water transport and the high membrane expression of various nutrient transporters. Therefore, to avoid excessive shrinkage or swelling, enterocytes, like most cell types, have developed efficient mechanisms to maintain osmotic balance.This chapter reviews selected model systems that can be used to investigate cell volume regulation in intestinal epithelial cells, with emphasis on the regulatory volume decrease (RVD), and the methods available to study the compensatory redistribution of (organic) osmolytes. In addition, a brief summary is presented of the pathways involved in osmosensing and osmosignalling in the intestine.

Introduction

Intestinal epithelial cells, like most cell types, are constantly exposed to a variable osmotic stress caused by the uptake or release of osmotically active substances (amino acids, sugars etc.), the formation or degradation of macromolecules (proteins, glycogen), hormone-induced alterations in cellular metabolism or changes in the osmolarity of the surrounding fluid. Because of the relatively high water permeability of the plasma membrane, osmotic imbalances will immediately trigger a redistribution of cellular water and, consequently, induce a rapid change in cell volume. To protect the cells against the potentially deleterious effects of alterations in size, almost all cell types have developed compensatory mechanisms to prevent excessive shrinkage or swelling and to restore their original volume (reviewed by 1,2,3,4,5,6). In general, adjustment of the intracellular osmolarity is achieved by the activation of transport pathways in the plasma membrane, leading to a net accumulation (regulatory volume increase or RVI) or loss (regulatory volume decrease or RVD) of osmotically active substances. Whereas the RVI involves the net uptake of NaCl through stimulation of Na^+/H^+- and Cl^-/HCO_3^--exchangers or the activation of $Na^+/K^+/2Cl^-$- and Na^+/Cl^--symporters, the RVD largely depends on the release of KCl through K^+- and Cl^--selective ion channels or by the activation of K^+/Cl^--symporters. In addition, an organic osmolyte release pathway was found to be activated in response to osmotic swelling in a number of cell models, resulting in the release of small organic molecules such as taurine and betaine (For reviews see: 7; 8; 9).

Properties of the ion channels and transporters activated during RVD in intestinal epithelial cells

Hypo-osmotic stimulation of intestinal epithelial cells will result in a rapid increase in cell volume and a subsequent activation of specific K^+ and Cl^- channels, leading to a net efflux of KCl, which promotes the loss of cellular water and restoration of the original cell volume. In cultured Intestine 407 cells , recovery of cell size is achieved within a 1-2 minute period after applying a hypo-osmotic stimulus [10,11]. For isolated intact intestinal crypts, however, considerable longer periods are needed to obtain full volume recovery [12,13]. Whereas the K^+ conductance involved has been identified as a Ca^{2+}-dependent K^+ channel, sensitive to Ba^{2+}, quinidine, tetraethylammonium and charybdotoxin [10,14,15,16], the molecular identity of the anion channel (Volume-Regulated

[10,14,15,16], the molecular identity of the anion channel (Volume-Regulated Anion Channel, VRAC), despite its ubiquitous expression throughout the animal kingdom and its marked biophysical profile, has not yet been elucidated. Several potential candidates have been proposed, which include MDR-1/P-glycoprotein, ClC-2, ClC-3 and ICln, however, none of them meet all the electrical and pharmacological criteria of VRAC [17,18,19,20,21].

The cell swelling-activated chloride conductance has been studied in numerous different cell types and its electrical characteristics were found to be very similar in all models investigated. Key features of VRAC are: 1) a strong outward rectification and a prominent inactivation of the conductance at depolarising potentials, 2) a permeability sequence that corresponds to the Eisenman's sequence I ($SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^-$ > gluconate) and 3) inhibition by common Cl^--channel blockers such as 4-acetamido-4'-isothiocyanostilbene (SITS), 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB), diphenylamine-2-carboxylate (DPC) and niflumic acid [6,17,18,19,21.]. Finally, the hypotonicity-provoked VRAC activation in Intestine 407 cells was found to be potentiated, but not elicited, by Ca^{2+}-mobilizing hormones as well as by micromolar concentrations of ATP [22,23]. In contrast, millimolar concentrations of ATP as well as the purinoceptor antagonists suramin and Reactive Blue were found to inhibit VRAC [23]. This inhibition is most prominent at depolarising membrane potentials and does not involve purinoceptor activation and Ca^{2+}-signalling.

In addition to the activation of K^+ and Cl^- channels, the release of small organic osmolytes contribute significantly to the RVD response in many cell models. In a number of tissues and cell types, the organic osmolyte release pathway was found to be the major mechanism involved in cell volume correction [6-9]. In Intestine 407 cells, osmotic cell swelling was found to promote the release of taurine independently of the activation of VRAC. Unlike activation of the cell swelling-induced anion conductance, activation of the organic anion release pathway occurred only after a distinct lag time of approximately 30-60s [24]. In addition, the threshold for activation of taurine release was reached only at a relatively strong hypotonic stimulation. This suggests that, in Intestine 407 cells, the release of organic osmolytes acts a second line of defence.

Osmosensing and signalling

To date, very little is known about the molecular identity of the receptor involved in osmosensing in vertebrates. A specific class of osmoreceptors belonging to the family of histidine kinases has been identified in fungi, yeast and plants cells [25-27]. Histidine-kinases however, as a part of the so-called two-component systems are rarely expressed in mammalian cells. Several alternative mechanisms for osmosensing have been proposed, including macromolecular crowding, signalling via the extracellular matrix and integrin receptors, as well as activation of mechano-sensitive ion channels [3,6,17,28]. For intestinal epithelial cells, the role of integrins and their effects on cytoskeletal rearrangements are of a particular interest because of the sensitivity of VRAC in these cells for agents that affect the integrity of the cytoskeleton and by the observation of several groups that osmotic cell swelling is accompanied by a cytoskeletal reorganization [29-33].

Although differences may exist between cell types, several general features of cell signalling in response to hypo-osmotic stress have recently emerged. Especially the requirement of protein tyrosine phosphorylation for the activation of VRAC has been well documented. In the presence of tyrosine kinase inhibitors like herbimycin A, genistein or tyrphostins, the cell swelling-activated anion conductance was found to be largely reduced, whereas (per) vanadate inhibition of phosphotyrosine phosphatases, to reduce phosphotyrosine phosphatase activity, potentiated the anion efflux triggered by non-saturating hypo-osmotic stimulation [11,34-36]. Many hypotonicity-activated (targets of) tyrosine kinases have been reported over the last few years, including the focal adhesion kinase (FAK), receptor tyrosine kinases like the epidermal growth factor receptor, phosphatidylinositol-3-kinase and members of the families of SRC and MAP kinases (for review see 37). A direct activation of VRAC, however, has only been established for the SRC-like p56lck tyrosine kinase in Jurkat T lymphocytes. For these cells, strong evidence exists that p56lck is both essential and adequate for channel activation [38]. This notion is supported by our observation that in Intestine 407 cells, the hypotonicity-provoked anion efflux is largely reduced after treating the cells with damnacanthal, an inhibitor of p56lck (B.C.Tilly, unpublished results). In Caco-2 colonocytes, however, targeting c-SRC to caveolae was found to inhibit VRAC [39].

In addition to tyrosine kinases and/or phosphatases, the involvement of G proteins in the activation of the RVD has been reported. Indeed, activation of G

proteins by the intracellular administration of GTPγS rapidly induced an anion-selective current in several cell types, including human HT29cl19A colonocytes [35,40]. Furthermore, a regulatory role for the Ras-related Gprotein p21Rho in the activation of VRAC has been reported for Intestine 407 cells and various other cell types [31,35,41,42,43]. Most plausibly, p21Rho exerts its function through the induction of cytoskeletal remodelling. In Intestine 407 cells, the related p12Ras and its downstream target Raf-1, which are also activated during osmotic cell swelling, did not affect VRAC regulation but were found to be essential for activation of the ERK-1/2 MAP kinases [44]. Notably, although p21Rho is essential for VRAC regulation, recent studies in bovine endothelial cells have shown that Rho activation alone is not sufficient to induce opening of the channel [42]. The hypotonicity-induced release of organic osmolytes was not sensitive to tyrosine kinase or phosphatase inhibition and did not require p21Rho or PtdIns-3-kinase activity, indicating that the efflux is regulated independently of VRAC [24].

Osmotic cell swelling is often accompanied by an increase in vesicle (re-) cycling and exocytosis [45-48]. In Intestine 407 cells and several other cell types, an increase in volume leads to the extracellular release of ATP, an almost universal cellular response to mechanical stress, through a mechanism involving exocytosis [44,48,49]. Although extracellular ATP is not directly involved in the regulation of VRAC, purinergic receptor activation was found to trigger the hypotonicity-provoked stimulation of ERK-1/2 in Intestine 407 cells [23,44]. It has been reported that exocytosis contributes also to the development of the compensatory anion conductance by the recruitment of additional anion channels to the plasma membrane [50].

Osmotic cell swelling activates several distinct signalling pathways that are often also associated with (neuro-) hormone and/or growth factor-associated signal transduction. Whereas some of these cascades are coupled to the regulation of VRAC, others, like the ATP-provoked activation of ERK-1/2, are apparently not involved in channel regulation and, although speculative, may have a function in restoring cellular homeostasis and in maintaining cell viability. Importantly, with the notable exception of p56lck in Jurkat T cells, activation of these signalling molecules by itself is not sufficient to activate VRAC. Linking these widely utilized signalling modules to an independent, but yet unidentified, 'volume sensor' not only preserves the specificity of the response but also couples the RVD to pathways activated by hormonal stimulation, thereby facilitating the correction of small changes in cell volume that may occur during hormone-induced changes in cellular metabolism.

Model systems

To investigate cellular responses to osmotic challenges in intestinal epithelial cells, a variety of different model systems can be used that vary in their complexity and in their resemblance to native intestinal epithelium. The human Intestine 407 [51] and rat IEC-6 [52] cells are examples of relatively fast propagating epithelial cells, which can be easily maintained in culture using standard conditions. Although they grow as monolayers, the cells are not interconnected by junctional complexes and they do not develop into a semi-tight or "leaky" epithelium, making them unsuitable for transepithelial potential or current measurements in Ussing chambers. They are however, good model cells for patch clamp and radioisotope efflux studies, as well as for biochemical analysis of signal transduction cascades and optical imaging. In addition, as compared to many other enterocyte or colonocyte cell types, expression of proteins can be modified relatively easily using modern molecular genetic tools. Intestine 407 cells are very suitable for investigating cell swelling-activated anion currents because these cells lack expression of any other Cl^- conductances aside VRAC, such as Ca^{2+}- and voltage-sensitive Cl^- channels or cAMP/protein kinase A-sensitive CFTR Cl^- channels [10,11,53]. In addition, P-glycoprotein expression was not detected in the sub-clone used in our lab.

The colonocyte cell lines Caco-2 [54], HT29-cl.19A [55] and T84 [56,57] are all derived from lung metastasis of human colonrectal cancers. As compared to Intestine 407 and IEC-6 cells, they more closely resemble native enterocytes in that they express the cystic fibrosis transmembrane conductance regulator (CFTR, the chloride channel defective in cystic fibrosis patients), which is ubiquitously present in the apical membrane of intestinal epithelial cells, and, when cultured on a permeable support for at least 2 weeks, form semi-tight epithelia and develop junctional complexes. When grown on Costar Transwell™ polycarbonate cell culture inserts, these cell lines are not only suitable for Ussing chamber experiments, but can be used also to determine independently the isotope efflux from the apical and basolateral part of the cells.

Enterocytes, villi or crypts, isolated from freshly excised intestine, are preparations of the native epithelial cells lining the gut. Although villi and crypt preparations can be used to study changes in volume [12,13], for instance, using time-lapse video imaging, and to unravel the signalling pathways triggered by such a change biochemically, these models are less suitable to investigate ionic responses using physiological techniques such as radioisotope efflux assays or

patch clamping. This is partly due to an impaired cell viability, a short life span after isolation and difficulties in attaching to a substrate. Suspensions of villus epithelial cells however, have been used succesfully to investigate changes in cell volume by electronic cell sizing using a Coulter counter [58]. In addition, this preparation was found suitable to determine alterations in intracellular pH and $[Ca^{2+}]$, as well as to quantitate $^{22}Na^{+}$ uptake and $^{86}Rb^{+}$ efflux in hypotonicity or Na^{+}-nutrient stimulated cells [59-61].

Perhaps the most physiological model for intact intestinal epithelium is isolated muscle-stripped mucosa, which can be obtained from small laboratory animals [62]. This model can also be used to study and compare the different regions of the intestine [63,64]. In our laboratory, the preparation of muscle-stripped mucosa for short circuit measurements in Ussing chambers is performed as described by Andres et al. (1985). Briefly, under full anesthesia, the intestine is removed and subsequently flushed extensively with ice-cold modified Meyler solution (108 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 20 mM Hepes and 10 mM glucose, pH 7.4). Sections of approx. 3–4 cm were cut of the area of interest and placed on a glass rod. Using a blunt scalpel, a superficial longitudinal incision was made and the serosa and muscle layers were removed mechanically using tweezers. Thereafter, the tissue was sliced open, mounted on a circular tissue holder and put between the two Ussing chamber half cells. Both half chambers were perfused contineously with modified Meyler solution at 37°C and gassed with 95% O_2 / 5% CO_2 .

Measuring ionic responses

Whole cell patch clamp

The single electrode patch clamp technique allows a direct evaluation of the magnitude and the electrical properties of currents under voltage clamp conditions [65,66]. In its whole cell configuration, this method has been used to study volume-sensitive channels under well-defined (ionic) conditions in numerous cell models, leading to the identification of the K^{+} channels involved and to a detailed electrical characterization of VRAC (see above). Although whole cell patch clamping is a well-established technique, widely used by many different research groups around the world, and almost all of the equipment and software needed is available commercially, investigators who are planning to introduce this technique in their own laboratory are strongly adviced to get into contact with a lab specialized in membrane electrophysiology. This because

of the special requirements needed to isolate the setup from environmental electrical noise and mechanical vibrations. Because a high-resistance seal between the pipette and the cell membrane can be obtained easily when the cell has a smooth surface [65,67], it is much easier to study cultured cells at low densities than using fully differentiated enterocytes containing numerous microvilli and an apical mucous layer. As a consequence, isolated villi and crypts as well as stripped intact intestinal epithelium are less suitable for patch clamp analysis. In combination with fluorescence microscopy, patch clamping is very useful to study channel activation in cells transfected with fluorescently-tagged vectors, making it possible to identify cells with high expression levels. Notably, in contrast to the isotope efflux assays and short circuit measurements (described below), the RVD is not functional under whole cell patch clamp conditions and the cell swelling-induced currents last as long as the bathing solution remains hypotonic with respect to the pipette solution (cf see 19).

Whole cell recordings can be obtained after disruption of the membrane patch directly under the pipette by a mild negative pressure, creating a direct access between the cell interior and the microelectrode. Although this allow us to control the intracellular composition, it could also result in a dilution of essential cellular components and lead to a run down of the currents. To avoid diffusion of small molecules, but not ions, the perforated patch configuration [68] can be used, in which the membrane patch under the pipette is not disrupted but instead permeabilized using pore-forming antibiotics (nystatin, amphothericin).

To investigate VRAC activation in Intestine 407 cells, we used a bathing isotonic solution composed of 110 mM CsCl, 5 mM $MgSO_4$, 3.5 mM sodium gluconate, 12 mM Hepes, 8 mM Tris-HCl and 100 mM mannitol at pH 7.4 [23]. The intracellular pipette solution contained 110 mM CsCl, 2 mM $MgSO_4$, 25 mM Hepes, 1 mM EGTA, 1 mM Na_2ATP and 50 mM mannitol, pH 7.4. Patch pipettes were pulled from borosilicate glass (Clark Electromedical Instruments, Pangbourne, Berks, UK), heat polished and had a resistance of 2–3 MΩ. Whole cell current recording were made using a RK-300 amplifier (Bio-Logic, Claix, France) and digitized using a Digidata 1200 AD converter (Axon Instruments Inc., Foster City, CA), To monitor the development of the current, alternating step pulses (100-ms duration) from 0 to ±100 mV were applied every 30 s. Voltage-dependence of whole-cell current was monitored by applying step pulses (2-s duration, 7-s interval) from −100 mV to +100 mV with 25 mV increments (Figure 1). For command pulse control, data acquisition and analysis, pCLAMP 9 software (Axon Instruments, Union City, CA)

was used. All data were sampled at 5 kHz after being low-pass filtered at 500 Hz.

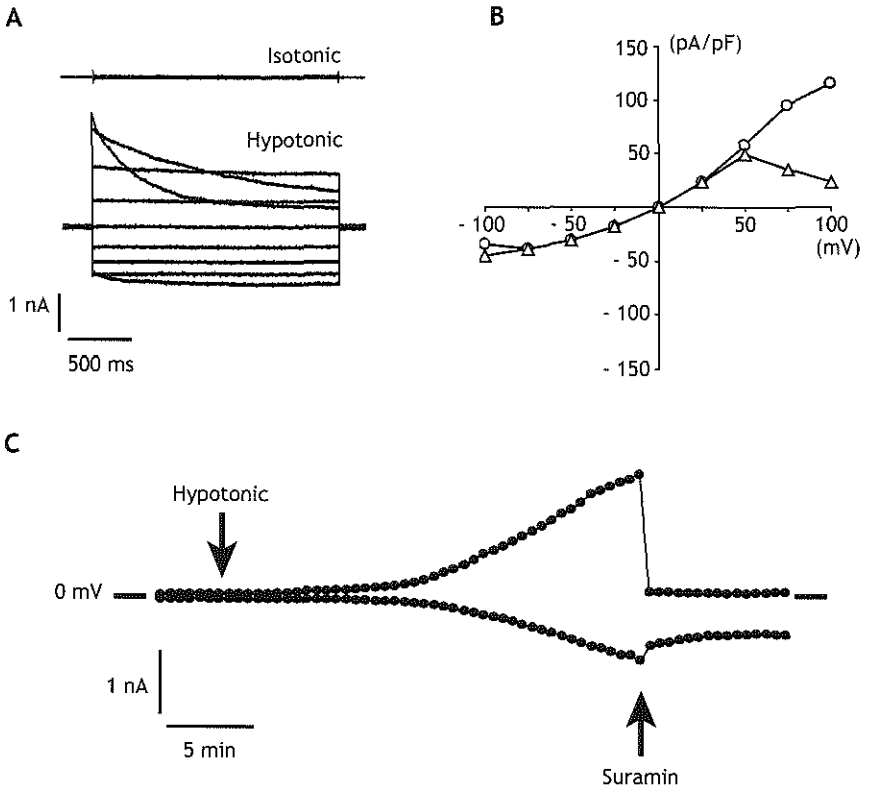

Figure 1: **Volume-sensitive chloride currents from Intestine 407 cells.** **A)** Whole cell Cl^- currents in control (isotonic) and hypo-osmotically stimulated cells in response to step pulses (2 s duration) from −100 mV to 100 mV (25 mV increments). **B)** Current to voltage relationship of the hypotonicity-provoked Cl^- currents immediately after the onset of the pulse (circles) and after reaching their plateau values (triangles). **C)** Time course of the hypotonicity-provoked anion currents at −100 and +100 mV as well as the voltage-dependent inhibition of the currents by suramin (100 μM).

Isotope efflux assay

The radioisotope efflux assay is a rapid and easy method to quantitate alterations in ionic conductances as well as to determine the release of small organic osmolytes from cultures of cells [24,69]. As a tracer for the chloride

conductance, either $^{36}Cl^-$ or $^{125}I^-$ or both can be used. Cation conductances can be evaluated by using $^{86}Rb^+$, $^{22}Na^+$ or $^{45}Ca^{2+}$ for respectively K^+, Na^+ and Ca^{2+} channel activity. An increase in the efflux of a specific isotope indicates the activation and opening of that particular channel, but does not give accurate information about the magnitude or the direction of the current. For instance, stimulation of $^{45}Ca^{2+}$-loaded Intestine 407 cells with bradykinin will result in a rapid and transient radioisotope efflux, indicative for a transient opening of Ca^{2+} channels, which is against the electro-chemical gradient for Ca^{2+} (22; Figure 2C). In addition, the efflux of tracer can occur through exchange with ions in the bath and does not require the presence of a shunt conductance, for example, an almost normal hypotonicity-provoked efflux of $^{125}I^-$ can be observed from Intestine 407 cells treated with phorbol esters to block the potassium conductance (Figure 2 A,B).

To perform isotope efflux assays, cells can be grown in tissue culture dishes, 12 or 24 multiwell plates or tissue culture treated polycarbonate filters (Costar Transwell™). Although optimal labeling conditions may vary per cell type, we routinely load our cells with 20-50 µCi/ml $^{36}Cl^-$, 0.5-1 µCi/ml $^{45}Ca^{2+}$, 5-10 µCi/ml $^{125}I^-$ and/or 0.5-1 µCi/ml $^{86}Rb^+$ in modified Meyler solution for 2 h at 37°C under a humidified atmosphere of 95% air / 5% CO_2. Prior to the experiment, the cells were washed 3 times with a low sodium isotonic medium containing mannitol (80 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 98 mM mannitol and 20 mM Hepes, pH 7.4). The radioisotope efflux from cells grown in wells was determined by consecutive replacement (1-2 minute intervals) of the medium (1 ml/well for 24 wells plates) with either isotonic or hypotonic medium at 37°C. Hypotonic buffers were prepared by adjusting the mannitol concentration. Residual isotope was determined at the end of the experiment by dissolving the cells in 0.5 M NaOH. Alternatively, the cells can be incubated for an additional 60 min in 1 ml of buffer to determine the amount of free diffusible tracer still present after full equilibration. This procedure is especially useful if a considerable amount of the tracer is immobilized intracellularly. When cells are grown on filter insets, both the efflux from the mucosa (apical efflux, upper compartment) and from the serosa (basolateral efflux, lower compartment) can be determined by replacing the medium in the upper compartment as described above and by simultaneously transferring the filter inset to a consecutive well containing fresh medium. Isotope efflux can be quantitated by ß-scintillation ($^{36}Cl^-$ or $^{45}Ca^{2+}$) or γ-scintillation ($^{125}I^-$ with or without $^{86}Rb^+$) counting. To compare

the magnitude of the efflux during the experiment, the data are expressed as "fractional efflux per minute" [69].

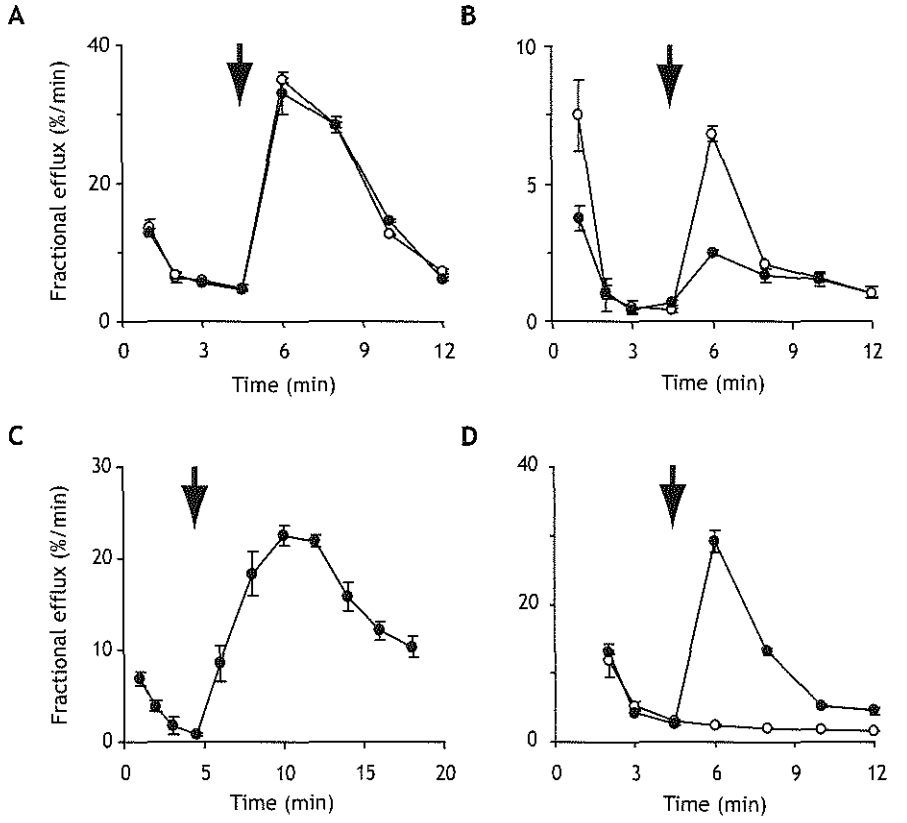

Figure 2: **Radioisotope efflux from Intestine 407 cells.** $^{125}I^-$ (A) and $^{86}Rb^+$ (B) efflux from isotope-loaded hypo-osmotically-stimulated control (open symbols) and phorbol 12-myristate 12-acetate-treated (PMA; 200 nM for 5 min; closed symbols) cultures. A marked inhibition of the $^{86}Rb^+$, but not the $^{125}I^-$, efflux was observed in PMA-treated cells. (C) Hypotonicity-provoked efflux of ^3H-taurine. (D) Efflux of $^{45}Ca^{2+}$ from isotope-loaded cells stimulated with bradykinin (closed symbols) or a hypo-osmotic (open symbols) medium. All data are expressed as mean ±S.E.M. for n=3. Arrows indicate time points of hypotonic stimulation (70% tonicity, **A-D**) or addition of bradykinin (1 μM, **D**).

A similar approach can be used to study the release of organic osmolytes. For Intestine 407 cells, loading for 2 h with 0.1 μCi/ml ^{3}H-taurine or ^{3}H-choline was found sufficient to get a reasonable intracellular accumulation [21]. To quantitate the actual amount of taurine released, medium fractions can be collected at the various time points and analyzed by reverse-phase HPLC after pre-column derivatization with *o*-phthaldialdehydeas as described previously.

Ussing chamber experiments

The "Ussing chamber" technique is particular convenient for studying ionic responses in intact muscle-stripped intestinal epithelium or in filter-grown monolayers of cultured enterocytes that have developed a junctional complex. This method not only allows a direct evaluation of the transepithelial potential or current but can also be used to measure changes in the epithelial resistance [reviewed by 70,71]. In comparison with patch clamp studies and radioisotope efflux assays, Ussing chamber experiments have the advantage that intact cells and epithelia, not impaled with micro-electrodes, are studied and that, unlike tracer studies, the actual transepithelial current, as well as its direction can be determined. A disadvantage of this method, however, is that the observed current reflects the sum of the magnitudes and directions of all individual anion and cation currents from both the mucosal (apical) or serosal (basolateral) sides. Therefore, to evaluate a specific current, inhibitors should be used or conditions should be chosen that nullify other contributing conductances.

Since the first description of the Ussing chamber, by the Danish physiologist Hans Ussing [72], a number of different designs have been reported, some available commercially, that fit pieces of isolated intestinal epithelium, filter inserts with cultured cells or even small pieces of tissue obtained from biopsies. In general, the chamber consists of 2 separate compartments connected by a small hole that can contain the tissue or the filter insert. Each compartment has two electrodes, one (platinum) for current injections and one for transepithelial voltage recording (calomel or Ag/AgCl electrode). Using an amplifier suitable for Ussing chamber studies, the transepithelial potential difference (PD) or the short circuit current (Isc, under voltage clamp conditions), as well as by injecting a current pulse of known magnitude the transepithelial electrical resistance (TEER) can be determined. The most suitable chamber designs have half cells, which can be perfused continuously and independently of each other, making changes in the composition of the serosal and mucosal medium possible, and are temperature controlled.

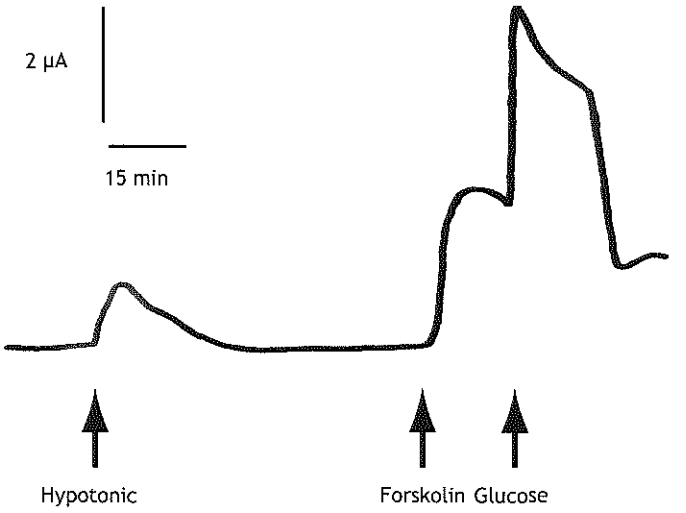

Figure 3: **Short circuit current recording of stimulated murine muscle-stripped ileum mounted in an Ussing chamber.** Arrows indicate time points of the shift to a hypotonic medium (70% tonicity, serosal and mucosal) or the addition of forskolin (10 µM, serosal), an activator of CFTR-mediated Cl^- secretion, or glucose (10 mM, mucosal), initiating glucose-coupled Na^+ uptake. Upward deflections represent a net current from the mucosal to the serosal side of the chamber.

To study transepithelial potentials and short circuit currents in murine or rat intestinal epithelium, small sections stripped mucosa were mounted in Ussing chambers (0.3 cm^2 exposed area) perfused with modified Meyler solution continuously gasses with 95% O_2 / 5% CO_2 at 37°C. The potential difference was measured using calomel electrodes (Radiometer, Copenhagen, Denmark) connected to each of the half chambers by KCl-agar bridges; current (1–10 µA) was injected through platinum electrodes. The electrodes were connected to a DVC-1000 Dual Channel Voltage/Current clamp amplifier (WPI, Berlin, Germany). Data were digitised using a Digidata 1322A AD converter (Axon Instruments, Union City, CA) and analysed using AxoScope software (Axon Instruments, Union City, CA). Two Ussing chambers were used simultaneously to record the PD or Isc. The PD was expressed relative to the potential of the serosal side of the tissue; an Isc current from mucosa to serosa was designated positive. The Isc was measured continuously with the transepithelial voltage set at the PD value.

To evoke hyperosmotically-induced changes in Isc, mannitol could be added to either the serosal and/or the mucosal side of the tissue. To avoid junction potentials however, it is better to study hypotonicity-provoked currents in chambers perfused with a low sodium and mannitol containing modified Meyler solution. A hypo-osmotic stimulus can than be applied by switching the perfusion medium to one containing less mannitol. The magnitude of the Isc current in response to mucosal glucose, a consequence of electrogenic Na^+-driven glucose uptake, will help the experimentator to evaluate tissue viability. Figure 3, shows an example of osmotically induced changes in Isc in murine ileum.

Fluorometric quantification of intracellular ion concentrations

To date, fluorescent probes are frequently used as easy and non-invasive tools to study changes in the intracelluar concentrations of ions or to determine alterations in membrane potential and intracellular pH in many different model systems. In addition, several of these indicators have been proven to be suitable for high-throughput quantitative studies. The use of fluorescent anion indicators has been boosted by search for pharmacological potentiators and correctors of F508delCFTR, the most common mutant CFTR chloride channel identified in cystic fibrosis patients. Most fluorescent anion indicators, such as SPQ and MQAE (Molecular Probes, Eugene, OR, U.S.A.), are methoxyquinolinium derivatives and have a rather poor plasma membrane permeability [73]. As a consequence loading of the cells with these probes may take many hours. Recently, a halide-sensitive derivative of Green Fluorescent Protein (YFP-H148Q/I152L) has been generated which could be expressed in target cells, thereby avoiding long periods of loading [74]. Although these indicators are very useful to study channel activation under isotonic conditions, the fluorescence signal is sensitive to changes in probe concentration, limiting their suitability for studies of ion concentration changes in response to changes in cell volume.

Concluding remarks

To date, a wide variety of model systems and techniques are available to study osmo-sensing and osmo-signalling in intestinal epithelial cells. However, despite many years of intensive research by multiple groups, important questions such as the identity of the volume sensor VRAC and the transporter/channel involved

in organic osmolyte release, remain to be resolved. In addition, our present knowledge about the processes linking the putative osmosensor to its effectors is rather limited. Perhaps newly emerging technologies, such as gene knockdown with small interfering RNAs (siRNA; cf. see 75) in either cells or intact animals, may help elucidating these questions.

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Chapter 3

Regulation of the Cell Swelling-Activated Chloride Conductance by Cholesterol-Rich Membrane Domains

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Abstract

Aim: The role of high cholesterol-containing microdomains in the signal transduction cascade leading to the activation of Volume-Regulated Anion Channels (VRACs) was studied.

Methods: Osmotic cell swelling-induced efflux of $^{125}I^-$ was determined in human epithelial Intestine 407 cells and in skin fibroblasts obtained from healthy controls or Niemann Pick type C (NPC) patients. Cellular cholesterol content was modulated by pre-incubation with 2-hydroxypropyl-ß-cyclodextrin in the presence of acceptor lipid vesicles.

Results: Osmotic cell swelling of human Intestine 407 cells leads to the rapid activation of a compensatory anion conductance. Treatment of the cells with cyclodextrin enhanced the response to submaximal hypotonic stimulation by approx. twofold, but did not further increase the efflux elicited by a saturating stimulus. In contrast, the volume-sensitive anion efflux was markedly inhibited when cholesterol-loaded cyclodextrin was used. Potentiation of the response by cholesterol depletion was maintained in caveolin-1 deficient Caco-2 colonocytes as well as in sphingomyelinase-treated Intestine 407 cells, indicating that cholesterol-rich microdomains are not crucially involved. However, treatment of the cells with progesterone, an inhibitor of NPC1-dependent endosomal cholesterol trafficking, not only markedly reduced the hypotonicity-provoked anion efflux, but also prevented its potentiation by cyclodextrin. In addition, the volume-sensitive anion efflux from human NPC skin fibroblasts was significantly smaller when compared to control fibroblasts.

Conclusions: The results support a model of regulatory volume decrease involving recruitment of volume-sensitive anion channels from intracellular compartments to the plasma membrane.

Introduction

Most mammalian cells respond to osmotic swelling by a rapid activation of compensatory mechanisms that encompass K^+ and Cl^- selective ionic channels as well as an organic osmolyte release pathway [for review see: 1-4]. As a consequence, osmolytes will leave the cells, driving the efflux of water, and promoting the restoration of cellular volume (regulatory volume decrease, RVD). In Intestine 407 cells, an epithelial cell line derived from human fetal jejunum, the osmo-sensitive K^+ conductance has been identified as the calcium-dependent intermediate K^+-channel (I_K) [5]. The molecular identity of the anion conductance in these and other cell types however, similar to the identity of the organic osmolyte release pathway, remains to be elucidated [6]. Besides the rapid release of osmolytes, osmotic cell swelling, similar to many other forms of mechanical stress, triggers the extracellular release of ATP through a mechanism which, in Intestine 407 cells, involves exocytosis [7,8].

Although the molecular identity of the Volume-Regulated Anion Channel (VRAC) has not been elucidated so far, its biophysical properties are well established and do not differ considerably between the cell models studied [6,9]. In addition, several components of the signalling cascade(s) leading to channel activation, such as tyrosine kinases and the Rho GTPase, have been identified [6,10,11]. Recently, a putative role for caveolae, cholesterol-rich microdomains of the plasma membrane involved in transmembrane signalling [for review see: 12], in the regulation of cell-swelling activated Cl^- channels has been demonstrated [13,14]. Trouet et al. [13] found that the volume-sensitive anion conductance was limited in caveolin-1 deficient cell types, but could be enhanced robustly by overexpression of the protein. In addition, transfection of calf pulmonary artery endothelial (CPAE) cells with mutant caveolin-1, thereby disturbing the formation of caveolae, was found to markedly reduce the hypotonicity-induced anion current [14]. Other studies suggest a direct effect of the lipid composition of the plasma membrane on the regulation of VRAC activity [15]. A decrease in plasma membrane cholesterol content enhanced the swelling-induced anion current whereas an increase in cholesterol content resulted in channel inhibition [15,16]. Based upon these results, a mechanism involving a shift in equilibrium between the channel open and closed states was suggested [15,16]. In addition, because alterations in the cholesterol content of the plasma membrane modulate the rate of endocytosis [17-20], the internalization of channel proteins may also be affected.

In the present study we investigated the putative role of cholesterol containing micro-domains of the plasma membrane in the regulation of the cell swelling-induced anion channels in cultured Intestine 407 cells. This cell line is particularly suitable for studying cell swelling-regulated Cl^- channels because other anion channels, i.e. Ca^{2+}-, voltage-activated or cAMP/protein kinase A-activated CFTR Cl^- channels, are not expressed in this cell line [10].

Materials and methods

Materials

Radioisotopes were obtained from Amersham Netherlands B.V. ('s Hertogenbosch, The Netherlands). Other chemicals were purchased from Sigma (St. Louis, MO). Human control and Niemann-Pick type C (NPC) skin fibroblasts were obtained from the European Cell Bank (Rotterdam, the Netherlands). High-density lipoproteins (HDL) and low density lipoproteins (LDL) were isolated from blood of healthy volunteers by sequential ultra-centrifugation using standard techniques [21].

Cell culture

Intestine 407 cells, Caco2 cells and human skin fibroblasts were routinely grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (Hepes; Intestine 407 and Caco2 cells only), 10% fetal calf serum, 1% non-essential amino acids, 40 mg L^{-1} penicillin and 90 mg L^{-1} streptomycin under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Hypotonic buffers were prepared by adjusting the concentration of mannitol and osmolality was assessed using a cryoscopic osmometer (Osmomat 030, Salm & Kipp B.V., Breukelen, The Netherlands).

Efflux assay

Monolayers of cells were loaded for 2 h with 5 µCi mL^{-1} $^{125}I^-$ in the absence or presence of 2% 2-hydroxypropyl-β-cyclodextrin (CD) and 1 mM phosphatidylcholine large unilaminar vesicles (PC-LUVs) and washed 3 times with isotonic buffer (80 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM Hepes, pH 7.4) prior to the assay. Isotope efflux was determined at 37°C by replacing the medium at 1-2 minute intervals. Hypotonic buffers were prepared by reducing the concentration of mannitol. Radioactivity in the

media was determined by gamma- radiation counting and expressed as fractional efflux per minute as described previously [22]. All experiments were performed in triplicate and repeated at least 3 times.

Filipin staining

Cultures of human control and NPC skin fibroblasts were fixated using 3% paraformaldehyde in phosphate-buffered saline (PBS) for 60 min. Thereafter, the cells were washed (PBS, 3 times), treated with 50 mM glycine in PBS (30 min) to quench paraformaldehyde and stained with filipin (40 µg mL^{-1} , 30 min). Epifluorescence was examined using an inverted Olympus IX50 microscope and images were acquired and analyzed using AnalySiS imaging software (Soft Imaging Systems, Münster, Germany).

Results

Stimulation of the volume regulated anion conductance by 2-hydroxypropyl-ß-cyclodextrin and LUVs

Treatment of Intestine 407 cells with 2-hydroxypropyl-ß-cyclodextrin (CD) in the presence of phosphatidylcholine large unilaminar vesicles (PC-LUVs) led to a reduction in total cellular cholesterol by approx. 50 %, whereas acceptor lipid vesicles alone were not effective (cholesterol content of control, CD/PC-LUVs- and PC-LUVs-treated cells; 90.2 ± 9.3, 47.7 ± 2.8 and 87.3 ± 10.8 nmol mg^{-1} protein respectively).

In CD/PC-LUVs treated cells, an approx. twofold increase in the fractional iodide efflux triggered by a mild hypotonic stimulus (80% tonicity) was observed (Figure 1). This stimulation of the response was not found when PC-LUVs were used in the absence of CD or when cholesterol-bound CD (0.1 mol mol^{-1})/ PC-LUVs were used (Figure 1). In contrast, these conditions resulted in reduced hypotonicity-provoked iodide efflux, indicating a diminished anion conductance. Upon CD/PC-LUVs treatment, the anion efflux was increased only slightly after stimulation with hypotonic stimuli that elicited a saturating response (≥ 30% reduction in osmolarity [23]), suggesting that cholesterol depletion potentiated VRAC activity but did not induce an additional efflux pathway distinct from VRAC (Figure 2).

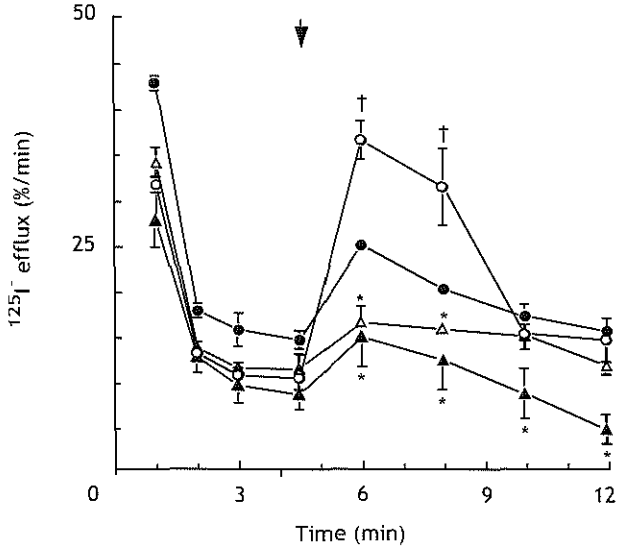

Figure 1: **Stimulation of the hypotonicity-provoked $^{125}I^-$ efflux by CD/ LUVs.** Control and CD/PC-LUVs treated cultures of $^{125}I^-$-loaded Intestine 407 cells were stimulated with a hypotonic medium (80% tonicity). Thereafter, the fractional radio-isotope efflux was determined as described under Material and method. Closed circles: untreated cultures; open triangles: cultures treated with PC-LUVs alone; open circles: cultures treated with PC-LUVs in the presence of CD; closed triangles: cultures treated with cholesterol-loaded CD (0.1 mol mol^{-1}) and PC-LUVs. Data are expressed as mean ±S.E.M. for n=3. Arrow marks time point of shift to a hypotonic medium. A significant potentiation or inhibition of the hypotonicity-induced efflux relative to the untreated control is indicated by † or * respectively ($p > 0.05$; Student's *t*-test).

The concept that modulation of the amount of plasma membrane cholesterol affects the volume-regulated anion conductance was corroborated by our finding that a reduction in cholesterol synthesis, using atorvastatin, likewise resulted in a potentiation of the response, whereas exposure to hydroxycholesterol led to a distinct inhibition (Table 1). Furthermore, treatment of the cells with isolated High Density Lipoproteins (HDL), involved in physiological transport of cholesterol from peripheral cells to the liver, mimicked the effect of atorvastatin on the swelling-induced efflux, whereas incubation with Low Density Lipoproteins (LDL), implicated in cellular cholesterol uptake, had no effect (Table 2). Notably,

the anion efflux through forskolin- or 8-bromo-cAMP activated CFTR chloride channels expressed in murine IEC-CF7 enterocytes or NIH-3T3 fibroblasts was not affected by CD/PC-LUVs pretreatment (results not shown).

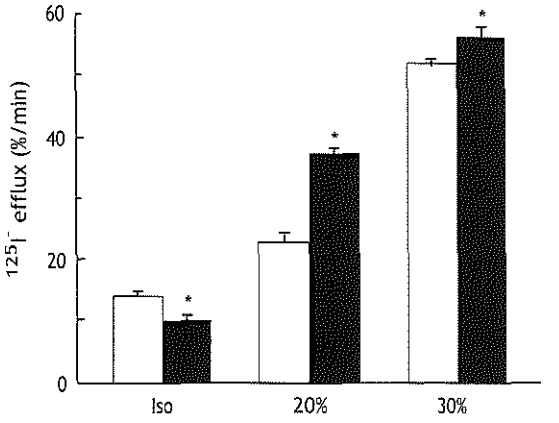

Figure 2: **CD/PC-LUVs-mediated potentiation of the hypotonicity-induced $^{125}I^-$ efflux.** Fractional $^{125}I^-$ efflux from control (open bars) and CD/PC-LUVs-treated (solid bars) cultures under isotonic (Iso) and hypotonic conditions (20% or 30% reduction in tonicity). Data are expressed as mean ±S.E.M for n=3. Asterisk indicates a significant difference with the untreated control ($p < 0.05$; Student's *t*-test).

Potentiation of VRAC involves recruitment from intracellular compartments

To investigate a putative role for cholesterol-containing microdomains of the plasma membrane, such as caveolae and/or cholesterol/sphingolipid-containing lipid rafts, in the regulation of VRAC, we studied the cell swelling-induced anion efflux in caveolin-1 deficient Caco-2 cells [13,24,25] as well as in sphingomyelinase treated Intestine 407 cells. As is clear from Figure 3, disruption of these microdomains did not affect the ability of CD/PC-LUVs to potentiate the hypotonicity-provoked anion efflux, suggesting that these domains do not play a critical role in VRAC regulation.

Table 1: Modulation of plasma membrane cholesterol

Reduction of tonicity (%)	Increase in fractional iodide efflux (% min^{-1})		
	Control	Hydroxycholesterol	Atorvastatin
20	11.7 ± 1.5	6.2 ± 0.4*	18.3 ± 1.5*
30	36.3 ± 0.6	27.2 ± 0.6*	n.d.

Cultures of Intestine 407 cells were treated with hydroxycholesterol (25 μM for 2 h) or with atorvastatin (10 μM, 20 h). Hypotonicity-induced increase in isotope efflux is expressed as mean ±S.E.M (n=3). n.d. = not determined. Asterisk indicates a significant difference with the untreated control ($p < 0.05$).

Table 2: Modulation of hypotonicity-provoked $^{125}I^-$ efflux by LDL and HDL

	Increase in fractional iodide efflux (% min^{-1})
Control	10.4 ± 0.4
CD/PC-LUVs	27.0 ± 0.8*
LDL	11.7 ± 0.9
HDL	18.1 ± 1.3*

Cultures of Intestine 407 cells were treated with either CD/PC-LUVs or isolated human LDL (2 mM, 2 h) or HDL (1 mM, 2h) prior to hypo-osmotic stimulation. Hypotonicity-induced (20% reduction in tonicity) increase in isotope efflux are expressed as mean ±S.E.M (n=3). Asterisk indicates a significant difference with the untreated control ($p < 0.05$).

Alternative targets for the action of CD/PC-LUVs are the recycling endosomes or retrosomes involved in intracellular cholesterol transport [26-28]. It has been established that trafficking of these organelles is inhibited in the presence of the steroid progesterone [29,30]. To investigate a putative role for endosomes in recruiting VRAC to the plasma membrane, cells were treated with 30 μM progesterone for 2 h. As shown in Figure4a, the cell swelling-induced increase in $^{125}I^-$ efflux triggered by a mild (20%) reduction in tonicity was only slightly affected in progesterone-treated cultures. In contrast, a marked inhibition was observed when a more severe hypotonic stimulation (30% reduction in tonicity) was applied, resulting in a slower development of the efflux and lower peak values Figure 4a). In addition, potentiation of the anion efflux by CD/PC-LUVs was largely inhibited (Figure 4b). These results suggest that the potentiation

of the Cl^- conductance, at least partly, depends on channel recruitment from endosomal vesicles.

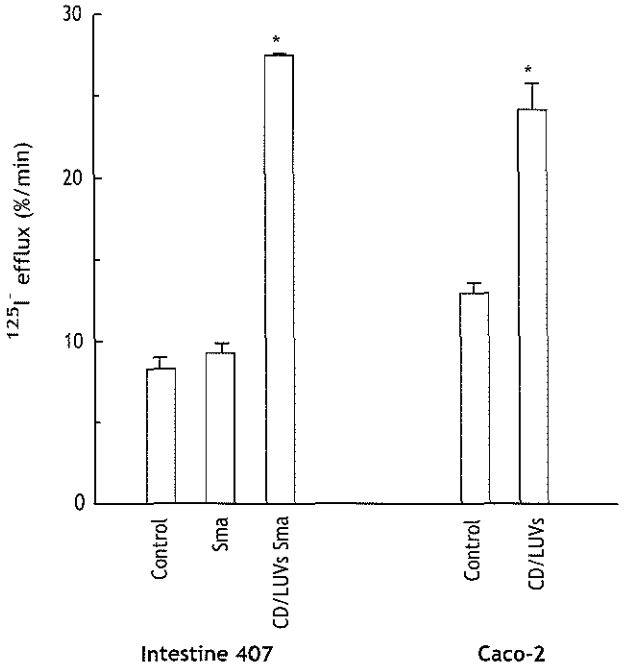

Figure 3: **Lipid rafts and caveolae are not crucially involved in the CD/LUVs potentiation of the cell swelling-induced anion efflux.** Potentiation of the hypotonicity-provoked efflux of anions by CD/LUVs from control and sphingomyelinase (Sma; 2 I.U. mL^{-1} for 30 min)-treated Intestine 407 cells and from caveolin-1 deficient Caco-2 cells (Mirre et al. 1996, Trouet et al. 1999, Patlolla et al. 2004). Because the anion efflux from Caco-2 cells is less responsive to hypo-osmotic stimulation (Trouet et al. 1999), the tonicity of the hypotonic buffer was reduced to 50% of the isotonic medium, allowing a better comparison with the Intestine 407 cells (which were stimulated with a 20% hypotonic medium). Data are expressed as the hypotonicity-induced increase in fractional iodide efflux relative to the isotonic control (mean ±S.E.M. for n=3). Asterisk indicates a significant difference with the control ($p < 0.05$; Student's *t*-test).

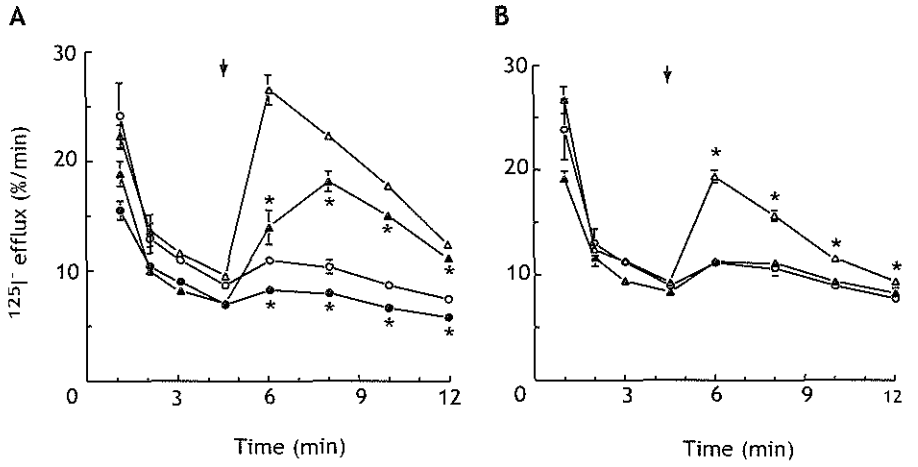

Figure 4: **Cell swelling-induced anion efflux in progesterone-treated cells.** (A) Control (open symbols) and progesterone-treated (30 μM for 2 h; closed symbols) $^{125}I^-$-loaded cultures of Intestine 407 cells were challenged with 20% (circles) or 30% (triangles) hypotonic media. (B) Fractional efflux from untreated (open symbols) and progesterone-treated (30 μM for 2 h; closed symbols) cultures stimulated with a 20% hypotonic medium. Circles: control cultures; triangles: CD/LUVs-treated cultures. Arrows indicate start of osmotic stimulation. Data are expressed as mean ±S.E.M. for n=3. Asterisk indicates a significant difference of the hypotonicity-provoked efflux relative to the untreated control ($p < 0.05$; Student's *t*-test).

Activation of VRAC is reduced in skin fibroblasts obtained from Niemann Pick type C patients

Endosomes involved in the transport of cholesterol to the plasma membrane or to other vesicular compartments contain the NPC1 transport protein, which is defective in patients with the Niemann-Pick type C disease [for a recent review see: 31]. Although the physiological function of the NPC1 protein remains to be established, mutations in the gene encoding this protein result in a massive intracellular accumulation of cholesterol. Using control and NPC skin fibroblasts, we studied the role of the NPC1-containing endosomes in the cell swelling-induced activation of VRAC. As shown in Figure 5, a dramatic increase in the amount of intracellular vesicles as well as in filipin staining of cholesterol was observed in NPC cells, indicating a massive accumulation of cholesterol into

endosomal compartments. Osmotic cell swelling of the NPC fibroblasts did not affect intracellular filipin staining, suggesting that these cholesterol-containing vesicles are rather immobile and do not migrate to the plasma membrane (Figure 6). However, the Niemann Pick C condition resulted in a significant reduction in the hypotoncity-induced fractional $^{125}I^-$ efflux when compared with control cells (Figure 7), supporting our notion that NPC1-dependent recycling of endosomes contributes to the recruitment of VRAC. Because the inhibition of the cell swelling-induced anion efflux in the mutant fibroblasts was only partial, it also indicates that a substantial portion of the VRAC channels already pre-exist in the plasma membrane or are recruited through (an) alternative pathway(s).

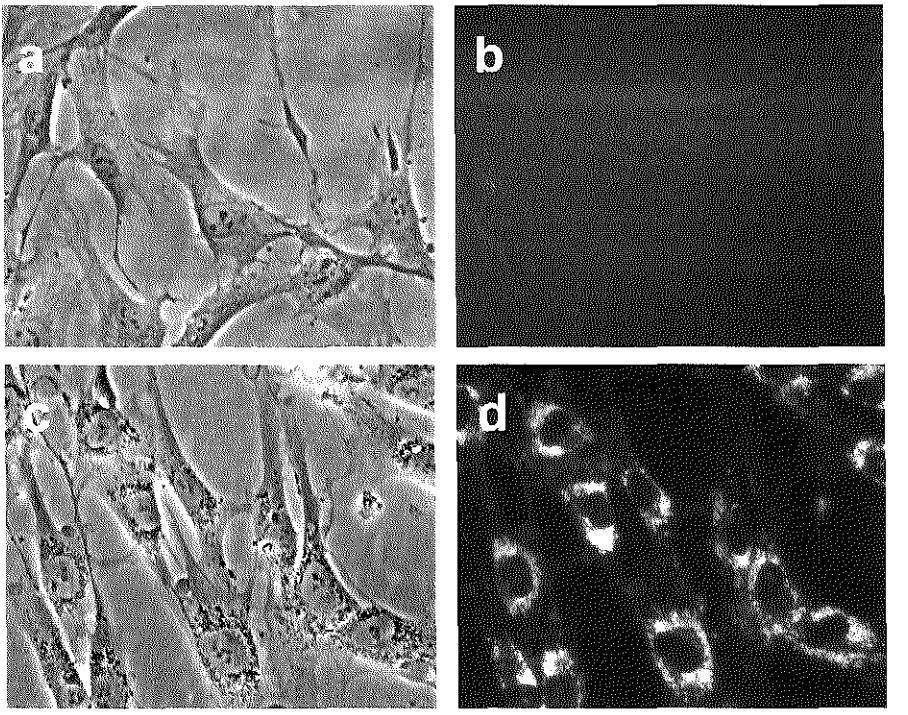

Figure 5: **Intracellular accumulation of cholesterol in cultured skin fibroblasts obtained from Niemann Pick C patients.** Phase contrast (**a**, **c**) and filipin fluorescence (**b**, **d**) images obtained from control (**a**, **b**) and Niemann Pick type C (**c**, **d**) human skin fibroblasts. Data are representative for fibroblasts obtained from 3 different control persons and 3 different NPC patients.

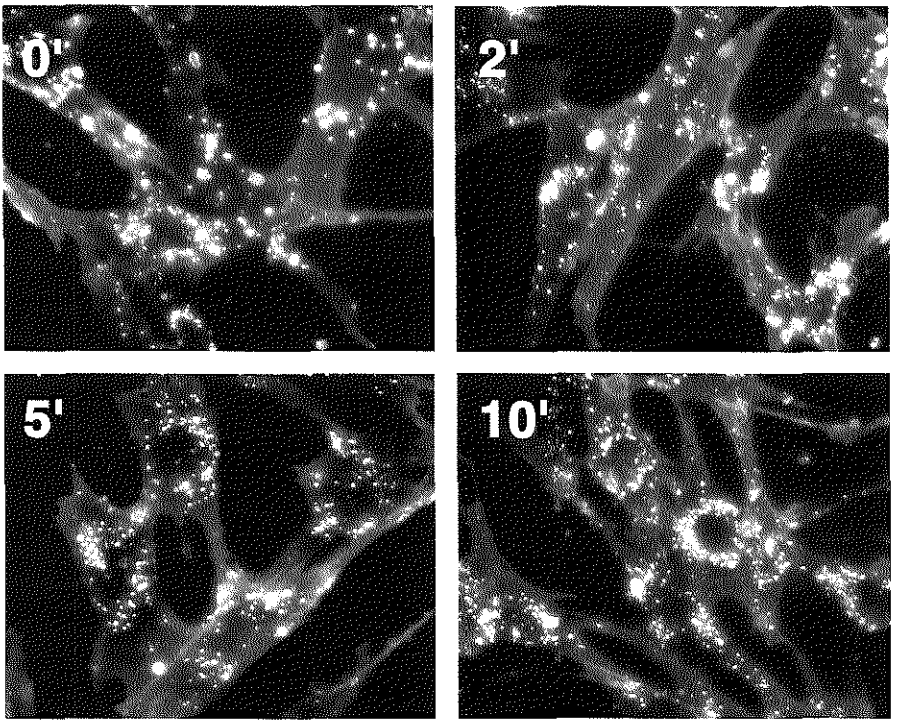

Figure 6: **Osmotic cell swelling does not affect intracellular cholesterol accumulation in Niemann Pick C type fibroblasts.** Fluorescence images of filipin-stained Niemann Pick C human skin fibroblast stimulated with a 30% hypotonic medium for 2, 5 or 10 minutes prior to fixation.

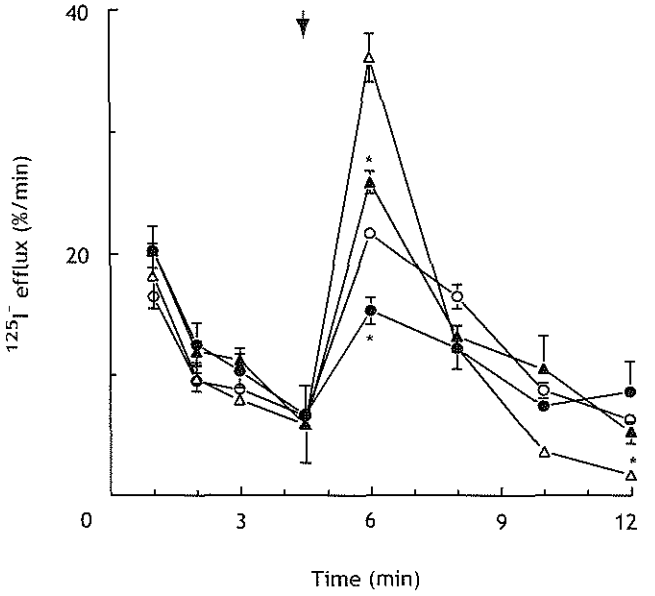

Figure 7: **Hypotonicity-provoked anion efflux from control and NPC skin fibroblasts.** Osmotic cell swelling-induced efflux from isotope-loaded control (open symbols) and NPC (closed symbols) skin fibroblasts. Circles and triangles represent stimulation with 20% or an 30% hypotonic medium respectively. Data presented are representative for fibroblasts obtained from 3 different control persons and 3 NPC patients and expressed as mean ±S.E.M. for triplicate incubations. Arrow marks the shift from an isotonic to a hypotonic solution. Asterisk indicates a significant difference in the hypotonicity-provoked efflux (20% and 30%) in NPC skin fibroblasts relative to control fibroblasts ($p < 0.05$; Student's *t*-test).

Discussion

Osmotic swelling of epithelial Intestine 407 cells rapidly activates a compensatory anion conductance through a signalling mechanism involving p21 Rho, protein tyrosine kinase(s) and/or phosphatase(s) and (a remodeling of) the F-actin cytoskeleton [10,11]. For CPAE cells, a crucial role for caveolae in the activation of VRAC has been reported, based on the observations that the magnitude of the cell swelling-induced Cl^- current was proportional to the level of expression of caveolin-1 [13,14]. In bovine endothelial cells however, treatment of the cells with CD, leading to a disruption of the caveolae [32], resulted in a potentiation of

the swelling-induced current [15,16]. Furthermore, using cholesterol analogues, Romanenko et al. demonstrated that the physical properties of the lipid bilayer (e.g. membrane fluidity, susceptibility to deformation, phospholipid ordering) play a key role in the regulation of VRAC. Modulation of ion channel properties by alterations in the lipid environment is not unprecedented but has also been reported also for Na^+, K^+ and Ca^{2+} conductances [33-37]. In addition, association of channel proteins with lipid microdomains such as lipid rafts or caveolae has been demonstrated for a number of distinct ion channels [for review see: 38].

As observed in bovine endothelial cells [15], the volume-sensitive anion efflux from Intestine 407 cells was robustly potentiated by treating the cells with CD in the presence of PC-LUVs. This potentiation was not observed when PC-LUVs were used in the absence of CD or when the cells were exposed to cholesterol-loaded CD. We cannot exclude that an increase in the open state probability of the channel, as has been reported for endothelial cells [15,16], contributes to the observed increase in the volume-sensitive anion conductance. However, our observation that the CD/PC-LUVs- induced potentiation of the cell swelling provoked $^{125}I^-$ efflux was completely absent in cells treated with progesterone, known to block trafficking of cholesterol containing vesicles to the plasma membrane [29], suggests that depletion of plasma membrane cholesterol promotes the recruitment of VRAC. Stimulation of exocytosis by osmotic cell swelling has been reported previously by our laboratory as well as by others [8,39-41] and, in Intestine 407 cells, is an essential step in the extracellular release of ATP [7,8]. Our present observations additionally suggest that exocytosis might also contribute to the development of the compensatory anion conductance. Several lines of evidence support a role for VRAC recruitment during the RVD response. First, the activation of the hypotonicity-provoked anion efflux was impaired in cells exposed to progesterone [cf. Figure 4a]. Second, compared with normal skin fibroblasts, the volume-sensitive anion conductance is smaller in skin fibroblasts obtained from Niemann Pick type C patients [cf. Figure 6] characterized by a specific defect in cholesterol transport to the plasma membrane [27,28]. It should be noted, however, that inhibition of exocytosis reduced the volume-sensitive anion efflux only partly, suggesting that other mechanisms, for instance a cell swelling-induced unfolding of plasma membrane invaginations [9], might also contribute to the recruitment of VRAC.

It has been well established that plasma membrane levels of cholesterol markedly affect endocytosis and the subsequent recycling of endosomes to the

membrane. Depletion of cholesterol was found to inhibit clathrin-coated pit budding and to increase the retention of receptors and glycosylphosphatidylinositol (GPI)-linked proteins in the plasma membrane [17-19]. *Vice versa*, an increase in cholesterol is known to stimulate endocytosis [20]. In addition to promoting exocytosis, osmotic cell swelling of Intestine 407 cells also resulted in a robust increase in the rate of endocytosis, starting after a distinct lag time of 2–3 min [8]. Most plausibly therefore, CD/PC–LUVs treatment may, in addition to VRAC recruitment, also result in a prolonged retention of the chloride channels in the plasma membrane which could contribute to the observed potentiation of the chloride efflux. Increased membrane retention may also explain the potentiating effect of CD/LUVs in caveolin-1 deficient Caco2 cells [e.g. see: 13,14]; although the total number of VRAC docking sites is apparently reduced in this cell line, a decrease in the rate of endocytosis may result in an increased volume-sensitive anion efflux.

In addition to alterations in vesicle trafficking, depletion of plasma membrane cholesterol has been reported to increase membrane stiffness [42] and to induce a reorganization of the F-actin cytoskeleton [42-44]. Both phenomena might have consequences for the development of compensatory conductances during the RVD, either by altering osmo-sensing or by modulating channel regulation. Especially the effects of CD exposure on the cytoskeleton are of interest because F–actin remodeling in response to osmotic cell swelling has been reported previously in several cell models, including Intestine 407 cells [11], and has been implicated in the regulation of VRAC [reviewed in: 1-4,6].

In conclusion, the results presented support a model in which the recruitment of volume-sensitive anion channels from intracellular compartments act as an important step in the mechanism of VRAC activation. Although the molecular identity of VRAC still remains an enigma, our observations imply that intracellularly localized Cl^- channels should be included as possible candidates in a search for the identity of Volume-Regulated Anion Channels.

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Chapter 4

Cholesterol Depletion and Genistein as Tools to Promote F508delCFTR Retention at the Plasma Membrane

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Abstract

Background/aims: F508delCFTR-, but not wtCFTR-, expressing fibroblasts resemble Niemann Pick type C cells in the massive intracellular accumulation of free cholesterol. The recruitment and activation of F508delCFTR by cholesterol depletion was studied.

Methods: Filipin staining, forskolin-stimulated anion efflux and FITC-dextran uptake were studied in control cells and fibroblasts treated with 2-hydroxypropyl β-cyclodextrin and phosphatidylcholine large unilamellar vesicles to deplete cellular free cholesterol.

Results: Treatment of F508delCFTR-, but not wtCFTR-, expressing fibroblasts with 2-hydroxypropyl β-cyclodextrin resulted in a reduction in cellular cholesterol and a potentiation of the forskolin-induced anion efflux. In addition, forskolin also promoted a massive increase in the rate of endocytosis in F508delCFTR fibroblasts, which was absent in genistein- or cyclodextrin-treated cultures.

Conclusion: The results not only suggest that reducing cellular cholesterol may serve as pharmacotherapeutic tool in the treatment of cystic fibrosis but also reveal a novel mechanism for genistein regulation of F508delCFTR, i.e. retention by inhibition of endocytosis.

Introduction

Cystic fibrosis (CF), the most common fatal hereditary disease among Caucasians, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. CFTR is a cAMP/protein kinase A- and cGMP/protein kinase G-II-regulated Cl^- channel [2,3] crucially involved in transepithelial salt and water transport. In the Western population more than 70% of the mutations in CFTR appear to be a deletion of a phenylalanine residue at amino-acid position 508 (F508delCFTR) [1,4], resulting in an incompletely processed protein, defective in its ability to traffic to the plasma membrane [5,6]. Although a small fraction of F508delCFTR protein may reach the plasma membrane and retain its function as cAMP-activated chloride channel [7-9], the reduced number of channels at the cell surface leads to dramatically impaired transepithelial transport of chloride [8]. In addition, although glycosylation of CFTR is not required for CFTR function [10], F508delCFTR exhibits a significant decrease in the open probability [11, 12] and a strongly reduced cell surface half-life as compared to wild-type [8,13, 14], which also contribute to the diminished Cl^- conductance and, consequently, water flux.

In a previous study, we investigated the role of cholesterol-containing micro-domains of the plasma membrane in the activation of Volume-Regulated Anion Channels (VRACs). Intestine 407 cells were treated with 2-hydroxypropyl β-cyclodextrin in the presence of Phosphatidylcholine Large Unilamellar Vesicles (CD/PC-LUVs). CD/PC-LUVs depletes plasma membrane cholesterol. Consequently, the endosomal recycling rate was accelerated to replenish plasma membrane cholesterol. As a result, the endocytosis was inhibited [15-17]. When Intestine 407 cells were depleted of cholesterol, the cell swelling-provoked anion conductance was increased approximately 2 fold [18]. From these studies, we proposed a model of VRAC regulation involving recruitment of channels from cholesterol containing intracellular compartments [18]. This model is supported by our observation that in Niemann Pick type C (NPC) fibroblasts lacking the functional NPC1 protein involved in cellular cholesterol transport, as well as in cells treated with an inhibitor of intracellular cholesterol transport, the cell swelling-induced anion efflux was strongly impaired [18]. In addition, experiments with Niemann Pick type C skin fibroblasts revealed that the volume-sensitive anion efflux could be restored by CD/LUVs-induced cholesterol depletion. (C.H.Lim and B.C.Tilly, unpublished results).

Recently, White et al. showed that F508delCFTR-expressing epithelial cells develop an NPC-like phenotype [19,20]. To investigate whether recruitment and activation of F508delCFTR could be (partially) restored by cholesterol depletion, analogous to the model proposed for VRAC regulation in Intestine 407 cells [18], the forskolin-stimulated anion efflux was studied in wtCFTR- and F508delCFTR-expressing murine 3T3 fibroblasts. The results clearly demonstrate that a reduction of cellular cholesterol promotes an increase in the forskolin-stimulated anion efflux from F508delCFTR-containing fibroblasts whereas the efflux from wtCFTR-expressing cells was not affected. In addition, the results suggest inhibition of endocytosis as a novel mechanism by which genistein is able to enhance CFTR activity in intact cells.

Material and methods

Materials

Radioisotope was obtained from Amersham Netherlands B.V. ('s Hertogenbosch, the Netherlands). FITC-conjugated dextran (MW=10,000 Da) and FM1-43 were from Invitrogen Molecular Probes (Leiden, the Netherlands). Other chemicals were purchased from Sigma (St. Louis, MO, USA)

Cell culture

Swiss 3T3 fibroblasts stably expressing human wtCFTR or F508delCFTR were a generous gift of Dr. Michael J. Welsh [21]. The cells were grown routinely as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. All experiments were carried out at 37°C.

Filipin staining

Cultures of cells were fixated using 3% paraformaldehyde in phosphate-buffered saline (PBS) for 60 min. Thereafter, the cells were washed three times (phosphate-buffered saline), treated with 50 mM glycine in PBS (30 min) to quench paraformaldehyde and stained with filipin (40 µg/ml, 30 min). Epifluorescence was examined using an inverted Olympus IX50 microscope and images were acquired and analysed using AnalySiS imaging software (Soft Imaging Systems, Münster, Germany).

FM1.43 staining

Cultures of cells were fixated using 3% paraformaldehyde in phosphate-buffered saline (PBS) for 60 min. Thereafter, the cells were washed three times (phosphate-buffered saline), treated with 50 mM glycine in PBS (30 min) to quench paraformaldehyde and stained with FM1.43 (1 μM, 30 min). Epifluorescence was examined using an inverted Olympus IX50 microscope and images were acquired and analysed using AnalySiS imaging software (Soft Imaging Systems, Münster, Germany).

Determination of cellular total free cholesterol

Cells were sonicated in PBS and cholesterol in the cell lysate was determined enzymatically using a commercially available kit (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's instructions.

Efflux assay

Monolayers of cells were loaded for 2 h with 5 μCi $^{125}I^-$ in the absence or presence of 2% 2-hydroxypropyl-β-cyclodextrin (CD) and 0.5 mM phosphatidylcholine large unilamellar vesicles (PC-LUVs). Prior to the assay, the cells were washed 3 times in isotonic buffer (130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES, pH 7.4). Isotope efflux was determined at 37°C by replacing the medium at 1-2 minute intervals. Radioactivity in the medium was determined by gamma- radiation counting and expressed as fractional efflux per minute as described previously [22].

FITC-dextran uptake

Cells on coverslips at 80-90% confluency were incubated for 10 min in a medium containing 0.5 mg/ml FITC-dextran (10,000 Da) in the absence or presence of forskolin (10 μM) and/or genistein (100 μM). After the incubation, cells were washed three times with ice-cold phosphate-buffered saline and fixed with 2% paraformaldehyde for 20 min. Using a 40X oil immersion objective (Axiovert 135M, Zeiss, Oberkochen, Germany), confocal images of 512 X 512 pixels (0.22 μm /pixel) were constructed by summation of 15 optical sections parallel to the substratum, each 1 μm apart. Endocytosis was quantified using KS400 software (Zeiss Oberkochen, Germany) by counting the number of fluorescent spots sized between 3 and 75 pixels, after subtraction of background fluorescence. Background fluorescence was determined by measuring the mean pixel intensity and adding five times its standard deviation of a circle drawn in the background.

Results

Intracellular accumulation of cholesterol in murine 3T3 fibroblasts expressing F508delCFTR

As compared to the parental and wtCFTR-expressing cells, F508delCFTR fibroblasts contain an enlarged intracellular area of vesicular structures, localized predominantly around the nucleus (Figure 1 A-C). Because White et al. recently showed that F508delCFTR-expressing epithelial cells develop a NPC-like phenotype, we studied the amount and cellular distribution of cholesterol in these fibroblast cell lines. Using filipin-treated cell cultures, accumulation of free cholesterol was found around the nucleus of F508delCFTR-expressing fibroblasts (Figure 1c), a phenomenon characteristic for NPC1-deficient cells [23-26]. In contrast, little if any filipin staining was observed in parental or wtCFTR expressing cells (Figure 1a, b). Determination of the total amount of free cellular cholesterol confirmed the increased accumulation of cholesterol in F508delCFTR cells (cholesterol content of parental, wtCFTR- and F508delCFTR-expressing 3T3 fibroblasts: 17, 18 and 24 µg/mg protein, respectively).

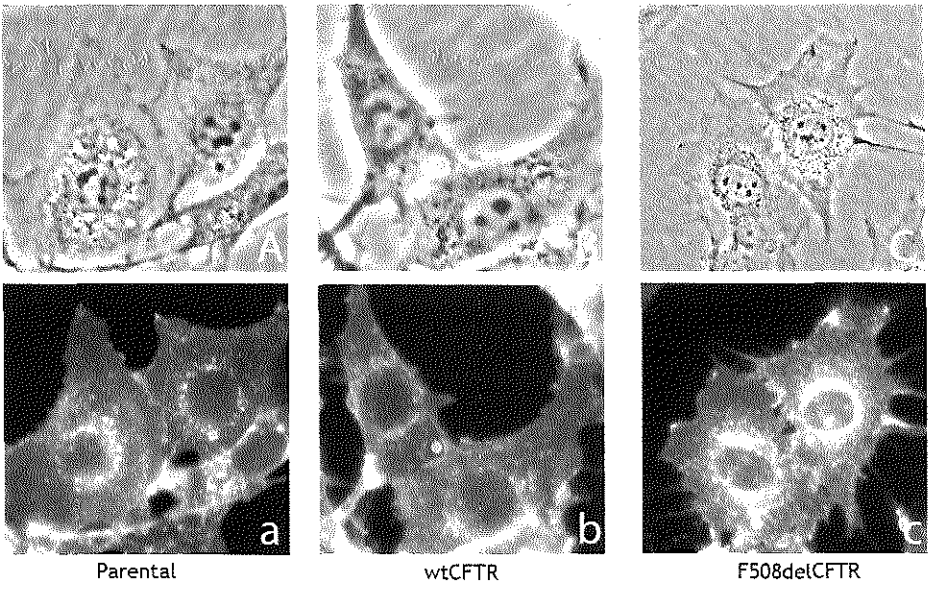

Figure 1: **Increased intracellular accumulation of cholesterol in fibroblasts expressing human F508delCFTR.** Cellular distribution of cholesterol in filipin-stained parental (**A, a**), wtCFTR- (**B, b**) and F508delCFTR- (**C, c**) expressing fibroblasts. Each image is representative of a total of 15 images taken from 5 individual experiments using 5 different batches of cells. **A-C**: phase contrast images; **a-c**: fluorescent images.

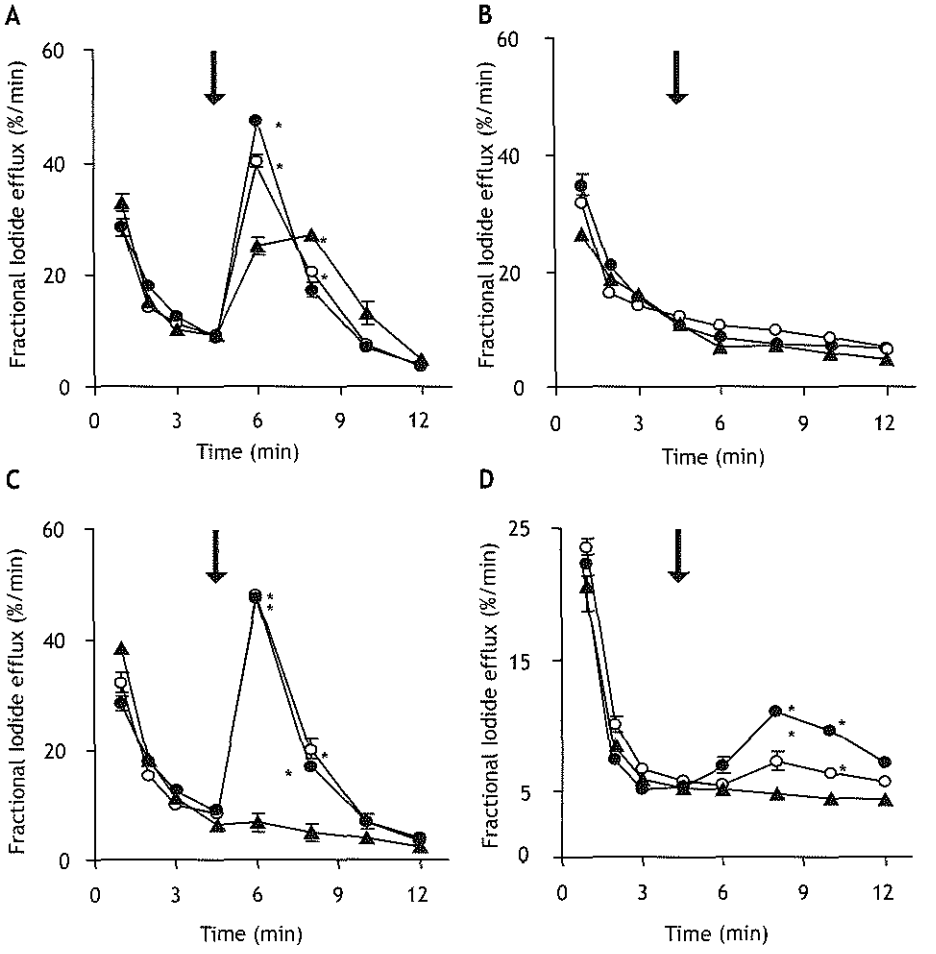

Figure 2: **CD/PC-LUVs treatment potentiate the forskolin- and genistein-induced anion efflux from F508delCFTR but not from parental or wtCFTR-expressing cells.** A) Fractional $^{125}I^-$ efflux from wtCFTR-expressing fibroblasts (A) stimulated with either forskolin (10 μM; open circles), genistein (100 μM; closed triangles) or both (closed circles). B - D) $^{125}I^-$ efflux from control (open circles) and CD/PC-LUVs-treated (closed circles) cultures of parental (B), wtCFTR- (C) and F508delCFTR- (D) expressing fibroblasts stimulated with forskolin (10 μM) and genistein (100 μM). Closed triangles represents the unstimulated control. Data are expressed as mean ±S.E.M for n=3. Arrow marks time point of forskolin and/or genistein addition. No error bar is given when the S.E.M. is smaller than the size of the symbol used. Asterisk indicates a significant difference from the control ($p < 0.05$).

CD/PC-LUVs treatment promotes rescue of F508delCFTR

Recently, we demonstrated that the osmotic cell swelling-induced activation of VRAC (Volume Regulated Anion Conductance) was impaired in both NPC1 fibroblasts as well as in cholesterol-loaded Intestine 407 epithelial cells [18]. In addition we found that treatment of these cells with CD/PC-LUVs markedly potentiated the ionic response, through a mechanism which most likely involved recruitment of addition channels by promoting vesicular transport [18]. To investigate the effect(s) of CD/PC-LUVs treatment on the anion efflux of the different clones of 3T3 fibroblasts, we first tested the effects of forskolin and genistein on wtCFTR fibroblasts. In line with previous studies, forskolin and genistein by themselves were able to promote the efflux of iodide from wtCFTR fibroblasts, simultaneous addition of both agonists, however, elicited the largest response (Figure 2A). As expected, genistein and forskolin did not provoke a $^{125}I^-$-efflux from parental 3T3 fibroblasts lacking CFTR (Figure 2B). In contrast, a slight increase in the anion efflux was observed after stimulating F508delCFTR fibroblasts with genistein and forskolin (Figure 2D). This response was enhanced by at least two-fold in F508delCFTR cells treated with CD/PC-LUVs. As compared to low temperature rescue of F508delCFTR function, a 2 h period of CD/LUVs treatment enhanced F508delCFTR activity to a level of approx 50% of an overnight incubation at 26°C (results not shown). Notably, the response to genistein and forskolin was not increased in CD/PC-LUVs-treated wtCFTR cells (Figure 2C). These results suggest that CD/PC-LUVs treatment, analogous to its effect on the recruitment of VRAC, is able to increase surface expression of F508delCFTR.

Cholesterol accumulation is reduced in CD/PC-LUVs-treated F50delCFTR fibroblasts

To evaluate whether cholesterol depletion is able to promote vesicle movement to the plasma membrane, the effects of CD/PC-LUVs on the intracellular distribution of cholesterol was studied in the different cell models. As shown in Figure 3A, accumulation of cholesterol was largely reduced in F508delCFTR-expressing cells treated with CD/PC-LUVs, but not in the parental cell line or in wtCFTR fibroblasts. Notably, incubation of the three different cell types with the fluorescent membrane probe FM-1.43 did not show any difference in the amount or distribution of intracellular vesicular compartments nor did it reveal a decrease in staining after CD/PC-LUVs treatment (Figure 3B). Taken together, these results suggest that a distinct intracellular compartment exists

in F508delCFTR-expressing cells, containing free cholesterol and at least some F508delCFTR, that can be accumulated at the plasma membrane by reducing plasma membrane cholesterol.

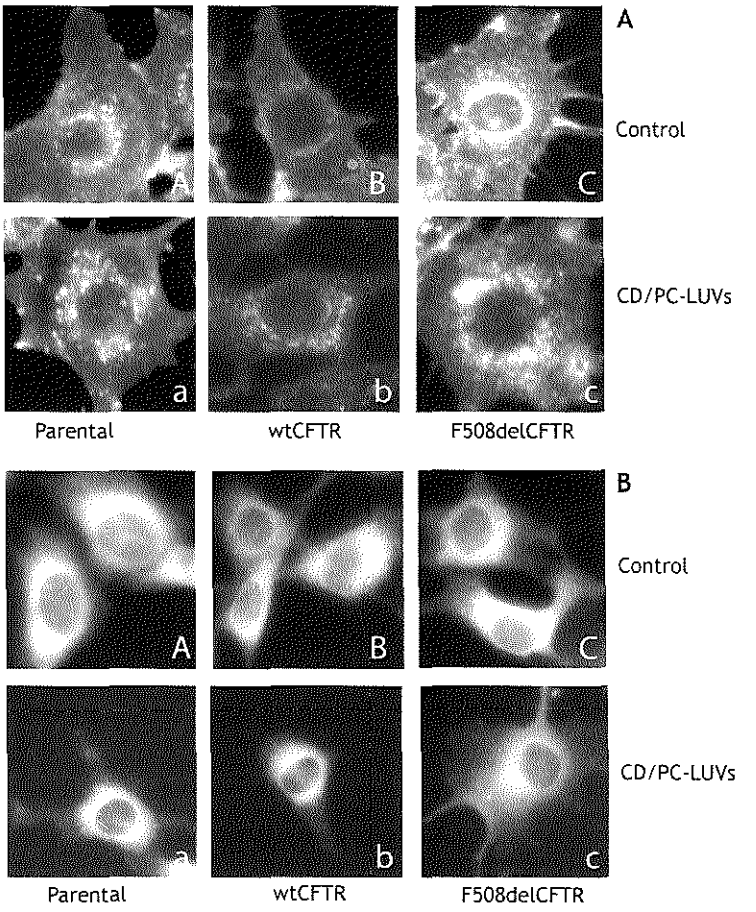

Figure 3: **Reduction of intracellular cholesterol in F508delCFTR fibroblasts after CD/PC-LUVs treatment.** Filipin staining **(A)** was compared with FM-1.43 staining **(B)**. Images of parental **(A, a)**, wtCFTR **(B, b)** and F508delCFTR **(C, c)** fibroblasts were taken before **(A - C)** and after **(a - c)** CD/PC-LUVs treatment. Each image is representative of a total of 9 images taken from 3 individual experiments.

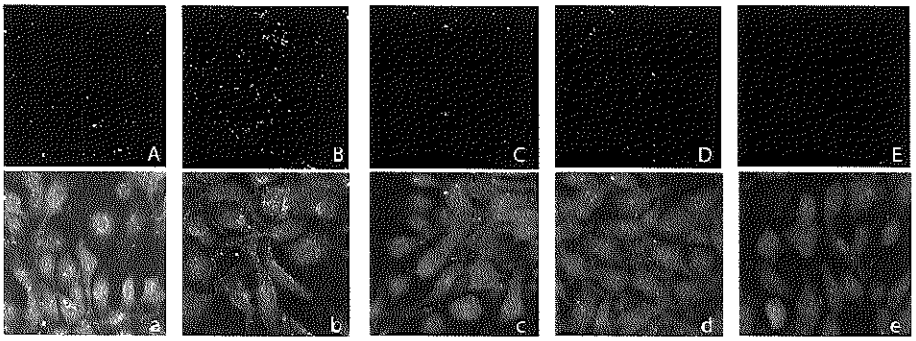

Figure 4: **Modulation of the rate of endocytosis by forskolin and genistein in F508delCFTR cells.** Endocytosis was determined by the uptake of FITC-conjugated dextran. Control cells (**A,a**) were compared with treated cells. Cultures were treated with 10 μM forskolin (**B, b**), 100 μM genistein (**C, c**), forskolin and genistein (**D, d**) or CD/PC-LUVs (**E, e**) as described under "Materials and Methods". **A-E**: fluorescence images. **a-e**: fluorescence merged with phase contrast images. Each image is representative of a total of 9 images taken from 3 individual experiments.

Regulation of endocytosis in 3T3fibroblasts by forskolin and genistein

To further pursue a putative role for vesicle cycling in the regulation of plasma membrane expression of F508delCFTR, the magnitude of endocytosis was studied by quantitating the cellular uptake of fluorescently labelled dextran. In F508delCFTR-expressing fibroblasts, the uptake of FITC-dextran was robustly increased by stimulating the cells with forskolin (Figure 4 and Table 1). In contrast, forskolin appears to reduce endocytosis in both parental and wtCFTR cells (Table 1). Surprisingly, the uptake of FITC-dextran was almost completely abolished in both control and forskolin-stimulated F508delCFTR cells treated with genistein (Figure 4 and Table 1). A comparable effect of genistein was also observed in parental and wtCFTR fibroblasts (Table 1). Similar to genistein treatment, incubation with CD/PC-LUVs largely prevented the uptake of dextran in both parental, wt-CFTR expressing and F508delCFTR-expressing cells (Figure 4 and Table 1).

Table 1: Modulation of endocytosis in parental, wtCFTR- and F508delCFTR-expressing fibroblasts

	Treatment	Parental	wtCFTR	F508delCFTR
Control	-	50 ± 1	64 ± 4	22 ± 1
	Forskolin	24 ± 5*	20 ± 4*	113 ± 5*
	Genistein	10 ± 2*	18 ± 5*	4.7 ± 0.3*
	Forsk. + Gen.	9 ± 2*	13 ± 2*	7 ± 3*
CD/PC-LUVs	-	7 ± 5*	11 ± 1*	2.0 ± 0.3*
	Forskolin	1.3 ± 0.2*	11 ± 2*	4 ± 1*
	Genistein	1.3 ± 0.9*	0.7 ± 0.1*	0.7 ± 0.2*
	Forsk. + Gen.	9 ± 2*	1.0 ± 0.2*	1.0 ± 0.3*

Endocytosis was determined by the uptake of FITC-conjugated dextran and quantitated as described under "Materials and methods". Data are expressed as the number of fluorescent spots (mean ±S.E.M for n=3). Concentrations used: forskolin, 10 μM; genistein, 100 μM. Asterisk indicates a significant difference from the untreated control ($p < 0.01$).

Discussion

In this study, we have demonstrated that a CD/PC-LUVs-mediated reduction in cellular cholesterol boosted the forskolin-provoked anion efflux from F508delCFTR-expressing cells approximately 2 fold. Modulation of ionic conductances by alterations in the lipid composition of membranes is not unprecedented and has been reported for VRAC as well as for several cation channels [15-17,27,28]. At least two different mechanisms can underlie the observed increase in the forskolin-stimulated efflux: (1) a modulation of the activity of the channel protein itself, altering the channel open and closed state [27,29], and (2) an increase in the number of channels in the plasma membrane by recruitment from intracellular compartments and/or by a prolonged retention in the membrane [15-17]. It is now well established that cholesterol-containing microdomains play an important role during the process of endocytosis. Indeed, depletion of plasma membrane cholesterol using CD/PC-LUVs largely inhibits endocytosis [15,16] and, *vice versa*, increasing membrane cholesterol is found

to promote endocytosis [30]. Although we do not entirely exclude the possibility that the open channel probability of F508delCFTR may also be affected by CD/PC-LUVs treatment, both the reduction of intracellular filipin staining and the inhibition of FITC-dextran uptake upon CD/PC-LUVs treatment indicate that additional channels are incorporated and/or retained into the plasma membrane in F508delCFTR-expressing fibroblasts. A similar mechanism has recently been proposed for the activation of a compensatory Cl^- current in osmotically stimulated Intestine 407 cells [18].

Both wild-type and mutant CFTR are internalised by clathrin-dependent endocytosis [31-33], although it seems that the rate of internalisation is higher for mutant CFTR [14,34]. Using dominant-negative mutants of Rme-1, a protein involved in endosomal cycling, it has been demonstrated that at least part of the CFTR escapes lysosomal degradation and recycles back to the plasma membrane [35]. Endocytotic trafficking of CFTR is regulated by several members of the Rab family of GTPases, including Rab 4 and 11, leading to recycling of the channels to the plasma membrane, and Rab 5, which promotes channel endocytosis [14]. Transport towards the membrane is inhibited in F508delCFTR-expressing cells but can be rescued by reduced temperature [6,36], chemicals chaperones like DMSO, glycerol [37,38] and proteasome inhibitors [14], most likely by promoting proper folding or stabilization of the mutant protein.

In this study, we showed that F508delCFTR-expressing fibroblasts, like several other F508delCFTR expressing cell models [19,20], have a NPC-like phenotype, i.e. a marked intracellular accumulation of cholesterol. Although cholesterol-containing vesicles are present throughout the cell, a marked accumulation of cholesterol was observed around the nucleus. This predominant perinuclear staining likely reflects the most important cellular area of CFTR synthesis, maturation and/or degradation, but may be additionally enhanced by the increased cell thickness at this region. The direct relation between F508delCFTR expression and cholesterol accumulation has not been fully established yet, however, White et al. reported that alterations in the regulation of NOS2, STAT1, SMAD3, and Rho A protein expression, which occur in NPC1 cells, are also observed in these CF models, suggesting that similar regulatory mechanism are involved [19]. Notably, cholesterol accumulation and an increase in Rho A expression was also observed in cells defective in ATP-binding cassette protein A1 (ABCA1), a cholesterol transporting ABC-transporter protein structurally related to CFTR [39]. Recently, the NPC1 protein has been proposed to function as a lipid flippase

of late endosomes and to promote the generation of transport vesicles by membrane deformation. The normal function of NPC1 is affected by the absence of NPC-2, a cholesterol binding protein, in such way that the cytosolic factors necessary for vesicle budding were inefficiently recruited, thereby disrupting vesicular formation and finally leading to the accumulation of many lipids [40]. In addition, increased cholesterol accumulation by itself can impair F508delCFTR trafficking by a direct inhibition of Rab 4, one of the Rab G proteins involved in CFTR recycling [25].

CFTR is activated by cAMP-dependent activation however, this process poorly activates F508delCFTR. The isoflavone genistein is able to potentiate CFTR by a direct binding to NBD-2 [41-43]. Our efflux assay demonstrated that genistein could augment the cAMP-dependent activation in CFTR-expressing fibroblasts. In F508delCFTR-expressing cells, neither genistein nor forskolin alone could induce a significant increase in the conductance after the CD/PC-LUVs treatment. However, the combination of both stabilized and potentiated the anion conductance. The cAMP-dependent activation is known to stimulate trafficking of CFTR to the plasma membrane in control cells [32,33,44,45] but not in CF cells [45]. Our dextran experiment demonstrates that forskolin stimulates endocytosis in F508delCFTR-expressing fibroblasts but not in parental and wtCFTR-expressing cells. This is in line with previous observations that the half-life of F508delCFTR in the plasma membrane is reduced [13,14,34] and with the findings of Bradbury et al. that elevated levels of cAMP fail to inhibit fluid phase endocytosis in F508delCFTR expressing cells [46]. This study also demonstrates for the first time that the genistein, which has previously been found to inhibit endocytosis in some model systems [47,48], can fully counter act the forskolin-induced FITC dextran uptake (Figure 4). This novel finding suggest that the beneficial effect of genistein on CFTR-mediated Cl^- secretion may result from a dual action: potentiation of the CFTR Cl^- channel by binding to CFTR and stabilizing the channel in its open state [41-43] and promotion of its retention in the plasma membrane through inhibition of endocytosis.

In this study we showed that treatment of F508delCFTR-expressing fibroblasts with CD/PC-LUVs potentiated the forskolin-induced anion efflux approximately 2 fold. We were however, unable to detect an increase in the amount of fully glycosylated mature CFTR (C-band) in CD/PC-LUVs-treated cells by Western blotting (results not shown). This indicates that CD/PC-LUVs treatment does not promote maturation of F508delCFTR but instead stimulates F508delCFTR trafficking to the membrane or, more likely, promotes channel retention.

Using mouse models, Dorin et al. demonstrated that 5 % mature CFTR is sufficient to restore intestinal chloride transport to 50% of normal level and to reach a 100% survival [49]. Furthermore, 8% of mature protein was found to correspond with normal cAMP-mediated chloride secretion in caecum and 40% residual cAMP-stimulated jejunal chloride secretion [50,51]. Because the forskolin-induced efflux in cells treated with CD-LUVs is approx. 10-15 % of the maximum response observed in CFTR expressing cells (Figure 2D) it would be of interest to further investigate the potential beneficial effects of a drug-mediated decrease in cellular cholesterol, for example by using atorvastatin, on the rescue of F508delCFTR in a more physiological model.

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Chapter 5

Osmotic Cell Swelling-Induced Recruitment of Volume-Regulated Anion Channels to the Plasma Membrane

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Abstract

In Intestine 407 cells, the hypo-osmotic activation of Volume-Regulated Anion Channels (VRACs) was found to be accompanied by the rapid and robust onset of exocytosis and, after a distinct lag period of several minutes, by an increase in the rate of endocytosis [1]. Inhibition of exocytosis, by treating the cells with N-ethylmaleimide or *C. botulinum* neurotoxin F, significantly reduced the hypotonicity-provoked anion efflux, whereas inhibition of endocytosis, using chlorpromazine, resulted in a significant increase of the basal efflux. These results suggest that vesicle cycling plays a role in the regulation of VRAC. To further pursue this notion, the role of Rab GTPases in the activation of VRAC was studied. Human Intestine 407 cells were transfected with plasmids encoding fluorescently tagged wildtype (WT) or dominant negative (DN) Rab4, Rab5 or Rab11 proteins. Both inhibition of exocytosis, by expressing Rab11DN or Rab4DN, and stimulation of endocytosis, by introducing Rab5WT, strongly reduced the volume-sensitive anion efflux. Stimulation of exocytosis however, using Rab11WT, did not increase the hypotonicity- provoked anion efflux but resulted in a considerable rise in basal efflux. This increase in basal conductance was also observed when Rab5DN was expressed. Taken together, our data suggest that a significant fraction of the volume-sensitive Cl^- channels is localized intracellularly and is recruited to the plasma membrane upon osmotic cell swelling. Consequently, anion channels localized primarily in intracellular compartments can no longer be excluded as candidates in the search for the molecular identity of VRAC.

Introduction

Due to the high water permeability of the plasma membrane, an osmotic imbalance of either physiological or pathological origin will rapidly induce a redistribution of water and, consequently, result in cell swelling or shrinkage. Osmotic cell swelling is accompanied by a fast activation of Volume-Regulated Anion Channels (VRACs). Despite its ubiquitous expression and its marked biophysical characteristics, as studied by the whole cell patch clamp technique in many different cell types, the molecular identity of VRAC still needs to be resolved. Several candidates have been proposed, including p-glycoprotein, ClC-2, ClC-3, CaCC and ICln, none of them however, comply with all of the electrical and pharmacological criteria [2,3]. In addition to the activation of compensatory conductances, osmotic cell swelling is accompanied by exocytosis, and, after a distinct lag time, by endocytosis [4].

It is now well established that the lipid composition of the plasma membrane can affect the activity of a number of ion channels, including VRAC [5-9]. This could be due to altered physical properties of the bilayer that shift the equilibrium between the channel open and closed state [6,10] or, indirectly, by affecting membrane retention [7-9]. Recently, we have shown that the cell swelling-induced activation of VRAC is markedly reduced in skin fibroblasts derived from patients suffering from the hereditary Niemann Pick type C cholesterol storage disease [11]. These patients are defective in NPC1, resulting in massive intracellular accumulation of cholesterol and an impaired endosomal cycling [11-14]. In addition we demonstrated that depletion of cellular cholesterol, by treating cells with 2-hydroxypropyl-β-cyclodextrin in the presence of acceptor lipid vesicles, increased the volume-sensitive anion efflux of Intestine 407 cells by approximately 2 fold. This potentiation was absent when trafficking of cholesterol containing vesicles to the plasma membrane was blocked by progesterone [11]. Taken together, these results suggested that VRAC recruitment to the plasma membrane may contribute to the activation of the compensatory conductance [11].

Exocytosis depends on the movement and the subsequent docking and fusion of vesicles with the plasma membrane [15,16]. Among the various proteins that participate in these processes are Rab type of GTPases, involved in directing vesicle transport, and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, responsible for docking and fusion (for review

see: [17,18]). Approximately 60 Rab proteins are encoded by the human genome [19,20] of which several have been implicated in the regulation of ion channels. Whereas membrane expression of the epithelial Na^+ channel (ENaC) has found to be regulated by Rab4 and Rab27a, both Rab5 and Rab11 are involved in the cell surface retention of wildtype and F508delCFTR [21]. In addition, Rab11 has been reported to diminished the Transient Receptor Potential TRPV5/6 mediated Ca^{2+} influx [22].

To further pursue a putative role for vesicle cycling and channel recruitment during cell volume regulation, we studied the role of Rab5, involved in clathrin-coated endocytosis [23], and Rab 4 and Rab11, both involved in a rapid and direct recycling route from the early endosomes back to the cell surface [24,25], in the activation of the cell swelling-induced anion efflux. Taken together, our data not only support a model of cell swelling-induced recruitment of VRAC from intracellular stores, but also suggest new molecular candidates for VRAC.

Material and methods

Materials

Radioisotope ($^{125}I^-$) was obtained from Amersham Netherlands B.V. ('s Hertogenbosch, the Netherlands). Wild-type and dominant negative Rab constructs expressed as DsRed fusions (Rab5), CFP fusions (Rab4) or GFP fusions (Rab11) were a generous gift from Prof. Dr. R. Pagano and Dr. D. Marks (Mayo Clinic College of Medicine, Rochester, USA). *Clostridium botulinum* neurotoxin F and the Chariot™ protein delivery kit were obtained from respectively Calbiochem (La Jolla, CA, U.S.A.) and Active Motif (Rixensart, Belgium). FITC-conjugated dextran (MW=10,000 Da) and TRITC-conjugated dextran (MW=10,000 Da) were purchased from Invitrogen Molecular Probes (Leiden, the Netherlands). All chemicals were purchased from Sigma (St. Louis, MO).

Cell culture and transfection

Intestine 407 cells were routinely grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM Hepes, 10% fetal calf serum, 1% non-essential amino acids, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Cells were transfected with rab encoding plasmids using Lipofectamine Plus Reagent (Invitrogen, Breda,

the Netherlands) and used 48 h after transfection. *Clostridium botulinum* toxin F was introduced intracellularly using the Chariot™ protein delivery kit according to the manufacturer's instructions.

Efflux assay

Confluent monolayer of cells were loaded with 5μCi/ml $^{125}I^-$ for 2 h in modified Meyler solution (108 mM NaCl, 4.7 M KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.8 mM $NaH_2PO_4H_2O$, 0.4 mM Na_2HPO_4, 10 mM glucose and 20 mM HEPES, pH 7.4). Prior to the assay, the cells were washed 3 times with isotonic buffer (80 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM HEPES, pH 7.5). Isotope efflux was determined at 37°C by replacing the medium at 1–2 minute intervals. The cells were stimulated with 20% or 30% hypotonic medium. Hypotonic buffers were prepared by adjusting the concentration of mannitol. $^{125}I^-$ Radioactivity in the medium was determined by γ- radiation counting. Data were expressed as fractional efflux per minute as described previously [26].

Dextran uptake

Cells grown on coverslips (80-90% confluency) were incubated for 10 min in 40% hypotonic medium containing either 0.5 mg/ml FITC–dextran (MW 10,000 Da) or 0.5 mg/ml TRITC–dextran (MW 10,000 Da). After the incubation, the cells were washed three times with ice-cold phosphate-buffered saline and fixed with 2% paraformaldehyde for 20 min. Confocal images of 512x512 pixels were constructed by summation of 15 optical sections parallel to the substratum and each 1 μm apart.

Confocal Immunofluorescence

The localization of dextran and/or Rabs in Intestine 407 cells was examined using a confocal microscope (Zeiss, LSM510META) with a 40X oil immersion objective (Axiovert 135M, Zeiss, Oberkochen,Germany).

Results

The hypotonicity-provoked anion efflux is reduced after inhibition of exocytosis

Osmotic cell swelling not only activate compensatory Cl^- and K^+ conductances, but also rapidly induces exocytosis [4]. To investigate a putative role for exocytosis in the regulation of VRAC, Intestine 407 cells were incubated with the sulfhydryl reagens N-ethylmaleimide (NEM), leading to inactivation of SNAP-25, one of the SNARE proteins involved in vesicle docking and fusion [18,27]. As shown in Figure 1, the hypotonicity-induced $^{125}I^-$ efflux activation was largely abolished in NEM treated cells, suggesting that upon osmotic stimulation additional channels are recruited and inserted into the membrane by vesicle fusion.

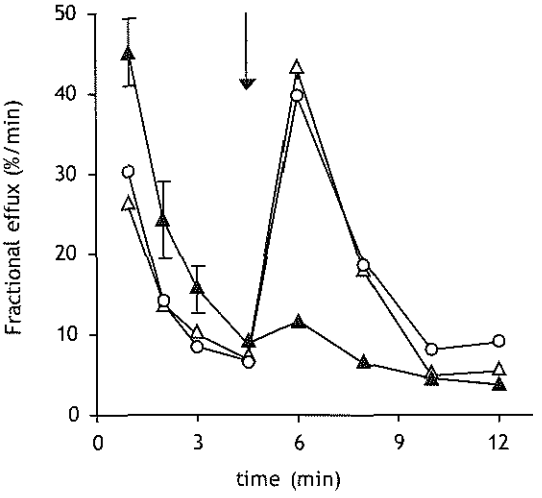

Figure 1: **Inhibition of the osmotic swelling-induced iodide efflux by N-ethylmaleimide.** Control Intestine 407 cells (open circles) and cells treated with 1 mM N-ethylmaleimide ((20 min, closed triangles) were stimulated with a 30% hypotonic medium. Addition of NEM together with the hypo-osmotic stimulus (open triangles) did not reduce the iodide efflux, indicating that NEM does not act as a direct blocker of VRAC under these conditions. Arrow indicates the shift to a 30% hypotonic medium. Data are expressed as mean ±S.E.M. for n=3. No error bars were given when the S.E.M. is smaller than the size of the symbol used.

To further substantiate our notion that recruitment of VRAC from endosomal compartments may be part of its regulation, the metalloprotease *Clostridium botulinum* neurotoxin F was introduced into Intestine 407 cells using the Chariot™ protein transfection kit. Neurotoxin F specifically cleaves the vesicle associated SNARE protein VAMP2, leading to a blockade of vesicle fusion and subsequent of exocytosis. As shown in Figure 2, the volume-sensitive anion efflux was substantially reduced in cells treated with neurotoxin F, again supporting our notion that exocytosis is involved in the development of the compensatory anion efflux (Figure 2A). Because the reduction of the volume-sensitive efflux could be due to an inhibition of regulated exocytosis or due to interference with constitutively cycling of VRAC containing vesicles, the cells were treated with neurotoxin F for various time points. As shown in Figure 2B, an equal, approximately 50% reduction of the osmo-sensitive anion efflux was observed after either 2 h or 4 h of neurotoxin F treatment, suggesting that the reduction of the cell swelling-activated anion release by neurotoxin F results from an inhibition of regulated exocytosis rather than a reduction in the rate of constitutive vesicle cycling. Prolonged treatment of the cells with neurotoxin F (20 h) only slightly further reduced the volume-sensitive anion efflux (Figure 2B). Partial inhibition of the swelling-provoked anion efflux was also observed in Brefeldin A treated cells (Figure 3).

After a distinct lag period, the swelling-provoked exocytosis in Intestine 407 cells is accompanied by an increase in the rate of endocytosis [1]. We therefore investigated the effects of modulation of endocytosis on the magnitude of the hypotonicity-induced anion efflux. Treatment of the cells with chlorpromazine, to inhibit clathrin-dependent endocytosis, only slightly affected the hypotonicity-provoked anion efflux but did significantly increase the basal, unstimulated efflux (Figure 3).

Taken together, the results suggest the existence of an intracellular and neurotoxin F-sensitive pool of VRAC in Intestine 407 cells that is rapidly recruited to the plasma membrane upon hypo-osmotic stimulation.

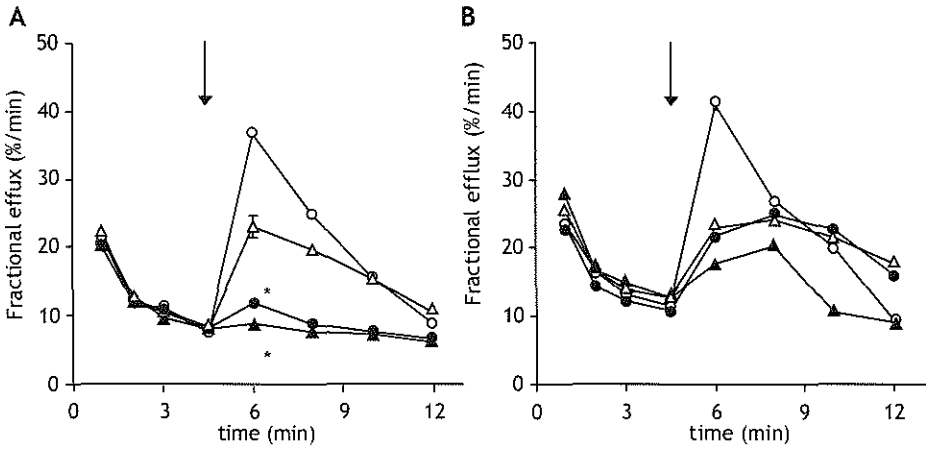

Figure 2: **Osmotic cell swelling-induced iodide efflux in control and *C. botulinum* neurotoxin F treated cells.** (A) Fractional $^{125}I^-$-efflux from control (open symbols) and neurotoxin F-treated (closed symbols) was quantitated after stimulation with a 20% (triangles) or 30% (circles) hypotonic medium. Asterisk indicates a significant decrease in the peak value of the efflux relative to the untreated control ($p < 0.05$; Student's *t*-test) (B) Hypotonicity-induced $^{125}I^-$ efflux (30% hypotonicity) from control (open circles) cultures and cultures treated with neurotoxin F for 2, 4 or 20 h (closed circles, open triangles and closed triangles, respectively). Peak values of 2, 4 and 20h were all significantly lower than the control ($p > 0.001$; Student's *t*-test). The hypotonicity-provoked iodide efflux after 2h and 4h of neurotoxin F treatment did not differ significantly (p-value: 0.25; Student's *t*-test). A small but significant decrease in the efflux between 2 an 20 h as well as between 4 h and 20 h treatment was observed (p-values: 0.004 and 0.005, respectively). Data are expressed as mean ±S.E.M. for n=3. Arrow marks the time point of shift to a hypotonic medium. No error bars were given when the S.E.M. is smaller than the size of the symbol used.

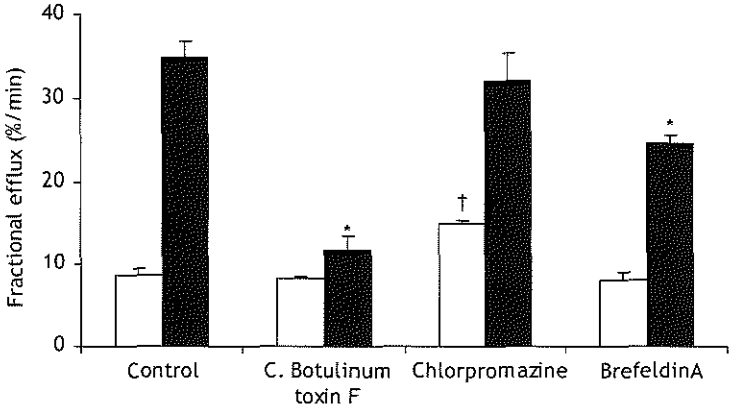

Figure 3: **Effects of pharmacological inhibition of exo- or endocytosis on the cell swelling-induced iodide efflux.** The basal (open bars) and hypotonicity-provoked efflux (solid bars) of control cells were compared with the efflux from *C. botulinum* toxin F, chlorpromazine (25 µg/ml, 1 hour) or Brefeldin A (50 µg/ml, 4 hours) treated cells. Both *C. botulinum* toxin F and Brefeldin A partially inhibited the volume sensitive anion efflux. Chlorpromazine had no effect on the anion efflux but resulted in significantly higher basal efflux. Cells were stimulated with a 30% hypotonic medium. † $p < 0.05$ relative to the efflux from unstimulated control cells; * $p < 0.05$ relative to the efflux from hypotonicity-stimulated cells.

Rab type GTPases are involved in the regulation of VRAC

Rab proteins are known to control vesicular trafficking at various stages in the endocytic cycle. Whereas Rab5 directs transport from the plasma membrane to the early endosomes, both Rab4 and Rab11 control the recycling to the cell surface [23-25]. To further persue the involvement of vesicle cycling in the regulation of the volume-sensitive anion conductance, the roles of Rab4, -5 and -11 GTPases were studied. Intestine 407 cells were transfected with plasmids encoding wild-type or dominant negative Rab4, -5 or -11 proteins fused to CFP (Rab4), DsRed (Rab5) or GFP (Rab11). Confocal immunofluorescence microscopy showed that Rab4WT, Ran5WT and Rab5DN were all predominantly concentrated in vesicular structures around the nucleus (Figure 4). Whereas Rab11WT staining was punctuate and widely dispersed throughout the cytosol, Rab11DN had a more vesicular and prenucleair localization (Figure 4). Rab4DN fluorescence in the cytoplasm, however, was rather diffuse (Figure 4).

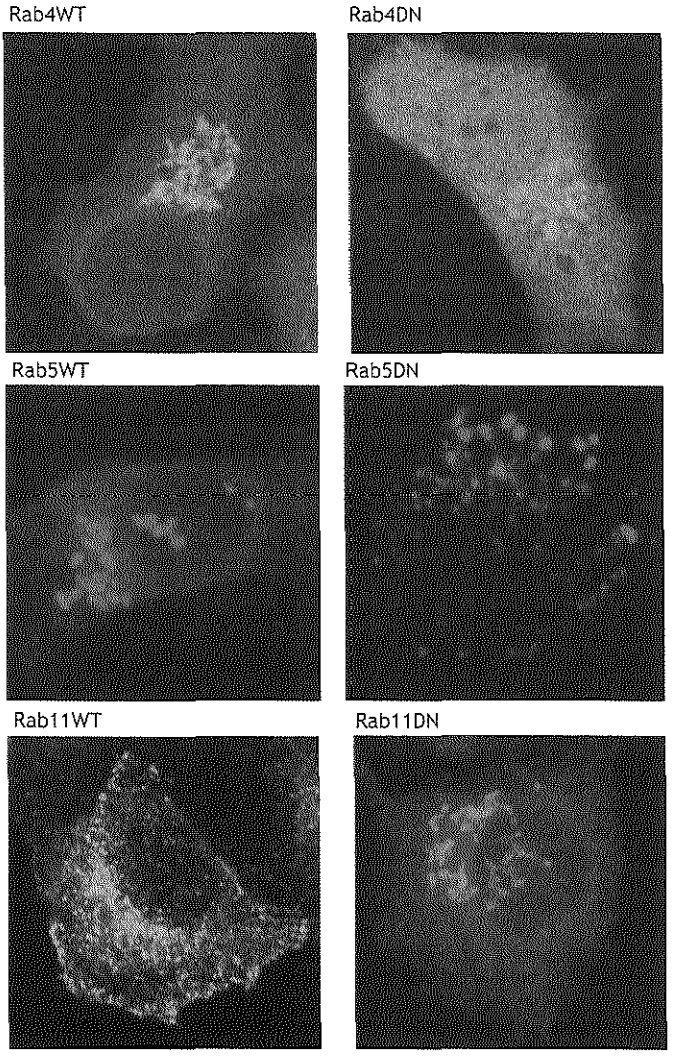

Figure 4: Cellular localization of Rab proteins. Intestine 407 cells were transfected with wildtype (WT) or dominant negative (DN) plasmids encoding CFP-tagged Rab4, DsRed-tagged Rab5 or GFP-tagged Rab11 proteins. (see page 173 for color figure)

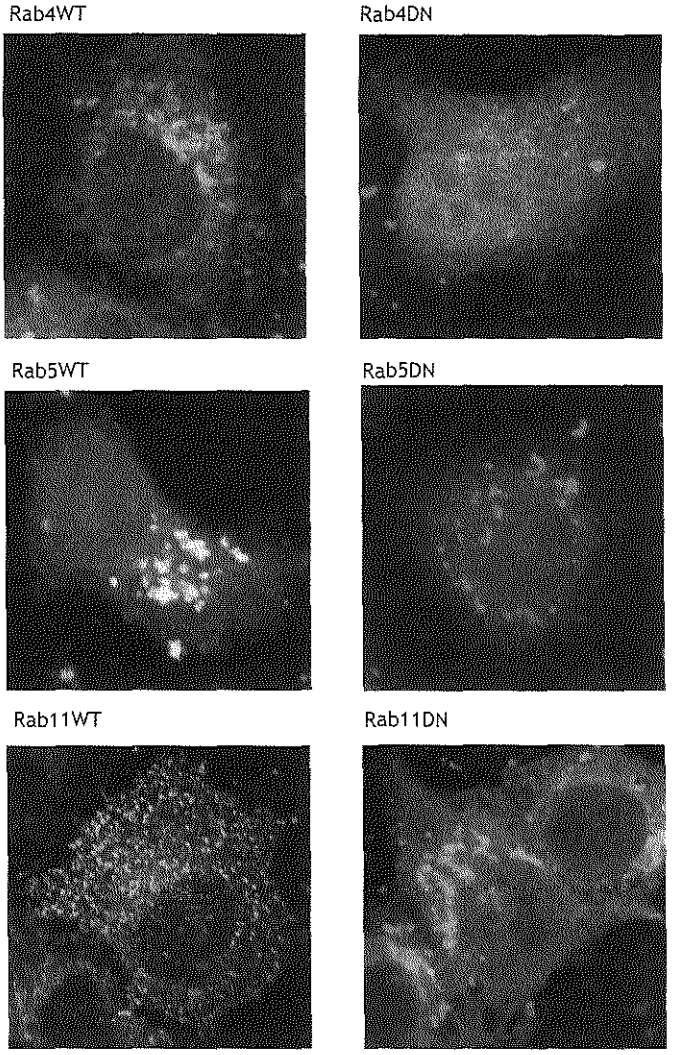

Figure 5: **Co-localization of Rab proteins and dextran.** Cells transfected with wild-type (WT) or dominant negative (DN) plasmids encoding CFP-tagged Rab4, or GFP-tagged Rab11 proteins were incubated with TRITC-labelled dextran MW 10,000 (0.5 mg/ml, 10 minutes). Cells transfected with wildtype (WT) or dominant negative (DN) plasmids encoding DsRed-tagged Rab5 proteins were incubated with FITC-labelled dextran. Only Rab5 WT was found to be localized close to dextran containing endosomes. (see page 174 for color figure)

Rab transfected cells were briefly incubated with fluorescently labeled dextrans as a marker for endocytic vesicles. A clear co-localization was observed only in cells expressing Rab5WT but not in cells transfected with Rab5DN (Figure 5). In addition, no co-localization was also absent in cells transfected with either wildtype or dominant negative Rab4 or Rab11 (Figure 5).

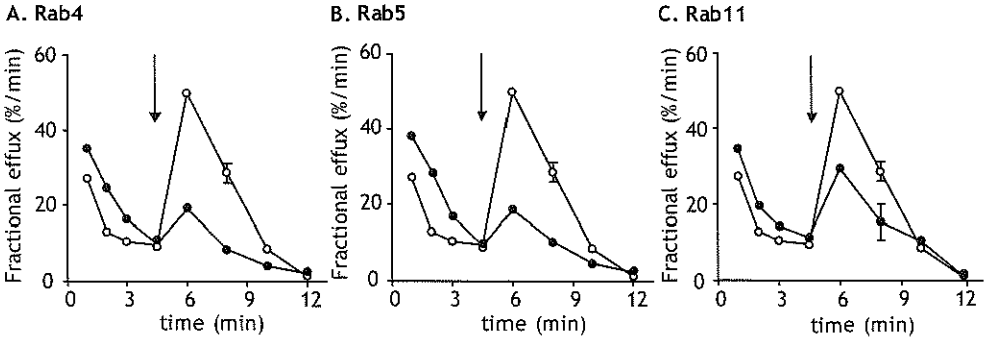

Figure 6: **Effects reduced membrane retention on the cell swelling provoked anion efflux.** Hypotonicty-provoked $^{125}I^-$- efflux from control (**A-C**; open circles) and Rab4DN-(**A**, closed circles), Rab5WT- (**B**, closed circles) and Rab11DN- (**C**; closed circles) expressing cells. Arrow indicates the shift to a 30% hypotonic medium. Data are expressed as mean ±S.E.M. for n=3. No error bars were given when the S.E.M. is smaller than the size of the symbol used.

To evaluate a functional role for Rab proteins in the regulation of VRAC, the hypotonicity-provoked anion efflux was studied in cells transfected with the different Rab encoding plasmids. In an attempt to reduce the plasma membrane expression of VRAC, Rab4DN and Rad11DN as well as Rab5WT transfected cells were used, leading to either an inhibition of Rab4 and -11 mediated exocytosis or to a stimulation of Rab5 facillitated endocytosis (Rab5WT expressing cells). As has been shown for NEM- and neurotoxin F-treated cells (Figures 1-3), inhibition of exocytosis resulted in a marked reduction of the cell swelling-induced anion efflux (Figure 6). Stimulation of endocytosis however, using Rab5WT expressing cells, also resulted in an attenuated response. Surprisingly, promoting plasma membrane retention by blocking Rab5-mediated endocytosis with Rab5DN overexpression or by stimulating exocytosis using Rab4WT or Rab11WT expressing

cells did not increase the hypotonicity-evoked $^{125}I^-$ efflux but instead resulted in a somewhat diminished response in Rab5DN and Rab11WT cells (Figure 7). This reduction in the magnitude of the cell swelling-induced efflux was accompanied by an increase in the basal, unstimulated efflux (Figure 7).

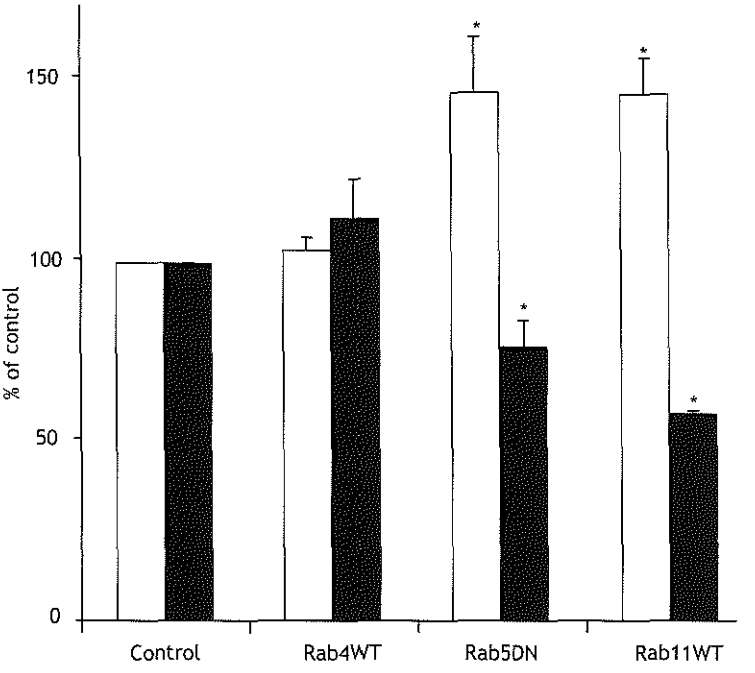

Figure 7: **Effects of increased membrane retention on the cell swelling provoked anion efflux.** Basal (open bars) and hypotonicity-provoked (solid bars) $^{125}I^-$–efflux from control, Rab4WT, Rab5DN and Rab11WT expressing cells. Cells were stimulated with a 30% hypotonic medium. Both basal and stimulated efflux are expressed as percentage relative to the fractional efflux of the control (mean ±S.E.M., n=3). Asteriks indicates a significant difference from ($p < 0.05$) the control.

Discussion

Osmotic swelling of Intestine 407 cells not only rapidly activates the release of osmolytes but was also found to stimulate exocytosis as well as endocytosis. In this study, we demonstrate that inhibition of exocytosis leads to a reduced efflux of anions, suggesting that at least part of the cell swelling-activated Cl^- channels are localized intracellularly and are recruited to the plasma membrane upon hypo-osmotic stimulation.

Involvement of vesicle cycling in the regulation of plasma membrane expression of chloride channels is not unprecedented. Recent evidence indicates that CFTR chloride channels undergo rapid endocytosis through a mechanism involving clathrin-coated vesicles [28-30]. Using dominant-negative mutants of Rme-1, a protein involved in endosomal cycling, it has been demonstrated that at least part of the internalized CFTR escapes from lysosomal degradation and recycles back to the plasma membrane [31]. Endocytotic trafficking of CFTR is regulated by several members of the Rab family of GTPases, including Rab5, which promotes channel endocytosis, Rab7, directing its movement to late endosomes and lysosomes, and Rab11, leading to recycling of the channels to the plasma membrane [21].

In Intestine 407 cells, inhibition of exocytosis, by treating the cells with agents that interfere with vesicle docking and fusion (NEM or neurotoxin F) or by transfection with dominant negative Rab4 or Rab11 to inhibit vesicle movement to the plasma membrane, resulted in a marked inhibition of the cell swelling-activated anion efflux (Figures 1, 2a and 6 respectively). Promoting exocytosis however, using cells overexpressing wild-type Rab4 or Rab11, did not potentiate the hypotonicity-induced anion efflux (Figure 7). In contrast, a moderate reduction of the response was observed in Rab11WT transfected cells (Figure 7). This reduction in the volume-sensitive efflux was accompanied by an increase in the anion permeability under basal, unstimulated conditions (Figure 7). A similar increase in the basal efflux was observed after inhibition of endocytosis using chlorpromazine or transfection with Rab5DN (Figures 3 and 7 respectively). These experiments suggest a redistribution of VRAC from intracellular compartments to the plasma membrane under these conditions. Notably, because the basal conductance was found to be increased, the results also suggest that freshly recruited VRAC is in an activated state.

The inhibition of the osmo-sensitive anion efflux observed in Rab4DN-, Rab5WT- or Rab11-DN overexpressing cells as well as in cells treated with neurotoxin F was incomplete. Although we cannot exclude that this is due at least in part to a suboptimal transfection efficiency, the observation that a chemical compound NEM reduced the response to an osmoshock by at most 60% also, suggests the existence of two distinct populations of channels, a neurotoxin-sensitive intracellular pool and a population of VRACs residing in the plasma membrane. Because prolonged treatment of Intestine 407 cells (20 h) with neurotoxin F did hardly reduce the swelling-induced efflux any further, it is apparent that the rate of constitutive recycling of VRAC containing vesicles is relatively slow in comparison with other chloride channels including CFTR [28-30].

In several model systems, an increase in intracellular free Ca^{2+} is able to trigger or modulate the activity of the transmembrane chloride conductance [32-36]. Indeed, although Intestine 407 cells do not express Ca^{2+}-sensitive anion channels, the hypotonicity-provoked anion efflux was found to be robustly potentiated by Ca^{2+}-mobilizing hormones such as bradykinin, histamine and ATP [37]. It has been demonstrated that a rise in $[Ca^{2+}]_i$ could promote exocytosis in Intestine 407 cells [1,38]. Therefore, it is tempting to speculate that exocytosis and VRAC recruitment in addition to direct effects on channel gating, underlies the mechanism involved in the potentiation of the osmo-sensitive conductance by Ca^{2+}-mobilizing hormones in Intestine 407 cells. This notion is supported by the observations that Ca^{2+} chelation did not dramatically affect the hypotonicity-induced anion efflux by itself, but strongly reduced exocytosis and the hormone-induced potentiation [1,37]. Whereas the hypotonicity-provoked $^{125}I^-$ efflux was reduced in BAPTA-AM loaded cells, it should be noted that an increase in $[Ca^{2+}]_i$ by itself was unable to activate VRAC [37]. This indicates that besides channel recruitment, an additional signal, for instance reduced ionic strength or macromolecular crowding (for review see [39]), is required.

Taken together, our results suggest that recruitment of volume-sensitive anion channels contribute to the development of the RVD response. This also provides a plausible explanation for our previous finding [37], that the Ca^{2+}-mobilizing hormones are able to potentiate the volume-sensitive anion conductance in Intestine 407 cells that do not express I_{ClCa}. To date, the molecular identity of VRAC has not yet been established. In the light of our finding of cell swelling-induced VRAC recycling it is tempting to postulate a major role for predominantly intracellularly localized anion channels as putative molecular candidates for VRAC.

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Chapter 6

Expression of putative chloride channel proteins in Intestine 407 cells

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Abstract

Intestine 407 cells lack the expression of chloride conductances other than the cell swelling-activated anion conductance. In the search for a molecular candidate for the Volume-Regulated Anion Channel (VRAC), we selected these cells for studying the expression of proteins that have recently been recognized as putative chloride channels. From RT-PCR studies the expression of bestrophin (hBest1), the human homologue of *tweety* (TTYH3), and the primarily intracellularly localized chloride channels ICln, CLIC1, CLIC2 and CLIC4 were demonstrated.

Introduction

Chloride is by far the most abundant anion in animals and its transmembrane movement is facilitated by a variety of Cl--selective channels. Cl- channels are regulated by such divers mechanisms as direct ligand-induced gating, activation by changes in the membrane potential or in cell volume, increases in the intracellular concentrations of Ca^{2+} or cyclic nucleotides as well as through phosphorylation of the channel protein itself. The physiological role of chloride channels is not limited to the regulation of cellular excitability, transepithelial salt and water transport, (vesicular) pH and cell volume. It is likely that these channels are involved also in more general biological processes such as cell growth, apoptosis and cell migration [1-4].

Using the whole cell patch clamp technique, the biophysical and pharmacological characteristics of the Volume-Regulated Anion Channel (VRAC) have been extensively studied and were found to be very similar in many different model cells used [3,5]. Its molecular identity however still remains to be elucidated. Several candidates have been proposed, however, none of them meets all the electrical and pharmacological criteria [4-6]. Especially CLC-3 has been put forward as the molecular equivalent of VRAC [7-9], mostly because transfection of HeLa cells with CLC-3 antisense DNA was found not only to reduce both the cell swelling-activated anion current but also to abolish the RVD response [10]. Although CLC3 resembles VRAC in many of its characteristics, some of its properties, including protein kinase C regulation, channel rectification and inhibitor sensitivity, were found to be at variance with VRAC [11-15]. Recent studies however, have clearly demonstrated that the volume-sensitive chloride conductance is still present in Clcn3 -/- mice lacking CLC3, arguing against the crucial involvement of CLC3 in cell volume regulation [16,17].

Recently, we provided evidence for the existence of a pool of intracellular localized VRACs that is recruited to the plasma membrane upon hypo-osmotic stimulation (e.g. see Chapter 5). Recruitment from intracellular compartments is not unprecedented, ICln, a ubiquitously expressed putative chloride channel protein, has been found in ealier studies to translocate from the cytosol to the plasma membrane upon hypo-osmotic stimulation. [18]. Intestine 407 cells do not express voltage-, calcium-, cAMP/protein kinase A (CFTR)-activated anion channels in the plasma membrane [19]. Instead, only the volume-sensitive chloride conductance has been observed. This property renders this cell line an

excellent model for studies towards the elucidation of the molecular identity of VRAC. In this study, we investigated the expression of several newly recognized chloride channels in Intestine 407 cells.

Material and methods

Materials

Primers for ICln were kindly donated by Dr. M.Paulmichl (Paracelcus University, Salzburg). CLIC primers and siRNAs were generous gifts from Dr. B.Ponsioen and Dr. W.H.Moolenaar (NKI, Amsterdam). Primers for hTTY1-3 and hBest1 were designed using the published sequences from the human gene bank at the National Center for Biotechnology Information (NCBI). Primer sites were selected around intron splice sites.

Cell culture

Intestine 407 cells were routinely grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM N-2-hydroxy-ethylpiperaz ine-N'-2-ethanesulfonic acid (Hepes), 10% fetal calf serum, 1% non-essential amino acids, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O2 and 5% CO2 at 37°C.

RNA isolation, amplification, and analysis

Total cellular RNA was isolated from Intestine 407 cells using an Ultraspec® total RNA isolation kit (Biotecx, Houston, TX, USA) according to the protocol provided by the manufacturer. First-strand cDNA synthesis was performed from 1 µg of total RNA using Promega M-MLV reverse transcriptase using the manufacturer's protocol and reaction buffers (Promega Benelux BV, Leiden, the Netherlands). The reaction was inactivated by raising the temperature to 70°C for 5 minutes, followed by the addition of 5 µg RNAse and 15 min. incubation at 37°C. PCR reactions were performed in a total volume of 25 µl containing 0.4 units of REDTaq DNA polymerase (Sigma, St. Louis, Mi), approx. 10 ng of total cDNA, 1X REDTaq PCR buffer, 2 mM MgCl2, 0.2 mM dNTPs, and 0.2 µM of each primer. The PCR program consisted of 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C, 40 seconds at 72°C and a final extension at 72°C for 10 minutes. The PCR products were subsequently analyzed on 1% agarose gel containing 0.2 µgml-1 etidium bromide.

Results and discussion

Expression of tweety homologues, bestrophin, ICln and CLICs in Intestine 407 cells

Expression of the recently recognized putative chloride channels was studied in Intestine 407 cells by RT-PCR. These channels include ICln, bestrophin (hBest1) and human homoloques of the *Drosophila* tweety (hTTYH1-3) as well as members of the CLIC family (CLIC1-6).

Tweety (dTTY1), a gene located in *Drosophila flightless* locus, is required for normal flight and is both structurally and functionally related to large conductance chloride channels [20]. The three human homologues of *tweety* (hTTYH1-3) possess 5 or 6 transmembrane segments and have been recognized as novel maxi Cl^- channels. Whereas both hTTYH2 and 3 were identified as Ca^{2+}-activated Cl^- channels by whole cell patch clamping, hTTYH1 was found to encode for a Ca^{2+}-independent and swelling-activated anion channel [20], making this channel a potential VRAC candidate. As clearly shown in Figure 1, only hTTYH3 expression was observed in Intestine 407 cells, which is remarkable because these cells do not express Ca^{2+}-activated Cl^--channels in their plasma membrane [19]. The absence of a detectable hTTYH1 expression however, makes it very unlikely that hTTYH1 channels are the molecular basis of the robust Cl^-conductance triggered by osmotic cell swelling in Intestine 407 cells.

Bestrophins belong to a family of proteins that consist of four transmembrane spanning domains and may form multimeric chloride channels that are inhibited by SH-specific reagents [21]. Furthermore, some members of the bestrophin family are sensitive to intracellular calcium and may represent Ca^{2+}-sensitive anion channels. Although bestrophins are recognized as novel Cl^--channels, little is as yet known about their physiological roles. Mutations in hBest1 result in a juvenile form of macular degeneration (Best disease) and channel disfunction, the relationship between the observed pathology and the altered ion channel activity however remains to be established [21,22]. In hBest1 transfected HEK cells, the volume-sensitive anion current was found to be dramatically increased, suggesting that hBest1 could serve as a molecular candidate for VRAC [23]. Expression of hBest1 was observed in Intestine 407 cells (Figure 2). Because of its apparent Ca^{2+}-sensitivity however, it seems unlikely that this channel may represent VRAC in these cells.

Both ICln and members of the CLIC family of anion channels are primarily localized in the cytosol but may translocate to the plasma membrane upon activation. Expression of ICln in *Xenopus* oocytes resulted in an outwardly rectifying anion current which closely resembles VRAC [24]. ICln interact with several other proteins and may serve as a "connector hub". In addition to its putative function as a Cl^- channel, ICln may be involved in several other regulatory pathways include regulation of cell morphology, angiogenesis and RNA processing (for review see: [25]). Like many other cell types, ICln is expressed in Intestine 407 cells (Figure 2). Because of the observed lethality of genetic knockdown of ICln in cell lines, *C. elegans* and mice it is difficult to investigate its direct involvement in cell volume regulation [26].

CLIC (chloride intracellular channel) proteins belong to a family of chloride channels whose physiological roles are yet unclear. The CLIC proteins have little homology to other chloride channels and exist in both a soluble cytosolic and a membrane-inserted form [27,28]. The proteins have some homology to glutathione S-transferases, suggesting that, like ICln, they may have additional cellular functions [29]. The CLIC homologue EXC-4 has been found to be critically involved in the formation of the excretory canal in *C. elegans*, which consists of a single elongated cell with an intracellular tube [30]. Loss of EXC-4 function results in the disruption of the tubular structure of the lumen by the formation of cystic enlargements, perhaps as a consequence of excessive swelling [30]. In Intestine 407 cells, expression of CLIC1, -2 and -4, but hardly any CLIC3, -5 and -6, was detected by RT-PCR (Figure 3).

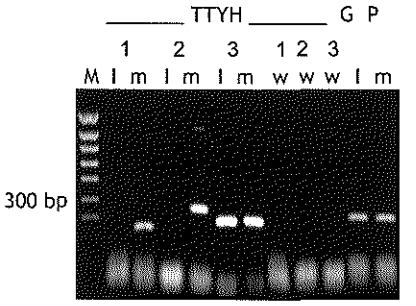

Figure 1: **Expression of the human homologues of the Drosophila tweety in human Intestine 407 cells and mouse brain.** mRNA expression of hTTY1, hTTY2 and hTTY3 in Intestine 407 cells (I) and in mouse brain (m). GAPDH (G) was used as a reference. All bands have the predicted length (M=marker).

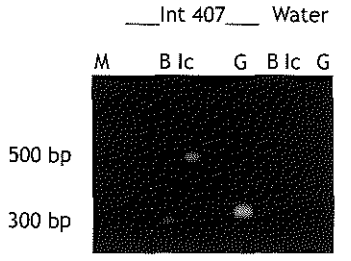

Figure 2: **Expression of bestrophin and ICln in Intestine 407 cells.** Expression of hBest1 (B) and ICln(IC) in Intestine 407 cells was determined by RT-PCR. GAPDH (G) was used as a reference.

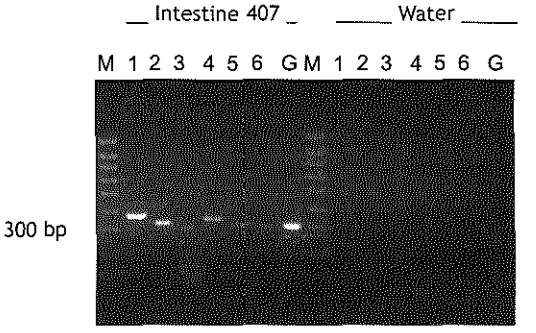

Figure 3: **Expression of members of the CLIC family of chloride channels in Intestine 407 cells.** Expression of CLIC1-6 in Intestine 407 cells. GAPDH (G) was used as a reference.

Conclusions

Although Intestine 407 cells lack most of the well established "classical" anion conductances, they do express several proteins that have recently been recognized as potential chloride channels. Especially members of the CLIC family, which are primarily localized intracellularly but are able to translocate to the plasma membrane, may serve as potential VRAC candidates. This notion is supported by our preliminary observation that the cell swelling-activated iodide efflux is reduced in cells treated with CLIC1/4 siRNAs (unpublished results).

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Chapter 7

Aquaporins are Required for the Hypotonic Activation of Osmolyte Release Pathways in Intestine 407 Cells

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Abstract

Osmotic swelling of human Intestine 407 epithelial cells rapidly activates the efflux of chloride and potassium ions as well as the extracellular release of organic osmolytes. Activation of these compensatory effluxes was largely abolished in cells treated with bivalent mercury ions. Surprisingly, hyposmotic activation of the Volume-Regulated Anion Channel (VRAC) under whole cell patch clamp conditions was only slightly affected by intra- or extracellular Hg^{2+}, suggesting the involvement of Hg^{2+}-sensitive aquaporins (AQPs) in osmotic cell swelling and/or the subsequent regulatory volume decrease (RVD). To further substantiate a role for AQPs during cell volume regulation, cells with reduced protein levels of one or more of the AQPs expressed (AQP1, 3 and 7 in our clone of Intestine 407 cells) were generated using a siRNA-based gene silencing technique. Whereas a knockdown of either AQP1, 3 or 7 resulted in a partial inhibition of the hypotonicity-provoked iodide, rubidium and taurine efflux, knockdown of all three AQPs resulted in an almost complete inhibition. AQP knockdown also markedly reduced the rate but not the extent of osmotic cell swelling and almost completely prevented the RVD response. In addition, the rapid and transient formation of membrane protuberances upon osmotic swelling of Intestine 407 cells was absent in AQP knockdown cells. The results not only indicate that the hypotonicity-evoked fast increase in cell volume as well as the formation of protuberances are facilitated by a rapid influx of water through AQPs, but also suggest that the rate of cell swelling is a critical determinant for the activation of the compensatory osmolyte effluxes and for the initiation of the RVD response in intact cells.

Introduction

Because of the relatively high water permeability of the mammalian plasma membrane, variations in the concentrations of osmotically active intracellular substances or in the osmolarity of the surrounding fluid will immediately lead to a redistribution of water. As a result, cells are continuously prone to changes in their volume. Because alterations in cell size may potentially be hazardous, almost all cell types have developed efficient mechanisms to maintain their volume within a certain range [1,2]. Upon osmotic cell swelling, Cl^--and K^+-selective channels are activated rapidly, leading to the net efflux of KCl. [1-3]. In addition, an organic osmolyte release pathway is activated, resulting in the release of small organic solutes such as betaine and taurine [4]. In Intestine 407 cells, the efflux of organic osmolytes was activated only after a distinct lag time of ~30-60 s and lasted for approximately least 8-12 min [5]. Subsequent to the release of these osmolytes, water will leave the cell and its original cell volume will be restored, a mechanism commonly referred to as regulatory volume decrease (RVD). Despite its ubiquitous expression and its marked biophysical characteristics, the molecular identity of the anion channel activated during the RVD (designated Volume-Regulated Anion Channels or VRAC) has not yet been elucidated. Several candidates have been proposed, but none of them comply with all the electrical and pharmacological criteria [6,7]. The K^+ conductance involved in the RVD response in Intestine 407 cells however, has been identified as a Ca^{2+}-dependent intermediate K^+ channel [8].

Water can move across the plasma membrane by diffusion through the lipid bilayer, as ion hydratation through ion channels [9] or through specific "water channels" or aquaporins (AQPs). The AQPs are a large family of integral membrane proteins widely expressed in prokaryotes, yeast, animals and plants [10,11]. At least 11 isoforms have been identified in mammalian tissues. AQPs are highly expressed in water-transporting organs such as the kidney, as well as in numerous other tissues and cell types including the choroid plexus, the eye lens, endothelium and erythrocytes [12-14]. In the gastro-intestinal tract, a role for AQPs in fat malabsorbtion, intrahepatic bile production, pancreatic secretion and saliva production has been proposed [13]. Whereas many mammalian AQPs are exclusively permeable to water, others (AQP3, 6, 7, 9, and 10) are also able to transport small polar molecules including glycerol and urea [15-19]. Interestingly, AQP6 was reported to conduct anions as well [20]. Recently, Kida et al. [21]

reported that functional AQP3 channels are essential for the RVD-driven water release from Intestine 407 cells after osmotic cell swelling [21]. These results prompted us to investigate the possible relationship(s) between AQPs and the regulation of the compensatory osmolyte fluxes. In line with the observations by Kida et al. [21], we found that the RVD response is impaired in cells lacking functional AQPs. The results also demonstrate, however, that all three osmolyte release pathways (i.e. for Cl^-, K^+ and taurine) are almost fully inhibited after AQP knockdown in intact cells grown as monolayers. In addition, the rate of osmotic swelling was dramatically reduced in cells lacking AQPs. Taken together, the data suggest that a fast increase in cell volume is a prerequisite for the activation of these compensatory fluxes in Intestine 407 cells. The results therefore indicate that knockdown of AQPs prevents the RVD response by the failure of the cell to activate the osmolyte release pathways but not by abolishing its capacity to accumulate water.

Methods

Materials

Radioisotopes (^{3}H-Taurine, $^{125}I^-$, $^{86}Rb^-$ and $^{36}Cl^-$) were purchased from GE Healthcare Netherland. (Zeist, the Netherlands). Enhanced chemiluminescence (ECL) Detection kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Intestine 407 cells were routinely grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM Hepes, 10% fetal calf serum, 1% non-essential amino acids, 40 mgl^{-1} penicillin and 90 mgl^{-1} streptomycin under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C.

RNA isolation, amplification, and analysis

Total cellular RNA was isolated from Intestine 407 cells using an Ultraspec® total RNA isolation kit (Biotecx, Houston, TX, USA) according to the protocol provided by the manufacturer. First-strand cDNA synthesis was performed from 1 µg of total RNA using Promega M-MLV reverse transcriptase using the manufacturer's protocol and reaction buffers (Promega Benelux BV, Leiden, the Netherlands).

The reaction was inactivated by raising the temperature to 70°C for 5 minutes, followed by the addition of 5 µg RNAse and 15 min. incubation at 37°C.

AQP PCR primers were designed using the published sequences from the human gene bank at the National Center for Biotechnology Information (NCBI). Primer sites were selected around intron splice sites. The amplifications were performed using the primer sets listed in Table 1.

PCR reactions were performed in a total volume of 25 µl containing 0.4 units of REDTaq DNA polymerase (Sigma, St. Louis, Mi), approx. 10 ng of total cDNA, 1X REDTaq PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.2 µM of each primer. The PCR program consisted of 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C, 40 seconds at 72°C and a final extension at 72°C for 10 minutes. The PCR products were subsequently analyzed on 1% agarose gel containing 0.2 $\mu g ml^{-1}$ etidium bromide. The sequence was verified using dye termination sequencing.

AQP gene silencing

Human AQP mRNA sequences were obtained from the NCBI website. Single-stranded small interfering RNA (siRNA) oligonucleotides were designed using the Ambion online tool for siRNA design (http://www.ambion.com/techlib/misc/siRNA_finder.html) and checked for significant homology with stretches from other human genes by performing a BLAST search at www.ncbi.nlm.nih.gov/BLAST. Two complementary DNA oligonucleotides containing the selected sequence, a hairpin sequence and the reverse complement of the selected sequence, were designed, annealed and ligated into the pSilencer 3.1-HI hygro vector (Ambion, Austin, Texas, USA). Intestine 407 cells were transfected with the siRNA plasmids using Lipofectamin Plus (Invitrogen, Breda, the Netherlands). A list of sense and anti-sense primer sets is presented in Table 2.

Table 1: List of primer sequences

Primer	Product size bp (base pair)	Primer sequence
AQP0	394	5'-TGTCTTCTTTGGGCTGGGGTCCTC-3' 5'-ATTCCGCCTCTCGTCGTATGT-3'
AQP1	283	5'-CCCTGGGCTTCAAATACC-3' 5'-CGGCCAAGCGAGTTCCCAGTCAG-3'
AQP2	386	5'-TCCATGAGATCACGCCAGCAGA-3' 5'-GCGAGCGGATTCAGCCATTACC-3'
AQP3	381	5'-CCTTTGGCTTTGCTGTCACTCTGG-3' 5'-CTCGGGGGACGGGGTTGTTGTAAG-3'
AQP4	244	5'-TTCTGGCCATGCTTATTTTTGTTC-3' 5'-CACTGGGCTGCGATGTAGA-3'
AQP5	406	5'-CGCTGCTCCGGGCTTTCTTCTACG-3' 5'-GCAGCCAGGACCGCCCCCACGAT-3'
AQP6	444	5'-ACGGTGGGGGCTGCTCTGCTTTAT-3' 5'-TGCCCCTGTCCCCACCTCTA-3'
AQP7	448	5'-CTACACGGCCATTCTCCACTTTTC-3' 5'-ACCCCGATGATGACCACGAGGATG-3'
AQP8	299	5'-GTGGGTGGCAGGTGGCGAGTGT-3' 5'-CCCCCGAGCAGCTGTGAGACC-3'
AQP9	357	5'-AACTGCTGATCGTGGGAGAAAATG-3' 5'-CAACCAAAGGGCCCACTACAGGAA-3'
AQP10	449	5'-GGTCAAGCTCCCCATTTACATC-3'5'- GGAGGCCAGCACCAGGAGAGTC-3'

AQP PCR primers were designed based on the published sequences from the human gene bank in the National Centre for Biotechnology Information (NCBI) by comparing chromosomal DNA with cDNA. Primer sites were selected around intron splice sites.

Efflux assay

Monolayer of cells were loaded for 2 h with the isotope (5μCiml-1 $^{125}I^-$, 0.5 μCiml^{-1} $^{86}Rb^+$, 5 μCiml^{-1} $^{36}Cl^-$ or 0.1 μCiml^{-1} ^{3}H-taurine). Prior to the assay, the cells were washed 3 times with isotonic buffer (80 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$,

1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM HEPES, pH 7.5). Isotope efflux was determined at 37°C by replacing the medium at 1-2 minute intervals. Hypotonic media were prepared by adjusting the concentration of mannitol. Radioactivity in the medium was determined by γ- ($^{125}I^-$ and $^{86}Rb^+$) or β-(^3H and $^{36}Cl^-$) radiation counting. Data were expressed as fractional efflux per minute.

Table 2: siRNA sequence

siRNA	Sequence
Scram	GATCCCGTCTCTGCTTCACTGGTTGATTCAAGAGATCAACCAGTGAAGCAGAGATTTTTTGGAAA AGCTTTTCCAAAAAATCTCTGCTTCACTGGTTGATCTCTTGAATCAACCAGTGAAGCAGAGACCG
AQP1	GATCCCGAAGAAGCTCTTCTGGAGGTTCAAGAGACCTCCAGAAGAGCTTCTTCTTTTTTGGAAA AGCTTTTCCAAAAAAGAAGAAGCTCTTCTGGAGGTCTCTTGAACCTCCAGAAGAGCTTCTTCGG
AQP3	GATCCCGTGGCTTCTTTGACCAGTTCTTCAAGAGAGAACTGGTCAAAGAAGCCATTTTTTGGAAA AGCTTTTCCAAAAAATGGCTTCTTTGACCAGTTCTCTCTTGAAGAACTGGTCAAAGAAGCCACGG
AQP7	GATCCCGATGGGATCTCATGAACCCTTCAAGAGAGGGTTCATGAGATCCCATCTTTTTGGAAA AGCTTTTCCAAAAAGATGGGATCTCATGAACCCTCTCTTGAAGGGTTCATGAGATCCCATCGG

Human AQP mRNA sequence was obtained from the NCBI webside. Single-stranded siRNA (small interference RNA) oligonucleotides were designed according to the Ambion online tool for primer design.

BLAST (Basic Local Alignment Search Tool) search was conducted to confirm that the sequences are not consistent with other gene sequences.

Scram = Scrambled construct AQP3

Western blot

The cells were washed three times and lyzed in Laemli-sample buffer. Equal amounts of protein was loaded, separated electrophoretically by SDS-PAGE and subsequently transferred to a nitrocellulose membrane. The membrane was blocked with 3% non-fat dried milk in Tris-Tween-buffered saline (TTBS) for two hours. AQP1 and AQP7 antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA), anti-AQP3 antibody was obtained from Santa Cruz Biotechnology Inc. (SantaCruz, CA, USA). Immunoreactivity was detected using horseradish peroxidase- (HRP) coupled secondary antibodies and an Enhanced Chemiluminescence (ECL) Detection kit.

Time-lapse video imaging

Intestine 407 cells were transfected with siRNA at 50% confluency and used in the experiments after 48 hours of the transfection. Images were acquired every second for 4 minutes using AnalySIS imaging software (Soft Imaging System, Munster, Germany) and an inverted Olympus IX50 microscope.

Results

Inhibition of the cell-swelling provoked osmolyte effluxes by bivalent mercury ions

Hypo-osmotic stimulation of Intestine 407 cells results in the immediate opening of Cl^- and K^+ selective ion channels and the activation of an organic osmolyte release pathway. Treatment of the cells with $HgCl_2$ (10 µM) not only inhibited the cell swelling-induced $^{36}Cl^-$ efflux almost completely (Figure 1 A), but also abolished the hypotonicity-provoked release of organic osmolytes (Figure 1B). In contrast however, the cell swelling activated chloride current, as determined by using the whole cell patch clamp technique, was only slightly affected by the presence of $HgCl_2$ (Figure 2). Previously, we have demonstrated that the cell swelling-induced efflux of Cl^-, K^+ and organic osmolytes is regulated independently and involves different release pathways [5]. Because it is unlikely that low concentrations of $HgCl_2$ affect these pathways similarly, we postulated that mercury-sensitive AQPs might serve as potential targets. This notion is supported by our observation that the hypotonicity-provoked efflux of $^{36}Cl^-$ is no longer inhibited when $HgCl_2$ was added after the initial phase of cell swelling (i.e. 30 s after the onset of hypotonic stimulation, results not shown).

Expression of AQPs in Intestine 407 cells

To further evaluate a potential role for mercury-sensitive AQPs during osmotic cell swelling and the subsequent RVD response, expression of AQP0 - 10 was studied by RT-PCR. As shown in Figure 3, amplified fragments of the predicted length were found for AQP1, 3 and 7 in all experiments (n=5) performed, the highest expression level being observed for AQP1 and 3. In 2 out of 5 experiments, an additional low expression of AQP4 was also observed. No bands were ever observed when primers for AQP0, 2, 5, 6 and 8-10 were used. Expression of AQP1, 3 and 7 was confirmed by excising the corresponding band from the gel followed by DNA sequencing.

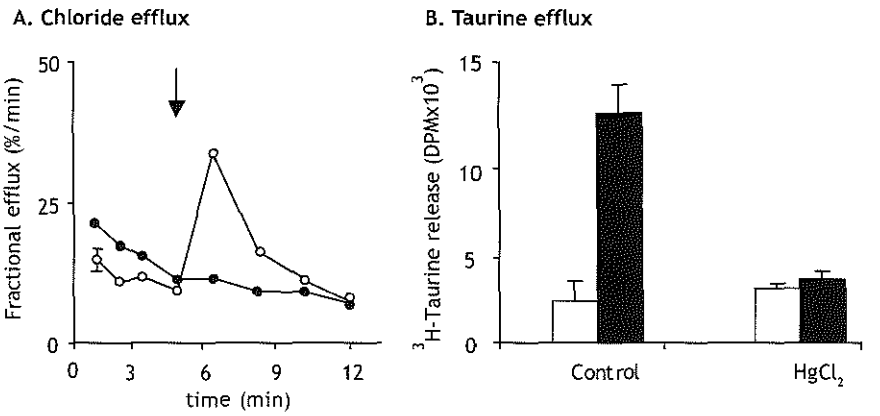

Figure 1: **Inhibition of the cell swelling-provoked chloride and taurine efflux by bivalent mercury ions.** A) Hypotonicity-provoked $^{36}Cl^-$ efflux in the absence or presence of $HgCl_2$. Open circles represent control and closed circles $HgCl_2$-treated (10 µM, 10 min) cultures. Arrow indicates the shift to a 30% hypotonic medium. B) Cell swelling-induced ^{3}[H]-taurine release. Open bars represent the efflux under isotonic, solid bars under hypo-osmotic (30% hypotonic) conditions. Data are expressed as mean ±SEM for n=3. No error bar is given when the SEM is smaller than the size of the symbol used.

AQP gene silencing

Cells with a reduced expression of AQP1, 3 and/or 7 were generated using a siRNA-based gene-silencing technique. Two days after transfection of Intestine 407 cells with siRNAs targeting either AQP1, 3 or 7, mRNA was isolated and AQP expression was analyzed by means of RT-PCR as described under "Materials and methods". As compared to control cells, AQP1, 3 and 7 mRNA expression was strongly reduced in siRNA treated cells (Figure 4A). In addition, expression of AQP1, 3 and 7 proteins was also markedly reduced in AQP-knockdown cells (Figure 4B). As shown in Fig 4B, siRNA treatment reduced both the nonglycosylated and the glycosylated forms of AQP1, 3 and 7 [22,23].

Reduced hypotonicity-provoked effluxes in AQP knockdown cells

To investigate a putative role for AQPs in the activation of hypotonicity-induced efflux of osmolytes, the cell swelling-provoked efflux of $^{125}I^-$ and $^{86}Rb^+$ were determined in control and AQP-knockdown cells. As compared to control cells, both the $^{125}I^-$ and $^{86}Rb^+$ efflux were markedly reduced after AQP1, 3 or 7 knockdown (Figure 5A, B), the $^{86}Rb^+$ efflux being slightly less affected than the $^{125}I^-$ efflux. In addition, because the hypotonicity-provoked release of organic

osmolytes was also found to be sensitive to $HgCl_2$ (see Figure 1), the efflux of taurine was studied in cells treated with AQP3 siRNAs. Like the release of iodide and rubidium, the taurine efflux was also inhibited after AQP3 knockdown (Figure 5C).

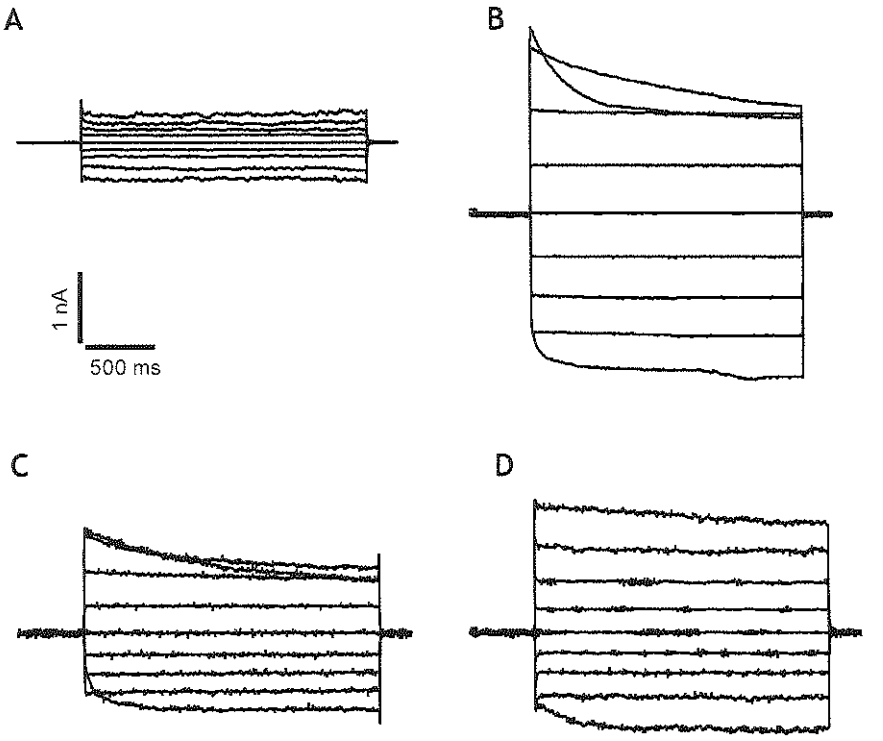

Figure 2: **The cell swelling-activated chloride current was only slightly affected by bivalent mercury ions under whole cell patch clamp condition.** Anion currents in response to step pulses from -100 mV to +100 mV (25 mV increments) from control cells (**A**, **B**) and cells exposed to extracellular (**C**; 10 minutes preincubation) or intracellular (**D**) $HgCl_2$ (10 μM). The cell swelling-induced anion conductance was provoked by a hyperosmotic (450m Osm) pipette solution (**B-D**). **A**: Isotonic control. Traces are representative of at least 5 independent experiments.

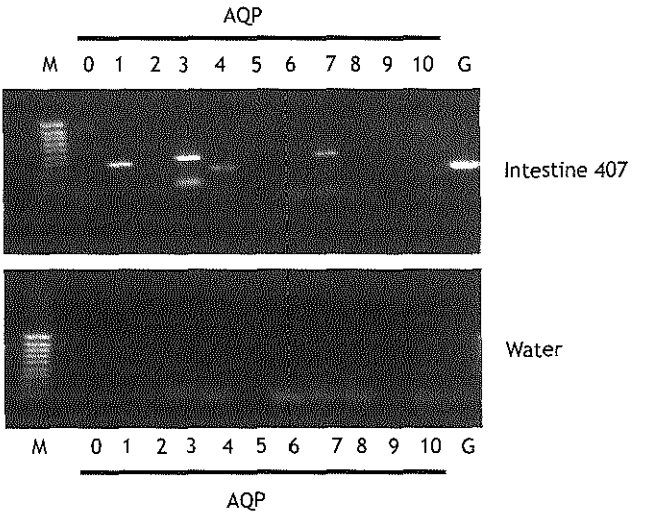

Figure 3: **Expression of AQPs in Intestine 407 cells.** Aquaporin 0-10 mRNA expression in Intestine 407 cells. The RT-PCR was repeated several times with different batches of cells and the products were checked by excising the band from the gel and sequencing. M = marker. G = GAPDH expression.

Notably, the efflux of $^{125}I^-$ and $^{86}Rb^+$ triggered by a hypo-osmotic medium were partly inhibited in cells with the reduced expression of AQP1, 3 or 7. To investigate whether a reduction of the expession of multiple AQP species could further decrease the osmo-sensitive anion efflux, double and a triple knockdown cells were generated. As shown in Figure 6, the cell swelling-induced $^{125}I^-$ efflux was significantly lower in AQP1-3 and AQP3-7, but not AQP1-7 knockdown cells. An almost complete inhibition however (reduced to less than ~ 10-15 % of the scrambled control) was observed after AQP1, 3, 7 triple knockdowns.

Hypotonicity-induced blebbing and cell swelling in AQP knockdown cells

To visualize hypotonicity-provoked changes in cell volume and morphology, images of monolayers of control and AQP1, 3, 7-knockdown cells were acquired each second for a period of at least 4 min after osmotic stimulation. As shown in Figure 7, hypo-osmotic stimulation of Intestine 407 cell swelling was accompanied by a transient formation of plasma membrane protuberances, which were absent in cells treated with AQP-specific siRNAs. AQP-knockdown cells however, are still able to increase their volume upon hypo-osmotic stimulation, but fail to exhibit

an RVD response (Figure 8A,C). A similar inhibition of the RVD response has been previously reported by Kida et al. [21] using cells treated with the sulfhydryl reagent methylmethanethio-sulphonate (MMTS) to inhibit AQP3. Importantly, although the AQP knockdown cells were able to increase their volume upon osmotic stimulation, the rate of cell swelling was markedly reduced as compared to control cells (Figure 8A, B). Especially in AQP1, 3, 7 triple knockdown cells, the initial rate of swelling was very low.

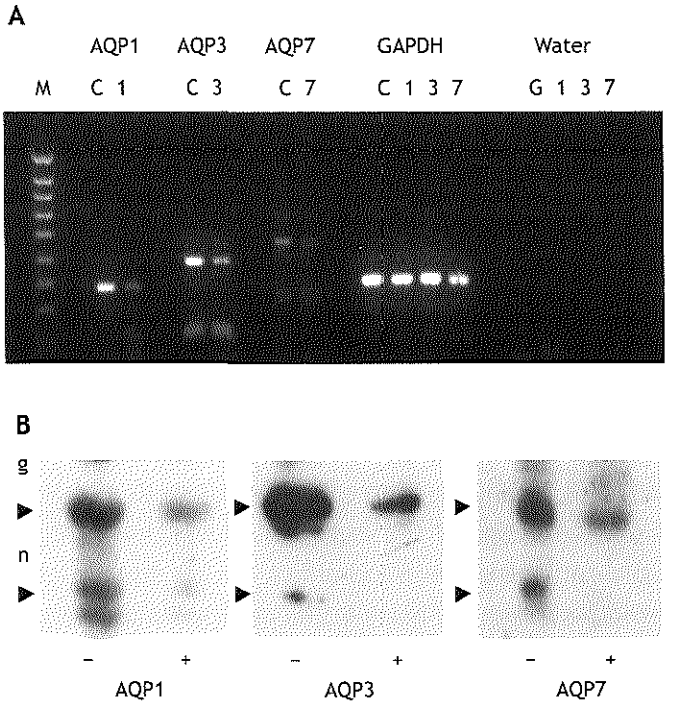

Figure 4: **AQP gene silencing.** **A)** mRNA expression of AQP1, AQP3, AQP7 and GAPDH in control and siRNA-treated cells. C = control cells, 1 = AQP1-siRNA-treated, 3 = AQP3-siRNA-treated and 7 = AQP7-siRNA-treated cells. **B)**: Protein expression of AQP1, AQP3 and AQP7 in control (–) and siRNA-treated cells (+). The core (non-glycosylated) protein is indicated by "n" (~28 kDa), the glycosylated species are indicated by "g" (~35kDa). The results are representative for 3 individual experiments using 3 different batches of cells.

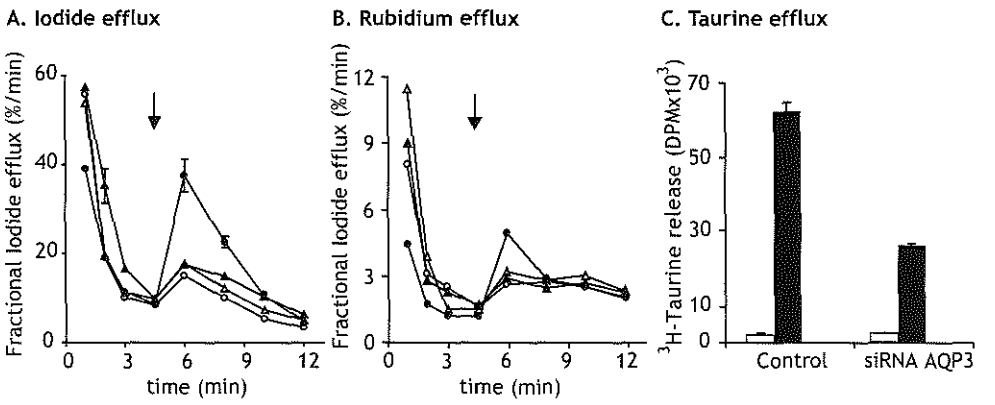

Figure 5: **Reduced hypotonicity-provoked efflux in AQP-knockdown cells.** Control and siRNA-treated Intestine 407 cells were loaded with $^{125}I^-$ (**A**), $^{86}Rb^+$ (**B**) or ^{3}[H]-taurine (**C**). **A, B**): Hypotonicity-provoked efflux of respectively $^{125}I^-$ and $^{86}Rb^+$ from control and AQP siRNA-treated cells. Open circles, closed triangles and open triangles represent AQP1, AQP3 and AQP7-knockdown cells, respectively. Closed circles represent the untreated control. Arrows indicate shift to a 30% hypotonic medium. **C**: Taurine efflux from control and AQP3 siRNA-treated cells. Open and solid bars represent the taurine efflux under isotonic and 30% hypotonic conditions respectively. Data are expressed as mean ±SEM for n=3. No error bar is given when the SEM is smaller than the size of the symbol used.

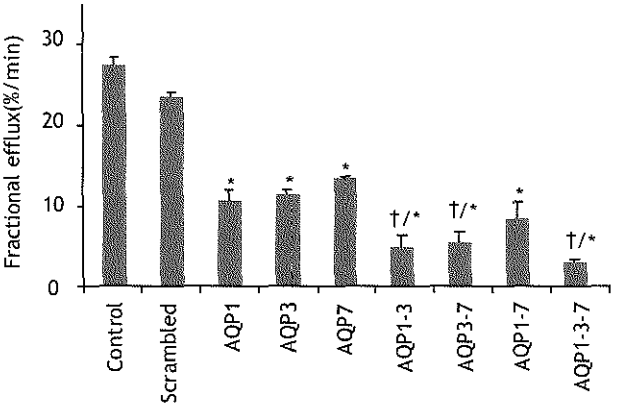

Figure 6: **Comparison of single and multiple knockdown of AQPs.** $^{125}I^-$ efflux from control and AQP siRNA-treated Intestine 407 cells was determined under 30% hypotonic conditions. Cells were transfected with plasmids expressing AQP1, AQP3 and/or AQP7 siRNA. Data are expressed as mean ±SEM for n=3. *) represents a significance difference from cells treated with scrambled AQP3 siRNA ($p < 0.05$). †) represents a significant difference from cells treated with AQP3 siRNA ($p < 0.05$). Control: mock-transfected cells.

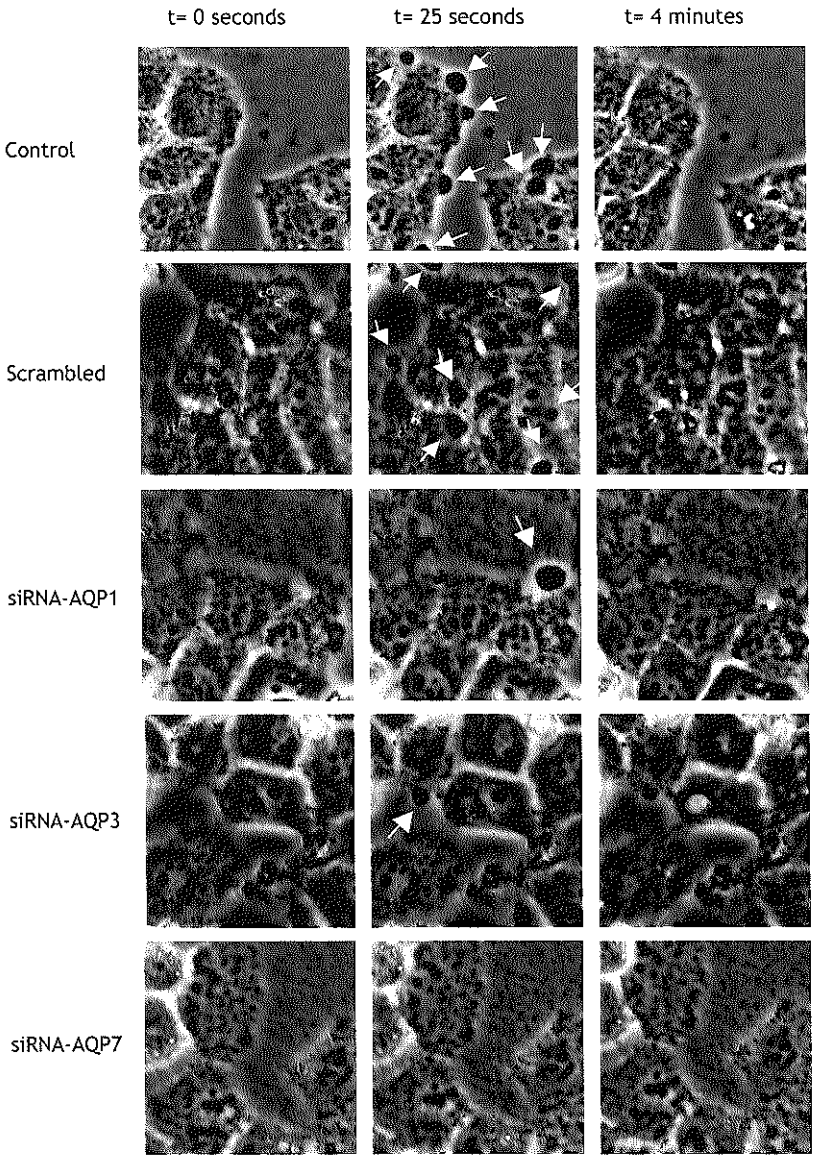

Figure 7: **Hypotonicity-provoked formation of membrane protrusions is absent in AQP-knockdown cells.** Control (upper panels) cells and siRNA-treated Intestine 407 cells (lower panels) were stimulated by 40% hypotonic buffer. Images were taken before stimulation (time = 0), and 25 seconds or 4 minutes after stimulation. Arrows indicate position of membrane protrusions. Each image is representative for 3 images taken from 3 individual experiments using 3 different batches of cells.

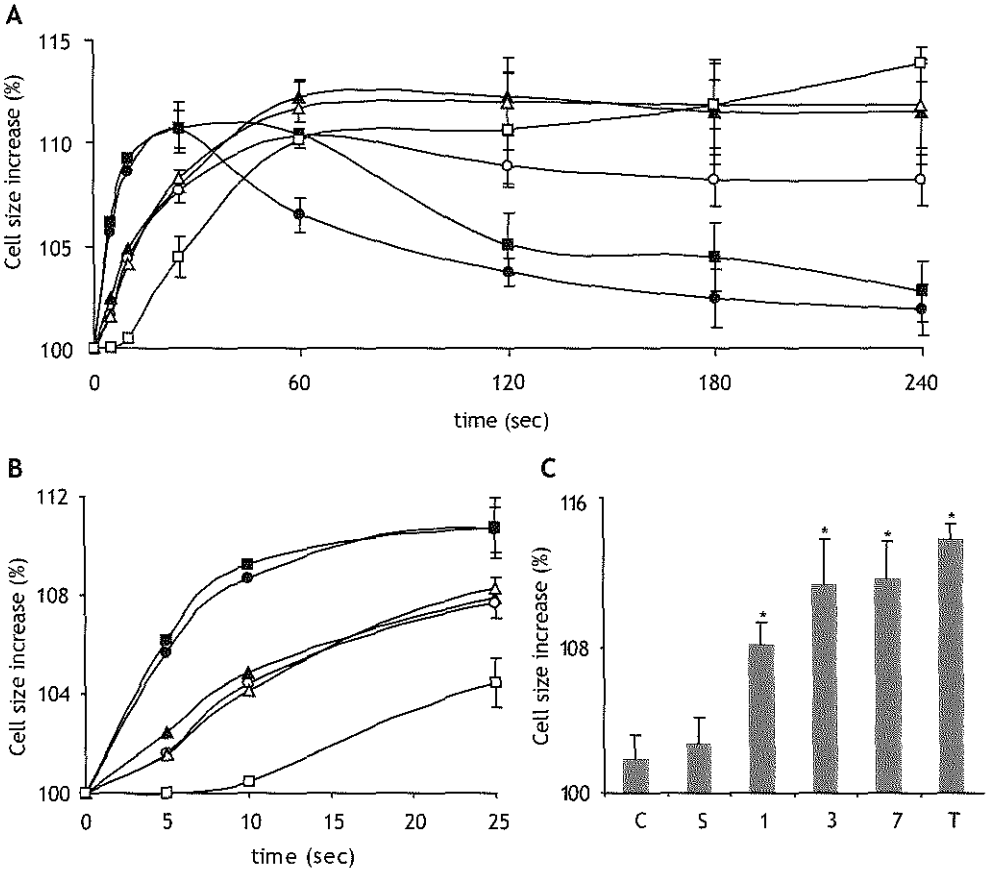

Figure 8: **Delayed swelling and impaired RVD response in AQP-knockdown Intestine 407 cells. A.** Control (closed circles), scrambled AQP3 treated (closed squares), AQP1-(open circles), 3-(closed triangles), 7-(open triangles) and triple-knockdown (open squares) Intestine 407 cells were stimulated with 40% hypotonic buffer. Cell size was measured before stimulation (t = 0 s) and up to 4 min after stimulation. Data are expressed as mean ± SEM (n=6) **B.** Enlargement, first 25 s after stimulation, of panel A. **C.** The increase in cell size after 4 minutes of hypo-osmotic stimulation (40% hypotonicity) relative to the initial cell size. C = control cells; S = cells transfected with scrambled AQP3 siRNA plasmid and 1, 3 and 7 = cells transfected with plasmids encoding respectively AQP1, AQP3 or AQP7 siRNA. Asterisk indicates a significance difference relative to the scrambled control (n=6, $p < 0.01$).

Discussion

In this study, we have shown that the hypotonicity-provoked efflux of $^{36}Cl^-$, $^{86}Rb^+$ and ^3H-taurine were inhibited after brief treatment of the cells with low concentrations of bivalent mercury ions. Because it is rather unlikely that low concentrations of Hg^{2+} similarly affect such diverse transporters as cell swelling-activated anion and cation channels and the organic osmolyte release pathway, a reduced rate of osmotic cell swelling through inhibition of mercury-sensitive AQPs was postulated. With the exception of AQP0 and AQP4, all AQPs have been found sensitive to bivalent mercury ions [24].

Surprisingly, in clear contrast to the $^{36}Cl^-$ efflux, the hypotonicity-provoked Cl^- current measured using the whole cell path clamp technique, was only moderately affected by Hg^{2+} treatment. Similar observations have been reported recently by Kida et al. [21]. Using the sulfhydryl reagent MMTS as an alternative way to inhibit aquaporin-mediated water fluxes, they found a fully active volume-sensitive Cl^- conductance under patch clamp conditions whereas, in intact cells, the RVD response was completely blocked. Notably, this inhibition was still present in gramicidin-treated cells, indicating that the K^+ conductance is not rate limiting [21]. Under whole cell patch clamp conditions, the anion conductance has been found to develop gradually over a period of several minutes and stays active as long as the surrounding medium remains hypotonic [25-27]. In intact cells however, the hypotonicty-provoked anion efflux starts almost instantaneously and lasts for only 2-3 minutes. Therefore, these observations suggest that, in intact cells, the rate of cell swelling is of crucial importance to trigger the compensatory anion efflux. Although speculative, the results may suggest the existence of two modes of VRAC activation, a fast mechanism that may involve channel recruitment [28] and a slower one, observed under patch clamp conditions, which might involve activation by membrane stretch, ionic strength and/or molecular crowding [1].

AQP expression may vary between the different clones of Intestine 407 cells available. The clone routinely used in our laboratory was found to express AQP1, 3 and 7, whereas another clone of this cell line expressed AQP3 only [21]. In our cells, all three members of the AQP family were required to fully activate the hypotonicity-provoken anion efflux and, *vice versa*, knockdown of all three AQPs was needed to maximally suppress the efflux. Notably, the $^{125}I^-$ efflux was found to be more affected by the knockdown of a singe AQP family member than the $^{86}Rb^+$ efflux (Figure 5A). In addition, we found that the iodide efflux was more

sensitive to MMTS treatment than the rubidium efflux (results not shown). These results indicate that, in line with early observations showing that the activation of a K^+ current precedes the Cl^- conductance [29], the efflux of cations is more sensitive to small changes in cell volume.

Hypo-osmotic stimulation of Intestine 407 cells grown as monolayers also induced an immediate and transient formation of membrane protuberances or blebs, which was absent in AQP knockdown cell. Membrane blebbing is often associated with apoptosis [30,31] and necrosis [31], but has also been observed during migration [32], mitosis [33] and spreading after plating [34]. Moreover, brief activation of ATP-gated $P2X_7$ receptors was also found to induce pseudo-apoptotic blebbing [35]. Using an annexin V based apoptosis kit, however, we were not able to detect any evidence for an increased induction of early stage apoptosis in osmotically stimulated Intestine 407 cells. The observed blebs are possibly formed as a result of a rapid increase in intracellular pressure due to the fast movement of water through AQPs. When the fluid driven expansion of the cell membrane is faster than the rate of actin polymerization, cytosolic fluid is squeezed through less rigid areas of the cortical cytoskeleton and blebs are formed [34,36,37]. This phenomenon could be enhanced by the cell swelling induces depolymerization of the actin cytoskeleton that has been observed in many different cell models [3,38-40]. A thin layer of actin however was found to surround blebs [34,41,42], which may play a role during the RVD associated retraction of the protuberances [31,34,41-43]. Alternatively, the blebs may represent cellular domains of increased membrane insertion. Because exocytosis has been found to occur rapidly upon osmotic swelling of Intestine 407 cells and recruitment of VRAC from intracellular compartments has been suggested [28,44], it is tempting to suggest that these blebs represent sites of VRAC insertion.

The rate of osmotic swelling was found to be reduced in AQP knockdown cells (Figure 8A, B), which coincides with a reduced activation of the compensatory osmolyte release pathways (Figures 5, 6) and an inhibition of the RVD response (Figure 8A, C). This suggests that rapid cell swelling is required to promote the efflux of osmolytes and to drive the RVD. To conclude, the results indicate that a high rate of cell swelling is a prerequisite for activating compensatory ion conductances and that the inability to promote the release of osmolytes underlies the absence of the RVD response in AQP siRNA-treated cells. Clearly further research is needed to identify the molecular mechanism by which changes in the rate of cell swelling are able to determine the efficacy of VRAC activation and the RVD response.

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Chapter 8

General discussion

General Discussion

This thesis focuses mainly on the regulation of Volume-Regulated Anion Channels (VRACs) and the search for its molecular candidates. The results described in this thesis demonstrate that rapid cell swelling, facilitated by water transport through aquaporins, is required for the activation of VRACs. Furthermore, our study also demonstrates that a significant fraction of the volume-sensitive Cl^- channels is localized intracellularly and is recruited to the plasma membrane by exocytosis upon osmotic cell swelling. Although the identity of VRAC still remains an enigma, the results of our study suggest that in the search for the molecular identity of VRAC, anion channels localized primarily in intracellular compartments should be considered as prime candidates.

1. VRAC recruitment and recycling

Cellular vesicle transport as well as transport protein function can be modulated by the lipid composition of the plasma membrane [1-4]. Treating the cells with CD/PC-LUVs, to deplete plasma membrane cholesterol, was found to result in enhanced exocytosis and reduced endocytosis [1-4]. As compared to control cells, the hypotonicity-provoked anion conductance was increased by approximately 2 fold in CD/PC-LUVs-treated Intestine 407 cells, indicative for an increase in the number of active and plasma membrane localized VRAC channels. This can be explained by an increase in the open state probability of the individual channels (as has been reported in references 5 and 6), by recruitment of additional (open) channels, by prolonged plasma membrane retention of channels [8] or by a combination of such mechanisms.

Caveolae and lipid rafts are distinct membrane areas with a high content of cholesterol [7,1]. Because the CD/PC-LUVs-mediated potentiation of the hypotonicity-provoked anion efflux was not changed in either caveolin-1 deficient or in sphingomyelinase-treated cells [8], a crucial role for caveolae and lipids rafts in the regulation of VRAC seems unlikely. Alternative cholesterol-sensitive membrane domains are the so-called recycling endosomes [9]. These organelles are involved in receptor cycling, non-secretory exocytosis as well as NPC-dependent recycling of cholesterol [9]. Skin fibroblasts of NPC patients, characterized by a massive intracellular accumulation of free cholesterol due to a specific defect in cholesterol transport [10,11], showed a much reduced volume-sensitive anion conductance compared as to the control [8], suggesting a role for

endosomes in the regulation of VRAC. This notion is supported by the observation that progesterone, a steroid known to block trafficking of cholesterol containing vesicles [12], not only reduced the cell-swelling induced anion efflux but also diminished its potentiation by CD/PC-LUVs [8]. Similar effects of cholesterol depletion were observed in mouse fibroblast expressing a trafficking mutant of CFTR (F508delCFTR), an epithelial chloride channel crucially involved in transepithelial salt and water transport [8]. Interestingly, F508delCFTR -expressing fibroblasts and several other F508delCFTR expressing cell models were found to display a NPC-like phenotype, i.e. a marked intracellular accumulation of cholesterol, reflecting a secondary defect in endosomal recycling [8,13]. Treating these cells with CD/PC-LUVs reduced the accumulation of cholesterol and potentiated the CFTR activity, as monitored by forskolin- and genistein- induced efflux of anions [8]. In F508del-CFTR expressing fibroblasts the rate of endocytosis was markedly reduced upon CD/ PC-LUVs treatment, as demonstrated by a diminished uptake of fluorescently labelled dextran [8]. Taken together, the results indicate that cholesterol depletion can be used to trigger anion channel recruitment and retention in the plasma membrane, thereby enhancing the capacity of the cell for anion secretion.

It is remarkable that different cystic fibrosis (CF) cell models display a NPC-like phenotype. In NPC cells, this phenotype is caused by mutations in NPC1, a protein that has been proposed to function as a flippase [14]. By moving lipids from the inner to the outer leaflet of the membrane, NPC1 urges the membrane to curve. The absence of functional NPC1 prevents the lipid redistribution and subsequent vesicle budding and finally leads to excessive cholesterol accumulation [14]. The mechanism by which accumulated F508del-CFTR perturbs lipid metabolism and vesicle budding, however, remains to be established. A comparable accumulation of cholesterol has been observed in cells defective in ATP-binding cassette protein A1 (ABCA1). ABCA1 is a cholesterol transport protein and structurally related to CFTR [15].

2. Putative involvement of intracellularly localized chloride channels

Exocytosis depends on adequate vesicle docking and fusion and is mediated by the so-called SNARE proteins. Binding of a v-SNARE to SNAP-25 and a t-SNARE forms a complex that finally leads to membrane docking and fusion [16]. Both NEM (inactivates SNAP-25) and *Clostridium botulinum* toxin F (cleaves v-SNARE proteins) are inhibitors of exocytosis [17,18] and were found to reduce the

volume-sensitive anion efflux [Chapter 5, this thesis]. These results further support our hypothesis that exocytosis and channel recruitment contribute to the development of the RVD response. In our model we propose the existence of two pools of VRAC channels, an intracellularly localized neurotoxin F-sensitive pool that is rapidly recruited to the plasma membrane upon hypo-osmotic stimulation and a toxin-insensitive pool with a low turn-over rate that is constitutively present in the plasma membrane [Chapter 6, this thesis].

Rab proteins (small GTPase) are among the numerous proteins involved in regulating cellular vesicle trafficking [19-22]. The role of Rab4, -5 and -11 in the regulation of VRAC was studied by expressing their active or dominant negative forms in Intestine 407 cells. Our results indicate that inhibition of exocytosis (by introducing Rab4DN or Rab11DN) or stimulation of endocytosis (Rab5WT) markedly reduced the volume-sensitive anion efflux, while stimulating exocytosis (Rab11WT) or inhibiting endocytosis (Rab5DN) resulted in higher basal efflux. These results indicate that modulation of exo- and endocytosis gives rise to a redistribution of VRAC and further support our notion that channel recruitment plays an important role in VRAC regulation. A role for Rab GTPases in the regulation of ion channels is not unprecedented. Rab proteins are known to modulate the surface expression of other channels such as F508del-CFTR [23] and ENaC [20,24].

3. The rate of swelling is important for RVD

For adequate transport of water through the lipid bilayer, diffusion alone is often not sufficient. Therefore, most cells express "water channels" or aquaporins (AQPs), which play a crucial role in (trans-) cellular water transport [25-29]. Brief treatment of Intestine 407 cells with low concentrations of bivalent mercury ions markedly reduced the osmolyte release pathways (i.e. for Cl^-, K^+ and taurine) triggered by hypo-osmotic cell swelling [Chapter 7, this thesis]. This inhibition is most likely caused by reduced osmotic cell swelling as consequence of a reduced influx of water through mercury-sensitive AQPs. At least three different AQPs are expressed in Intestine 407 cells: AQP1, 3 and 7 [Chapter 7, this thesis]. Knockdown of AQP by siRNA was found to markedly reduce the rate of osmotic cell swelling, however, a slow and steady increase in cell volume was still observed [25, Chapter 7, this thesis]. Surprisingly, activation of VRAC was found to be almost completely defective in AQP deficients cells, despite the observed increase in cell volume. Taken together, these results suggests that

the rate rather than the extent of cell swelling determines the activation of compensatory ion conductances and that the absence of RVD response in slowly swelling cells (resulting from AQP knock down) is caused by their inability to properly activate the osmolytes release pathways.

4. VRAC regulation by a rapid increase of intracellular water

The rate of water entry may in principle influence the regulation of ion channels through several distinct mechanisms. First, the plasma membrane may stretch upon cell swelling [30], leading to increased mechanical tension [31] and/or the release of stretch-induced messengers such as ATP that can regulate certain types of ion channels [32]. Nonselective stretch-activated cation channels for instance might contribute to the RVD by activating Ca^{2+}-sensitive K^+ channels through an increase of intracellular Ca^{2+} [32]. The activation of stretch-sensitive channels however requires a considerable amount of stretching [33]. Significant stretching was found to be obtained only when cell volume was increased by more than 60%. Notably, volume regulatory mechanisms are already activated by a 5%-10% increase in volume [34]. In addition, although osmotic swelling of Intestine 407 cells releases ATP into the medium, ATP alone was unable to directly activate VRAC [35]. Secondly, reduced macromolecular crowding and ionic strength, triggered by osmotic cell swelling, can markedly influence the thermodynamic and catalytic activities of enzymes that regulate transmembrane transport [36]. Although an increase in ionic strength might play a role during the RVI [37], our results in AQP deficient cells suggest that the rate of cell swelling but not an increase in cell volume by itself determines the activation of VRAC, arguing against a role for macromolecular crowding.

Finally, plasma membrane unfolding as result of the rapid increase in cell volume might expose additional and previously cryptic ion channels. Although we cannot exclude a contribution of the latter mechanism, the results in Chapter 5 strongly suggest a role for VRAC activation by vesicle fusion. How rapid changes in cell volume are translated into exocytosis remains an enigma.

5. Concluding remarks

Research into the signal transduction of cell volume regulation by many different groups has revealed that multiple signalling pathways may be involved [38-44]. These pathways include Ca^{2+} mobilization, (receptor) tyrosine kinases, MAP

kinases, G proteins like $p21^{rho}$, ATP release as well as PI-3-kinase and PKB (for reviews see: [33,45,46]). Although activation or inhibition of these pathways may modulate activity of VRAC channels, none of these signalling pathways and/or molecules was found to be sufficient to trigger its activation. A notable exception is $p56^{lck}$ which was found to directly activate VRAC in excised membrane patches of $p56^{lck}$ deficient J-Cam 1.6 cells [47]. Because most of these signalling pathways also affect vesicle cycling, it is tempting to speculate that these pathways are not directly involved in channel opening or closing, but act as modulators of the recruitment and/or endocytosis of VRAC, determining its cellular distribution.

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Summary

Summary

Any change in osmolarity, either of intracellular or extracellular origin, will initially result in swelling or shrinkage of cells. Because alterations in cell volume affect several important cellular functions including growth, necrosis, apoptosis and migration, it is of vital importance for a cell to be able to correct changes in its volume. In this thesis, we mainly focussed on the regulatory volume decrease (RVD) response triggered by osmotic cell swelling in epithelial cells. Upon osmotic cell swelling, potassium and chloride channels are rapidly activated. As a result, potassium and chloride will leave the cell, driving the efflux of water. This initial phase is followed by a delayed and prolonged release of organic osmolytes. Activation of these compensatory mechnisms is accompanied by a rapid increase in exo- and endocytosis and the extracellular release of ATP.

Although the activation of these compensatory mechanisms has been observed in many different cell models, the molecular pathways involved in osmo-sensing and -signalling are still largely unresolved as is the molecular identity of several of the transporters involved. The aim of this project was to study the regulation of Volume Regulated Anion Channels (VRAC) and to search for potential molecular candidates. **Chapter 2** summarize the different model systems and methods that can be used to investigate cell volume regulation in intestinal epithelial cells.

In **Chapter 3**, we report the role of recycling endosomes in the regulation of VRAC. The recycling endosomes are involved in the NPC1-dependent trafficking of cholesterol. Depleting plasma membrane cholesterol promotes exocytosis and inhibits endocytosis thereby potentiating the volume-regulated anion efflux. Notably, human skin fibroblasts with defective NPC1 protein showed, besides a massive intracellular accumulation of cholesterol, a reduced volume-sensitive anion conductance as compared to the control. From these experiments we concluded that exocytosis contributes to the development of the compensatory anion efflux by recruiting additional anion channels to the plasma membrane.

Cholesterol accumulation has also been observed in cultured cystic fibrosis (CF) cells. In **Chapter 4**, we continued our research on cholesterol dependent membrane cycling using murine fibroblasts expressing a trafficking mutant of the CFTR Cl^- channel, F508delCFTR. Depletion of plasma membrane cholesterol,

shown previously to promote exocytosis and to slow down endocytosis, was found to rescue F508delCFTR channels to the plasma membrane. A more detailed study of the role of exo- and endocytosis in the regulation of VRAC is described in **Chapter 5.** Compounds that interfere with vesicle docking and fusion, as well as (over)expression of several Rab proteins, were used to manipulate intracellular vesicular trafficking. Both inhibition of exocytosis and stimulation of endocytosis reduced the volume-sensitive anion efflux, while stimulating exocytosis and inhibiting endocytosis resulted in higher basal efflux. Together, the data suggest that a significant fraction of the volume-sensitive Cl^- channels is localized intracellularly and is recruited to the plasma membrane upon osmotic cell swelling. Therefore, anion channels localized primarily in intracellular compartments can no longer be excluded as VRAC candidates. **Chapter 6** shows an inventory of the intracellular Cl^- channels expressed in Intestinal 407 cells. Initial experiments put CLICs forward as novel potential VRAC candidates and as a channel family for future study.

In **Chapter 7**, we describe the role of aquaporins (AQPs) in cell volume regulation. In Intestine 407 cells, the AQP isoforms 1, 3 and 7 are expressed. In cells deficient in one or more AQPs, both the rate of cell swelling as well as the activation of the hypotonicity-provoked anion efflux were decreased. Notably, knockdown of AQPs only slowed down but did not prevent osmotic swelling. Our data indicate that AQPs are required for rapid cell swelling and that the swelling rate, rather than the extent of cell swelling, is a crucial factor in the activation mechanism of VRAC.

Samenvatting

Samenvatting

Elke verandering in de extra- of intracellulaire osmolariteit zal leiden tot veranderingen in het volume van cellen. Omdat deze verandering vitale cellulaire functies als groei, necrosis, apoptosis and migratie kunnen beïnvloeden, is het belangrijk dat cellen volume veranderingen snel en effectief kunnen corrigeren. In dit proefschrift beschrijven we vooral processen die te maken hebben met de gereguleerde volume afname (regulatory volume decrease, RVD), een respons die door celzwelling wordt geactiveerd. Bijna onmiddellijk na een toename van het celvolume worden kalium en chloride kanalen in de plasmamembraan geactiveerd. Hierdoor verlaat KCl en water de cel. Deze fase wordt gevolgd door een vertraagde maar langer aanhoudende efflux van organische osmolieten. Activering van deze compenserende mechanismen gaat samen met een snelle toename in exo- and endocytosis en met de extracellulaire afgifte van ATP.

Hoewel deze compenserende mechanismen in veel verschillende celmodellen zijn bestudeerd, zijn de moleculaire wegen die bij de activering betrokken zijn nog grotendeels onopgelost. Ook de moleculaire identiteit van een aantal van de betrokken kanalen en transporteurs is nog niet bekend. Het doel van dit project was om de regulering door celzwelling geactiveerde anion kanalen (Volume-Regulated Anion Channels, VRACs) te bestuderen en mogelijke nieuwe moleculaire kandidaten te vinden.

Hoofdstuk 2 geeft een overzicht van verschillende modelsystemen en methoden die gebruikt kunnen worden om celvolume regulatie in darmepiteelcellen te bestuderen.

In **Hoofdstuk 3** beschrijven we de rol van de recirculerende endosomen in de regulatie van VRAC. Recirculerende endosomen zijn betrokken bij het NPC1-afhankelijk intracellulair transport van cholesterol. Verlaging van cholesterol in de plasmamembraan resulteert in een stimulatie van exocytose en een remming van de endocytose. Het gevolg hiervan is dat de volume-gereguleerde anion efflux wordt gepotentieerd. Humane huidfibroblasten die een defect NPC1 eiwit tot expressie brengen vertoonden naast een massale intracellulaire cholesterol ophoping ook een verminderde volume gevoelige anion conductantie. Uit deze experimenten kunnen we concluderen dat exocytose een belangrijke rol speelt in

de activering van de compenserende anion efflux door middel van de rekrutering van additionele anion kanalen naar het plasmamembraan.

Een intracellulaire ophoping van cholesterol is ook waargenomen in cellen die het mutant CFTR Cl^-kanaal eiwit F508delCFTR tot expressie brengen. In **Hoofdstuk 4** beschrijven we onderzoek naar de cholesterol afhankelijke membraan (re)cycling in huidcellen waarin F508delCFTR tot expressie is gebracht. De F508del mutatie veroorzaak een eiwitvouwing defect waardoor het CFTR zijn bestemming in de plasmamembraan niet kan bereiken. Depletie van plasma membraan cholesterol, met als doel exocytose te stimuleren en endocytose te vertragen, bleek de aanwezigheid van F508delCFTR in het plasmamembraan te verhogen.

Een gedetailleerde studie naar de rol van exo- en endocytose in de regulatie van VRAC wordt beschreven in **Hoofdstuk 5**. Farmaca die de associatie en fusie van membraanblaasjes met het plasmamembraan beïnvloeden, en (over)expressie van Rab eiwitten, werden gebruikt om het intracellulaire membraantransport te manipuleren. Remming van exocytose en/of stimulatie van endocytose verminderde de celzwelling-geactiveerde anion efflux, terwijl stimulatie van exocytose en inhibitie van endocytose een verhoging van de basale efflux tot gevolg had. Tesamen suggereren deze resultaten dat een significant deel van de volume gevoelige Cl^- kanalen intracellulair is gelokaliseerd en bij celzwelling naar de plasmamembraan wordt gerekruteerd. Een belangrijke conclusie uit dit model is dat ook chloride kanalen met een voornamelijk intracellulaire lokalisatie als potentiële moleculaire kandidaten voor VRAC beschouwd moeten worden.

Hoofdstuk 6 geeft een overzicht van de expressie van een aantal eiwitten in Intestine 407 cellen die recent als chloride kanaal zijn geïdentificeerd. Uit deze oriënterende studie blijkt dat de voornamelijk intracellulair gelokaliseerde CLIC chloride kanalen mogelijke kandidaten voor VRAC zijn, en nader studie behoeven.

In **Hoofdstuk 7** wordt de rol van aquaporines (AQP, waterkanalen) bij de osmotische celzwelling en volume regulatie beschreven. In Intestine 407 cellen komen de isoformen AQP1, 3 en 7 tot expressie. Verlaging van de expressie van een of meer van deze AQPs, verminderde zowel de snelheid van celzwelling als de activering

van de compensatoire anion efflux, maar voorkwam de uiteindelijke toename van het celvolume niet. Deze resultaten tonen aan dat AQPs nodig zijn voor een snelle celzwelling. De snelheid van celzwelling, maar niet de mate van zwelling, bepaalt of de VRAC kanalen gerekruteerd en geactiveerd worden, waardoor de celzwelling gecorrigeerd wordt.

Acknowledgements

Acknowledgements

Everyone we encounter in our lives will have a certain level of influence on us. These influences, together with our decision and determination, will take us to where we are now. I began with my PhD, because I wanted to discover my capabilities. This ambition of mine began on the island called the drumming wave. I saw my mother left the group and out of fear that she would be lost, I followed her. Almost at the same time, my father's friend saw me leave the group and out of fear that I would be lost, he followed me. We did not find my mother and we were lost in the mountain. While we were trying to find our way back, he told me many things. Most of it I have long forgotten, but one I have always remembered: "Set your goal as high as possible and do your best. This way, you will never have any regrets because you have tried." Eventually, we reached the coast and while listening to the drumming sound of the waves, my mother found us.

Since that day, I set my goal as high as possible and I have always done my best. However, by doing my best alone, I would never have reached this point. There are many people who I have to thank. Inspiring teachers, nice colleagues, great friends and supporting family. Just by thinking about them, I realize how lucky I am.

First of all I will like to thank my promotor Professor Verrijzer. Besides being my promotor, I will like to thank you for showing interest in my career plans and giving me valuable advice.

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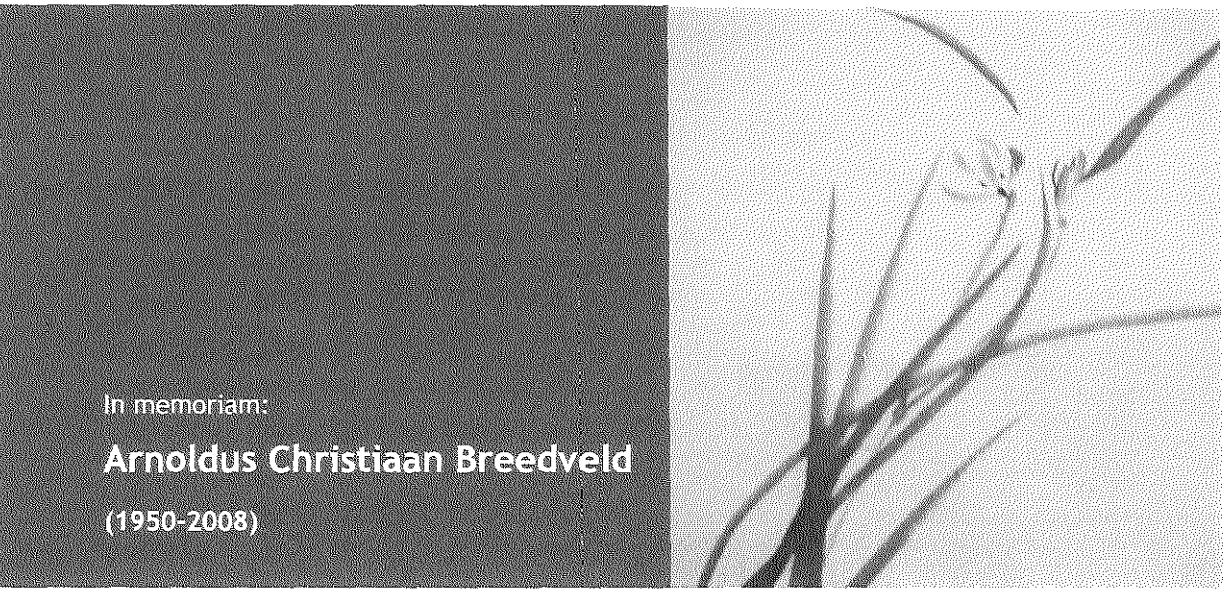
Not to forget the entire computer support team for solving every problem I encountered with my computer. Pim, from the entire team I have bothered you the most and yet you always welcome me with a big smile when I walk into your office. There were times when everyone, including me, gave up on the hope of solving the problem, but you will not stop until you have found a solution.

Michael, thank you for all the brownies. It is a privilege to be on your brownies delivery list.

Karl, thank you for all the great lunch and coffee breaks. Thank you for listening to me. I hope you will graduate soon so we can celebrate our graduation by going to the Japanese for sashimi and sake!

Bjorn, congratulations with your new lab in Italy. I will miss our conversation about politics, vacations and research during lunch and coffee.



In memoriam:

Arnoldus Christiaan Breedveld

(1950-2008)

So long as men can breathe or eyes can see,
So long live this, and this gives life to thee.

William Shakespeare

The first thought that came up to my mind when I have finally accepted the fact that you have left this world, is that you will not be able to be on my defence. And almost immediately I have decided to write a page for you in my thesis. This is the least I can do to thank you for treating me like your daughter and do everything for me what a father would do for his daughter.

I have many ideas, and most of them will remain just an idea if we have never met. When I told you about my ideas, you will always say: 'Christina, that is a great idea, you should do it!' and because of your encouragement, thoughts become deeds.

Now that I have almost completed my PhD, it feels like I have reached a crossroad. I am not quite sure where to go from here. But one thing I am certain of. When I have made a decision, your face will appear in my thoughts. With your usual big smile you will say: 'Christina, that is a great idea, you should do it!'

Acknowledgement and Curriculum vitae (Chinese version)

後記

真是一段很漫長的學習之路～
雖然不是在寒窗下，卻也是苦讀了十幾年。
我的運氣不錯，一路上都有好老師指導；縱使辛苦卻不艱難。
前一正子看蕭麗紅寫的 [白水湖春夢]。
讀到
一切所得皆因 [因**緣**聚會 聲過長空]。
讓我感觸良深。
因爲只靠我一個人是絕對無法完成這本論文的。
一種機緣加上父母的犧牲，讓我們從台灣移民到荷蘭。
感謝我萬能的媽媽一個人包辦了廚娘，朋友，伴讀 的功能。
感謝生下萬能媽媽的外婆。
感謝兩個當我保鏢兼司機的弟弟。
當然也不能忘了珍貴的搖錢樹爸爸。

我感恩我所擁有的。
並且希望能如媽媽所願;做個對社會有貢獻的人。

~聲過不長空~

作者簡介

林倩華
1978 年生於台灣台北
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於 2003 年取得荷蘭阿姆斯特丹自由大學生化系碩士學位
2008 年取得荷蘭鹿特丹大學,醫學研究院博士學位
並已有 3 篇作品發表於著名歐美科學雜誌

Publications

Publications

Christina H. Lim, Kees Schoonderwoerd, Wim J. Kleijer, Hugo R. de Jonge and Ben C. Tilly. 'Regulation of the cell swelling-activated chloride conductance by cholestero-rich membrane domains' *Acta Physiologica* (Oxf) (2006) **187**, 295-303

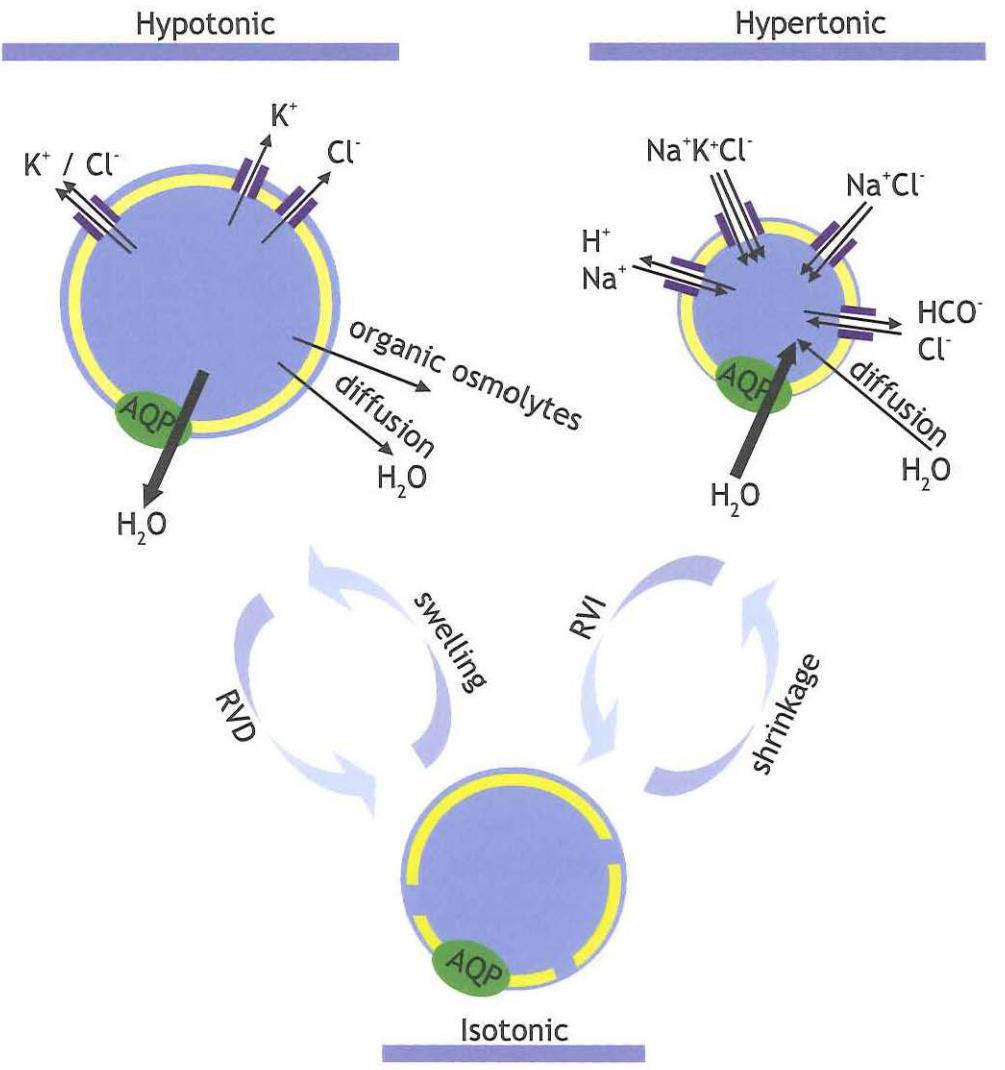
Christina H. Lim, Marcel J. Bijvelds, Alex Nigg, Kees Schoonderwoerd, Adriaan B. Houtsmuller, Hugo R. de Jonge and Ben C. Tilly. 'Cholesterol depletion and genistein as a tool to promote F58delCFTR retention at the plasma membrane' *Cellular Physiology and biochemistry* (2007) **20**, 473-482

Christina H. Lim, Alice G.M. Bot, Hugo R. de Jonge and Ben C. Tilly. 'Osmosignalling and volume regulation in Intestinal epithelial cells' *Methods in Enzymology* (2007) **428**, 325-342

Christina H. Lim, Sebastian F. Tomassen, Boris M. Hogema, Hugo R. de Jonge and Ben C. Tilly. 'Aquaporins are required for the activation of osmolyte release pathways in Intestine 407 cells' *Under revision*

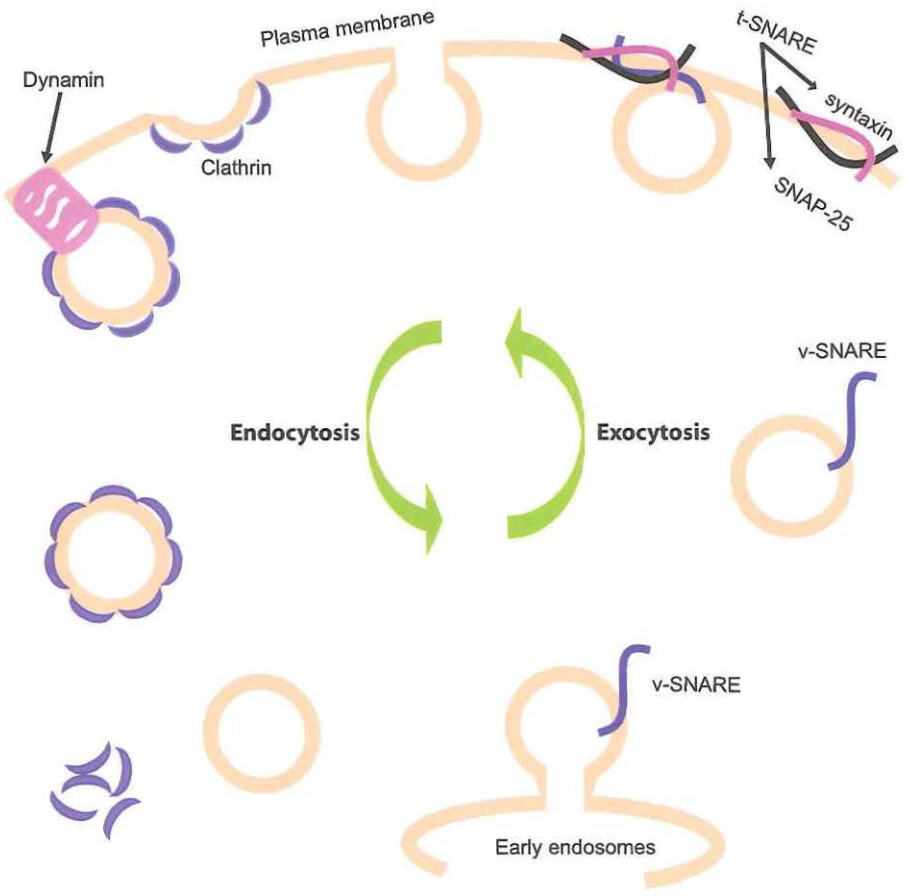
Christina H. Lim, Martina Wilke, Alex Nigg, Adriaan B. Houtsmuller, Hugo R. de Jonge and Ben C. Tilly. 'Osmotic cell swelling-induced recruitment of Volume-regulated anion channels to the plasma membrane' *In preparation*

Appendix
Full-color version

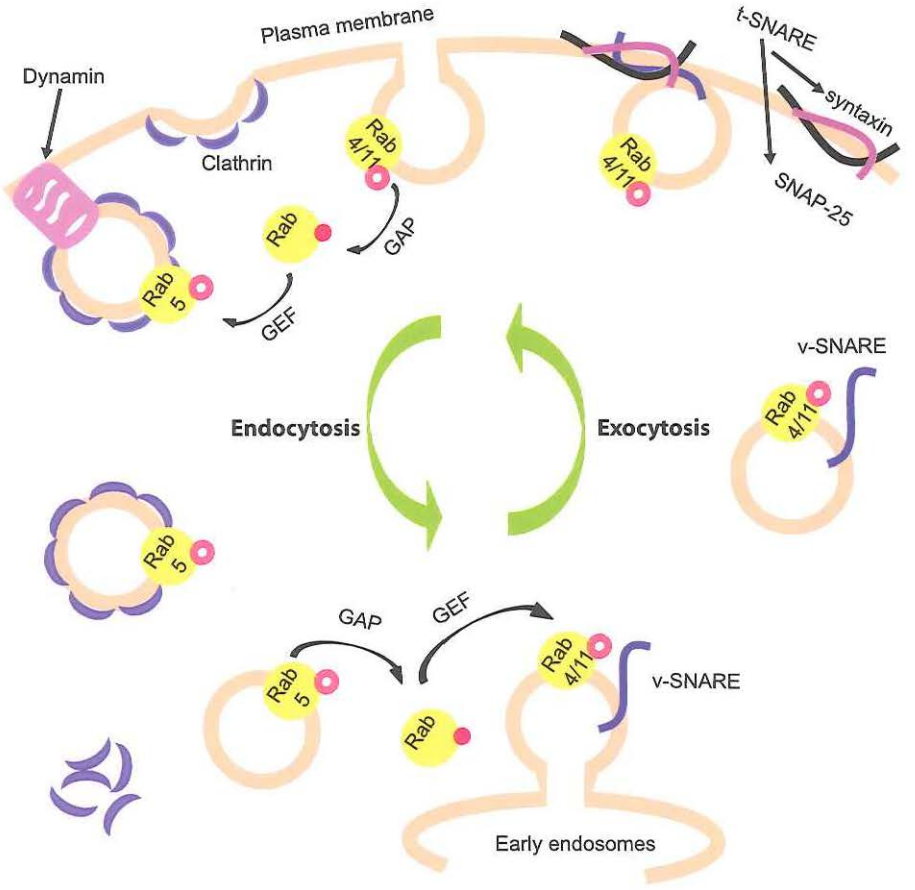


Chapter 1, figure 1: **Schematic illustration of cell volume regulation in mammalian cells.**

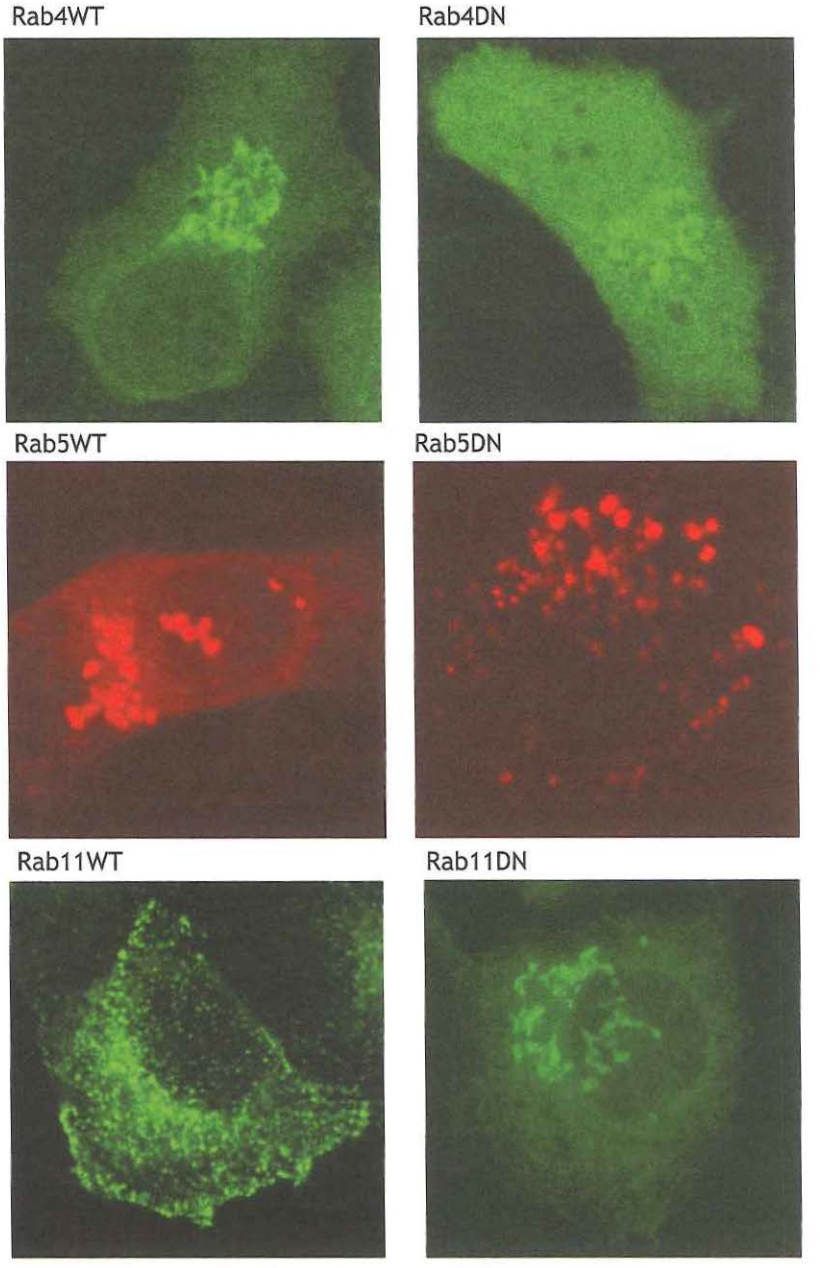
Alteration in extracellular osmolarity will result in redistribution of water, which rapidly leads to cell volume changes. Upon cell shrinkage, ion transporters are activated for the uptake of NaCl, facilitating water influx. This mechanism is commenly referred to as regulatory volume increase (RVI). Upon cell swelling, KCl and organic osmolytes are respectively released through ion channels and organic osmolytes release pathways, which will leads to water efflux, a phenomenon known as regulatory volume decrease (RVD).
(see page 19)



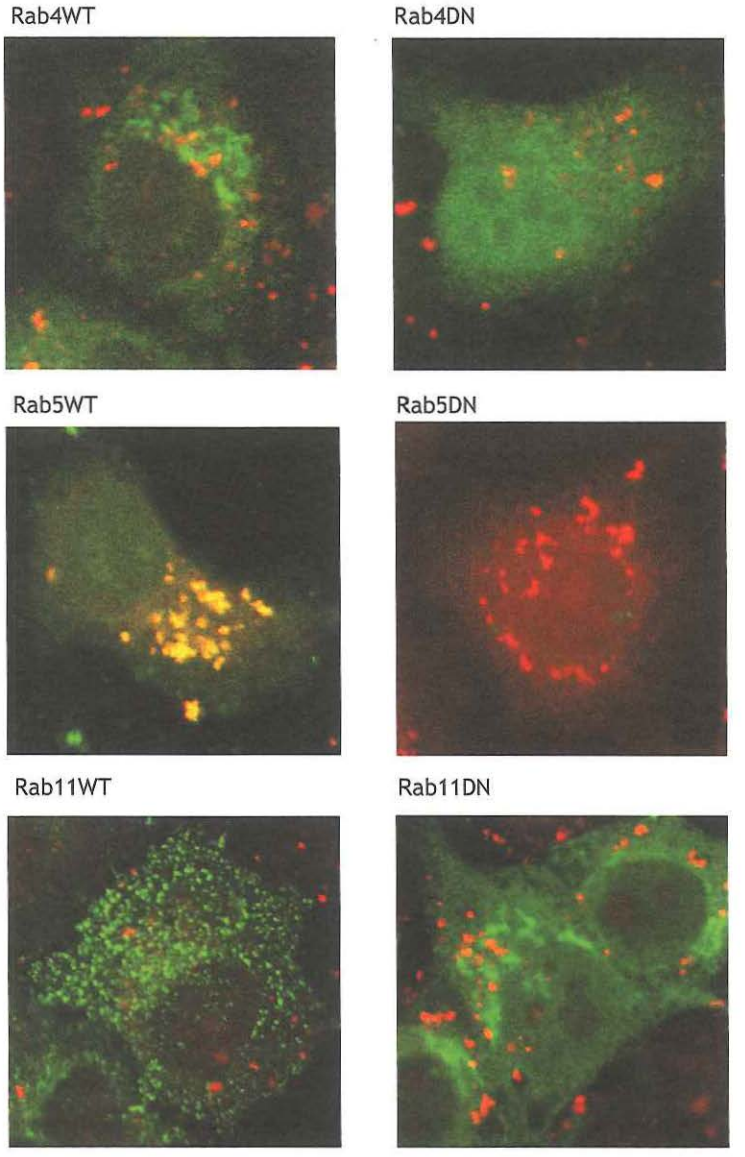
Chapter 1, figure 2: **Scheme of endo- and exocytosis.** Endocytosis begins with the assembly of clathrin coat, which leads to the formation of a vesicle. Dynamin mediates the release of vesicle from the membrane, allowing it to travel towards the early endosomes. Exocytosis is facilitated by the binding of v-SNARE to the t-SNARE. (see page 25)



Chapter 1, figure 3: **Rab proteins and their functions.** Rab5 is involved in the clathrin-coated endocytosis pathway. Exocytosis requires Rab4 and/or Rab11. Guanine nucleotide exchange factor (GEF) activates Rab by exchanging GDP (closed circles) for GTP (open circles). After vesicle fusion, a Rab GTPase hydrolyzes the GTP (open circles) to GDP (closed circles) resulting in a release of Rab proteins from the vesicle. (see page 26)



Chapter 5, figure 4: Cellular localization of Rab proteins. Intestine 407 cells were transfected with wildtype (WT) or dominant negative (DN) plasmids encoding CFP-tagged Rab4, DsRed-tagged Rab5 or GFP-tagged Rab11 proteins. (see page 102)



Chapter 5, figure 5: **Co-localization of Rab proteins and dextran.** Cells transfected with wildtype (WT) or dominant negative (DN) plasmids encoding CFP-tagged Rab4, or GFP-tagged Rab11 proteins were incubated with TRITC-labelled dextran MW 10,000 (0.5 mg/ml, 10 minutes). Cells transfected with wildtype (WT) or dominant negative (DN) plasmids encoding DsRed-tagged Rab5 proteins were incubated with FITC-labelled dextran. Only Rab5 WT was found to be localized close to dextran containing endosomes. (see page 103)

Curriculum Vitae

Chien-hua (Christina) Lim werd geboren op 22 februari 1978 te Taipei, Taiwan. Na het behalen van haar VWO diploma aan het Emmauscollege te Rotterdam in 1998, begon ze aan de opleiding Medische Biologie aan de Vrije Universiteit in Amsterdam. Tijdens haar eerste stage heeft ze mee gewerkt aan het onderzoek naar allergeen specifieke T cellen (Afdeling dermatologie, Vrije Universiteit Amsterdam). Gedurende haar tweede stage bij DSM (DSM Food Specialties, Afdeling Genetica) heeft ze mee gewerkt aan het ontwikkelen en optimaliseren van transformatie procedures voor een bacterie. In augustus 2003 is zij afgestudeerd. In januari 2004 begon zij haar aan haar promotie-onderzoek op de Afdeling Biochemie, Erasmus MC, Rotterdam alwaar het in dit proefschrift beschreven onderzoek is uitgevoerd.

Chien-hua (Christina) Lim was born in Taipei, Taiwan on February 22nd 1978. After she graduated from the Emmauscollege, Rotterdam in 1998, she started a Masters degree (MSc) in Medical Biology at the Vrije University in Amsterdam. During her first internship, she worked on *in vitro* detection of allergen specific T cells (Department of Dermatology, Vrije University Amsterdam). During her second internship at DSM (DSM Food Specialties, Department of Genetics), she helped with designing and optimalization of transformation procedures for bacteria. In January 2004, she started her PhD in the Department of Biochemistry, Erasmus MC, Rotterdam, doing research described in this thesis.