Molecular Basis of Cell Volume Regulation in Epithelial Cells

Moleculaire basis van celvolume-regulatie in epitheelcellen

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Abbreviations

G-actin	globular (monomeric) actin
F-actin	filamentous actin
cAMP	cyclic adenosine 3':5'-monophosphoric acid
AMP	adenosine mono-phospate
ARF	ADP ribosylation factor
ATP	adenosine 5'-triphosphate
AQP	aquaporin
AVP	arginine vasopressin
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester
bp	base pair
cdc42	cell division cycle protein 42 (Rho family member)
CFTR	cystic-fibrosis transmembrane conductance regulator
CHIP	channel forming integral protein
mCi	milli Curie
DAG	1,2 diacylglycerol
mDia	mammalian diaphanous protein
DIDS	4,4'-di-isothiocyanatostilbene-2,2'-disulfonic acid, disodium salt
DMEM	Dulbecco's modified Eagle's medium
dmso	dimethylsulfonyloxide
DTT	dithiothreitol
cDNA	copy DNA
E_A	Arrhenius activating energy
EDTA	ethylenediamine-N,N,N',N'-tetra-acetate
EGF	epidermal growth factor
EGTA	ethylene glycol-bis-(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular regulated kinase; MAPK
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutryic acid
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GDI	GDP dissociation inhibitor
GFP	green fluorescent protein
G-protein	guanosine nucleotide-binding regulatory protein
GTP	guanosine 5'-triphosphate
^{3}H	hydrogen isotope 3 / tritium
hepes	4-[2-hydroxyethyl]-1-piperazine etanesulfonic acid
^{125}I	iodium isotope 125
Lck (p56)	lymphocyte-specific protein tyrosine kinase
MAPk	p38 mitogen-activated protein kinase; ERK
MEK	mitogen-activated kinase; Erk-activating kinase
MEM	minimal Eagle's medium
MIP	major intrinsic protein
MTX	methotrexate
NEM	N-ethylmaleimide
NMDA	N-methyl-D-aspartate
NSF	N-ethylmaleimide-sensitive factor; NEM-sensitive fusion protein
OAT	organic anion-transporter (solute carrier family 22; SLC22A)
OCT	organic-cation-transporter (solute carrier family 22; SLC22A)
^{32}P	phosphate isotope 32
p	protein
PAGE	polyacrylamide gel electroforese
PBS	phosphate-buffered saline
PI-PLC	phosphatidylinositol-specific PLC
PI3k	phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase; protein kinase A
PKB/Akt/RAC	protein kinase B
PKC	protein kinase C
PLA	phospholipase A
PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 12-acetate
PtdOH	phosphatidic acid
TLC	thin layer chromatography
Rab	Ras genes from rat brain
Ras	Rat sarcoma oncogene homolog
Rac	Ras-related C3 botulinum-toxin substrate (rho family)
Rho (p21)	Ras homology (Rho family protein)
Rock	p160–Rho-associated coiled-coil–containing protein kinase
RVD	regulatory volume decrease
RVI	regulatory volume increase
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SH domain	Src homology domain
SH	sulfhydryl-group
SNAP	soluble NSF attachment protein
SNAREs	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
Src	sarcoma virus tyrosine kinase
TFP	trifluoperazine, calmodulin antagonist
TGN	trans Golgi network
TLC	thin layer chromatography
VRAC	volume-regulated anion channel
V_2-receptor	vasopressin type 2 receptor
wt	wild-type

List of activators and inhibitors used

A23187	Ca^{2+}-ionophore.
AACOCF$_3$	Arachidonyltrifluoromethyl ketone. Potent and selective inhibitor of cytosolic phospholipase A_2.
Apyrase	Nonspecific adenosine tri/biphosphatase (ATPase) that converts ATP into AMP and P_i.
ATP	Both a potentiator and an inhibitor of VRAC. Whereas micromolar concentrations of extracellular ATP (10-100 μM) were able to increase the conductance through VRAC, millimolar concentrations (>1mM) were found to effectively block the conductance.
C.botulinum neurotoxin F	zinc endopeptidase causing specific cleavage of vesicle associated SNARE protein VAMP/synaptobrevin.
C. botulinum exoenzyme C3	Enzyme that inactivates Rho A, B and C (not Rho D) through ADP-ribosylation.
BAPTA-AM	A cell permeable calcium chelator.
Brefeldin A	An inhibitor of vesicle transport between the ER and Golgi. It disturbes intracellular membrane flow and Golgi function.
4-BPB	4-Bromophenacylbromide, an inhibitor of the phospholipases A_2 and PLC.
1-butanol	A lineair alcohol, used to block the formation of phosphatidate by phospholipase D (PLD).
Chloroquine	Prevents the acidification of late endosomes and lysosomes, thereby inhibiting lysosomal degradation of proteins.
Cimetidine	Competitive antagonist of histamine H1 receptors. Cimetidine also inhibit the Organic Cation Transporter OCT1/2 and is a substrate for OAT3.
Cyanine-863	An Inhibitor of OCT2.
D609	Inhibitor of phosphatidylcholine (PC)-specific phospholipase C.
Decynium-22	An inhibitor of OCT2.
DIDS	Chloride channel blocker.
Genistein	An isoflavonoid that acts as a general inhibitor of tyrosine kinases.
GF-109203X	A selective inhibitor of protein kinase C.
Hg^{2+}	Able to bind to an SH group in the pore region of several aquaporins and to affect channel behaviour.
KT5926	A potent and selective inhibitor of myosin light chain kinase (MLCK).
LY294002	A specific inhibitor of phosphatidylinositol 3-kinase.
Methotrexate	An inhibitor of OATs.
NEM	Binds to the SNARE protein SNAP 25 and prevents vesicle fusion.
PACOCF$_3$	An inhibitor of Ca^{2+}-independent PLA_2 .
PD098059	MEK1/2 inhibitor, prevents the activation of Erk-type map kinases.
Phenobarbital	Promotes upregulation of the expression of OATP2, MRP3 and P-glycoprotein (Pgp).
Phloretin	An inhibitor of urea and glucose transport.
PMA	A phorbol ester able to activate several iso-enzymes of the protein kinase C (PKC) family.
Probenecid	Inhibits the secretion of weak acids by the nephron. Also a blocker of OAT1, 3 and 4.
Suramin	Purinoceptor antagonist and potent inhibitor of VRAC. Suramin also inhibits the cell-surface binding of various growth factors, including EGF, PDGF and TGF-β.
Tamoxifen	P-glycoprotein inhibitor and anion channel (VRAC) blocker.
TFP	Calmodulin antagonist.
Tunicamycin	A specific inhibitor of N-acetylglucosamine (GlcNAc) transferase, prevents protein glycosylation.

Quinidine	K^+- channel blocker, also an inhibitor of OCT1, 2 and N2, OAT3 and P-glycoprotein.
Quinacrine	A phospholipase A_2 inhibitor.
Verapamil	Ca^{2+}-channel blocker, also an inhibitor of OCT1 and Pgp.
Wortmannin	A specific inhibitor of phosphatidylinositol-3-kinase.
Y27632	A selective inhibitor of ROCK kinase.

An introduction to cell volume regulation

General introduction

This introductory chapter focuses on general concepts of salt and water homeostasis in mammalian cells as well as on the characteristics of the ion channels and transporters involved. In addition, basic physical principles underlying these physiological responses are briefly reviewed.

Principles of solute movement

All living cells have the ability to keep their intracellular osmolarity and ion composition within a narrow range. This defence mechanism allows the cell to let cellular processes, like enzyme activity, gene expression and DNA replication, function independently of (changes in) the extracellular environment. The basis for this phenomenon is the plasma membrane that separates the cell from its surroundings.

Diffusion

One of the most fundamental processes in nature is the spreading of solids, liquids, gases and solutes to distribute themselves uniformly in space. The reason for this movement is the thermal energy of the atoms that makes the molecules vibrate. The collisions between the molecules result in a net movement from regions with a high concentration to regions with a lower concentration.
Although lipid membranes are able to limit particle movement they are not perfect barriers. Small molecules can pass the membrane by simple diffusion (for example CO_2, O_2), other molecules, however, utilize specialized permeation pathways like channels or transporters.

Diffusion

Free moving molecules and atoms (that means they are not part of a crystal structure and no additional forces are involved) tend to distribute themselves over an as large as possible territory. This is formulated in the Second Law of Thermodynamics or Entropy law.

Osmosis

The diffusion rate of compounds through biomembranes depends on their size, charge, side groups and solubility in water and lipid. The diffusion rate is also a unique property of the membrane involved. Because biomembranes differ in composition, the exchange rate not only varies between organisms, tissues and cells but can even differ within a single cell. For example in the intestine, the

permeability characteristics of the luminal- and basolateral membrane differ, and this plays a crucial role in transcellular movement of nutrients from the lumen to the blood.
Biological membranes are considered to be semi-permeable. In general, only small molecules (ions and solvents) can permeate. If a semi-permeable membrane separates compartments with different solute concentrations, a net redistribution of the solvent (e.g. water) is initiated that will eventually counterbalance the difference in concentrations (osmosis).

Osmosis

When a selective membrane is present between two compartments with different concentrations of non-permeating solute(s), a redistribution of the solvent will occur to obtain the lowest free energy possible (a consequence of the Second Law of Thermodynamics).

Donnan equilibrium
This special situation arises when the membrane is not equally permeable for all ionic solutes present.

Gibbs-Donnan equilibrium

A system of dissociating charged complexes with at least one impermeable component gives rise to a partial redistribution of the permeable solutes.

The Donnan effect has several important consequences. Firstly, at equilibrium, there is an asymmetric distribution of permeable ions across the membrane resulting in an electrical gradient (membrane potential). As a result, the concentration gradient and the electrical gradient will push the potassium ions in opposite directions. Secondly, because of the presence of proteins intracellularly, there are as a whole more (osmotically-active) particles inside the cell then outside. Therefore, due to osmosis, cells have a tendency to increase their volume.

It will be clear that due to variations in intra- and extracellular concentrations the plasma membrane will be exposed to a varying osmotic force which can potentially lead to cell shrinkage or cell swelling and eventually cell rupture.

Evolution of cell volume regulation

Bacteria, fungi, algae and plants are less susceptible to changes in the osmolarity of their environment due to the presence of a rigid cell wall. This, however, prevents them only from cell swelling but not from shrinkage. Normally these

cells maintain their shape by the osmotic imbalance between the cytosol and their environment (turgor). Plants living near the sea prevent shrinkage by an increased intracellular osmolarity. Bacteria are also capable of changing their intracellular osmolarity in response to alterations in their environment (194). This ability makes them able to survive in a wide diversity of habitats.

Animal cells have flexible but fragile membranes which require osmotic volume regulation to prevent them from collapse by shrinkage or lysis by swelling. Most marine invertebrates are iso-osmotic with their environment. Their osmolarity matches the concentration of the surrounding seawater by adjusting the intracellular ion concentrations and/or the concentrations of organic osmolytes. During evolution, organisms developed the ability to regulate actively their internal osmolarity, making it possible to exploit brackish water habitats with a variable salinity (for example shrimps, barnacles, copepods, lobsters, crabs and woodlice). With an exception of the elasmobranches (sharks, dogfishes, skates and rays), the osmolarity of the body fluids in modern vertebrates is reduced as compared to seawater. All vertebrates have lowered their intracellular salt concentrations; the elasmobranches, however, remain iso-osmotic with their environment by the presence of urea or trimethylamine oxide (TMAO) in their body fluids (123).

Multicellular organisms with a closed circulation system are less dependent on the osmolarity of their environment. The cells are in contact with the interstitial fluid (ISF) which is a subcompartment of the extracellular fluid (ECF). The ECF includes interstitial fluid, body cavity fluids (e.g. coelom), plasma, lymph, fluid of bone and dense connective tissue and transcellular fluid (16). Blood and lymph are used to transport oxygen, carbon dioxide, food and waste to and from the cells. Composition of the ECF is controlled by respiratory, urogenital and digestive organs. To fulfill this function, individual cells of these organs contain a wide variety of transporters and channels in their plasma membranes.

General mechanism of volume regulation in vertebrates

Changes in volume may cause damage to the cell and result in a loss of its functions. Several mechanisms have been recognized to be involved in the recovery from rapidly occurring osmotic imbalances. These will be described below (**2.1** and **2.2**). In addition, cells are able to adapt to chronic changes in osmolarity.

Restoring cell volume is accomplished by a fast activation of potassium, sodium and chloride channels, exchangers and transporters. The osmotically active molecules are illustrated in Fig. 1. Depending on the difference between the intra-

and extracellular osmolarity two situations can be recognized: cell swelling, due to a relatively higher intracellular tonicity, and cell shrinkage, due to a lower intracellular tonicity. In general, two regulatory mechanisms have been recognized, the Regulatory Volume Decrease or RVD, activated upon osmotic cell swelling and the Regulatory Volume Increase or RVI, triggered by cell shrinkage (118, 189, 337).

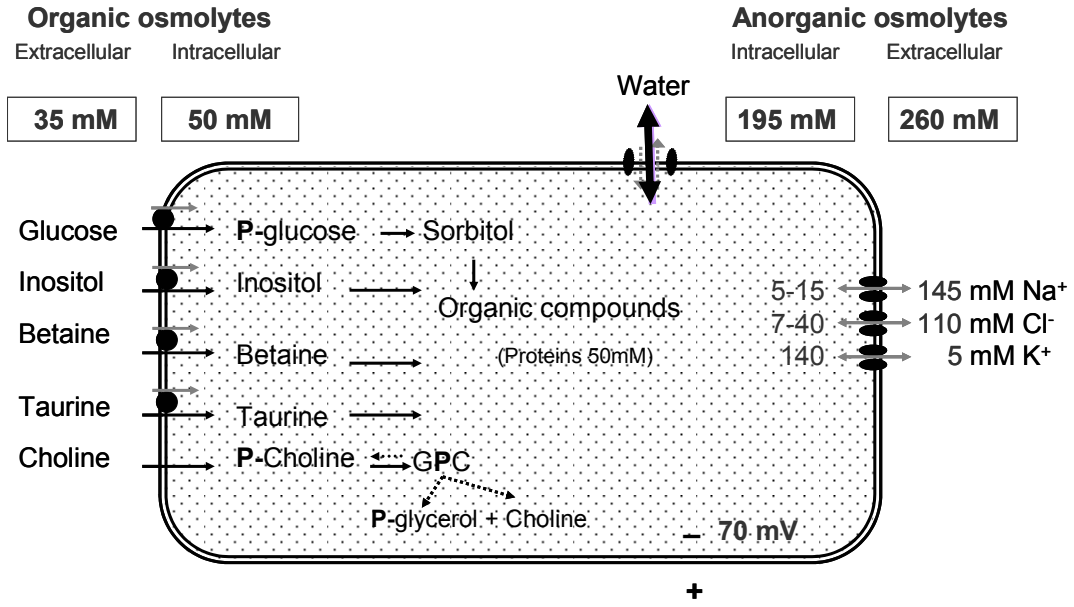

Figure 1. Schematic overview of the major osmotically active compounds.

Regulatory Volume Decrease

The transport systems activated by osmotic cell swelling can be divided into four different groups.

Firstly, the group of cation channels; they conduct positively charged potassium ions. In a number of cell models, activation of a calcium-activated K^+ channel seems to slightly precede the activation of Cl^- channels (97). The human hIK1 channel, an isoform of the intermediate-conductance calcium-activated potassium channel (IK) is proposed to play a role in volume regulation in epithelial cells. Inhibitors of these channels, and of the related small-conductance (SK) channels, significantly reduce the rate of the RVD (121, 333).

Secondly, the group of anion channels; they conduct negatively charged chloride ions. These chloride channels are activated concomitantly with the potassium channels. Resulting in the electro-neutral transport of KCl. Anion channels not only play an important role in cell volume regulation, they are also important determinants of the membrane potential and involved in transepithelial salt and water transport (211).

Thirdly, in selected cell models such as human erythrocytes, the K-Cl co-transporter has been recognized as the primary release mechanism for KCl (141). Finally, osmotic cell swelling results in the release of organic osmolytes (taurine, betaine, inositol) through a yet unidentified "organic osmolyte release channel". Figure 2 illustrates the cotransporters and channels activated as a result of swelling.

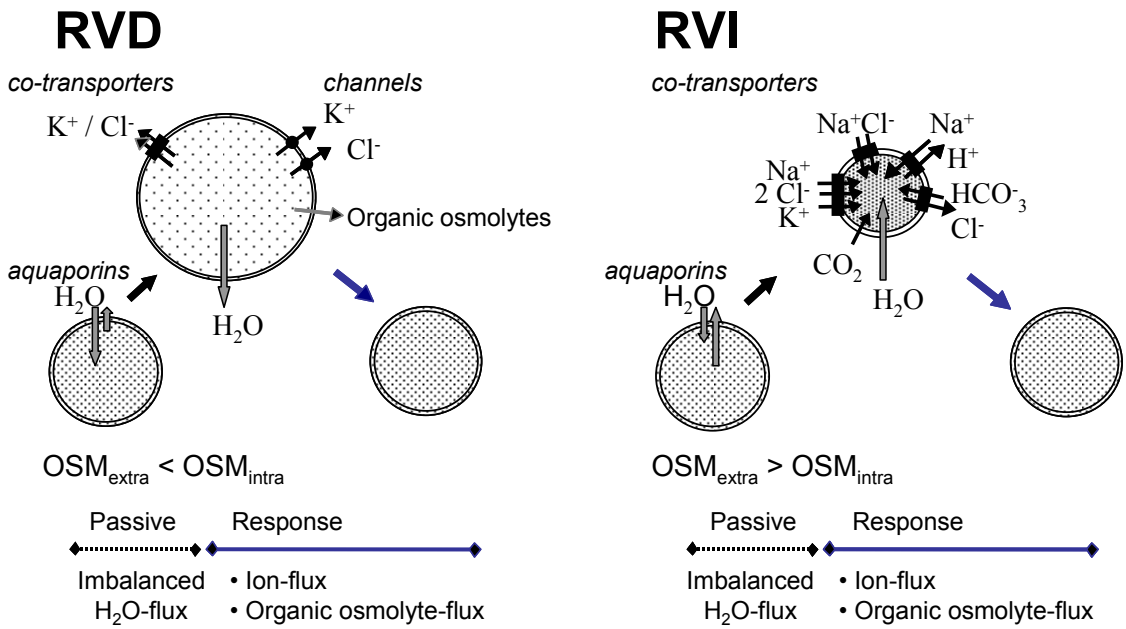

Figure 2. Regulatory Volume Decrease or RVD (Left) and the Regulatory Volume Increase or RVI (Right)
A decrease in extracellular osmolarity or an increase in intracellular osmolarity will result in a volume increase. This will immediately lead to the activation of mechanisms that result in the efflux of water till volume is restored. Ion channels are activated resulting in a loss of potassium and chloride. Additionally organic compounds can be released in some cell types by co transporters.
A decrease in cell volume lead to the activation of mechanisms that result in an influx of water. Mainly co-transporters are involved. Additionally, sodium and chloride channels can be activated. These processes result in an inwardly oriented osmotic force.

Regulatory Volume Increase

In the majority of cell types, RVI is mediated by the electro-neutral $Na^+,K^+,2Cl^-$ co-transporter or by the concerted activation of Na^+/H^+ and Cl^-/HCO_3^- exchange (140, 228). To date, two isoforms of the $Na^+,K^+,2Cl^-$ co-transporter ($NKCC_1$ and $NKCC_2$) have been reported (44, 70, 235, 262, 236). $NKCC_1$ is expressed ubiquitously and activated by cell shrinkage. The $NKCC_2$ is expressed exclusively in the kidneys; in the epithelial cells of Henle's loop and in the macula densa (189).
As yet, six mammalian isoforms of the Na^+/H^+ exchanger (NHE-1–6) have been characterized (for review see Refs: (37, 229, 331), all of them activated by cell shrinkage, except for NHE-3 which is inhibited (11, 125, 189). In contrast to invertebrates, organic osmolytes do not to play an important role during the RVI in mammals (189, 332). Figure 2 also illustrates the shrinkage induced channels and transporters.

The molecular identity of the channels activated during the RVD

In this chapter, putative molecular candidates for the channels involved in the efflux of K^+, Cl^- and organic osmolytes will be discussed. In addition, we will address the aquaporins, channels involved in water transport.

The potassium conductance

From a genetic point of view, the number of potassium channels that potentially can be expressed is rather large. For example the *Caenorhabditis elegans* genome contains at least 80 K^+ channel encoding genes and several of them are subject to elaborate alternative splicing (35, 185). Predictions for *Drosophila melanogaster* and humans are within the same order of magnitude.

Recently, it has been reported that in Intestine 407 cells only Ca^{2+}-dependent intermediate K^+ channel (IK) are expressed and activated upon osmotic cell swelling (224, 226, 333). In parotid glands however, the IK channels do not play an important role in cell volume regulation (208). In murine tracheal epithelial cells, the IsK (MinK) potassium channel seems to play a major role (160).
In other cell types, like osteoblasts and the bronchial epithelial cell line 16HBE14o-, activation of MaxiK-like channels has been detected (62, 338), whereas, in Ehrlich ascites tumor cells an mTASK-2 current was found (210).

The chloride conductance

Chloride channels are found in nearly every cell type known. They are involved in volume regulation, transport across epithelia, acidification of intracellular organelles, stabilization of cell membrane potential and signal transduction. Whole cell patch clamping of hypo-osmotically-stimulated cells reveals a robust anion conductance with marked electophysiological characteristics. These include [i] a prominent outwardly rectification; [ii] voltage-dependent inactivation at membrane potentials above 60 mVolts; [iii] a permeability sequence of $I^- > Cl^- > HCO_3^- >$ taurine > gluconate > aspartate, corresponding to Eisenman's sequence I (343) and [iv] sensitivity to the chloride channel blockers SITS and DCPIB (42). For review see Nilius (211). For a more complete list of inhibitors that affect VRAC see Table 1.

Although the biophysical characteristics of the Volume Regulated Anion Channel (VRAC) are well established, the molecular identity of the channel(s) still remains an enigma. Several membrane proteins have been proposed to function as VRAC

itself or as a crucial regulator of VRAC. These include the P-glycoprotein (90, 256, 257, 319), members of the ClC family ClC-2 (78, 173, 344) and ClC-3 (49) and ICl_n (200, 234). To date, evidence is provided for each of these proteins in selected cell models. Neither of them however, functions as an "universal" osmo-sensitive Cl^- channel or as its direct regulator (for review see (119, 211)).

Pharmacological agents / application	Compound
Antioestrogens	Clomiphen
	Nafoxidine
	Tamoxifen
Antimalarials	Mefloquine
	Quinine
Male contraceptive (but toxic)	Gossypol
Treatment of light astma	Chromones
Inflamtion suppressor, pain killer	Niflumic acid
Antiarrhythmic	Quinidine
T-type Ca^{2+} channel blocker	Mibefradil
Seeping sickness	Suramin against sleeping sickness and is a reverse transcriptase inhibitor
Inflammatory mediators	Arachidonic acid
Cyclooxygenase and lipoxy genase inhibitor	Nordihydroquaiaretic acid (NDGA); Chaparral
Band 3-mediated anion exchange inhibitor	4-Acetamido-4-isothiocyanostilbene (SITS)
	4-4′-Diisothiocyanatostilbene-2,2′disulfonic acid (DIDS) stilbene disulfonates
Cl^- channel blocker	Anthracene-9-carboxylic acid (9-AC),
	5-Nitro-2-(3-phenylpropylamino)-Benzoate (NPPB)
	4-4′-Diisothiocyanatostilbene-2,2′disulfonic acid (DIDS) stilbene disulfonates
	4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB) (42)
	Oxalon dye bis-(1,3-dibutylbarbituric acid)-pentamethine oxonol (diBA-5-C4)
	4-Acetamido-4-isothiocyanostilbene (SITS)
Mild PKA activator	1,9-Dideoxy-Forskolin
P_2Y receptor agonists / antagonist	Extracellular nucleotides; ATP
	Basilen blue (or Reactive blue) (ref 48)
	Intracellular Mg^{2+}
	Protons (refs 215, 217, 218, 264)
	Alixarenes

Table 1. List of established VRAC inhibitors.
The pharmacology of VRAC has been investigated more extensively than any of other Cl^- channel. Above table shows an overview of the inhibitory compounds (for a review see (211)).

The KCl-cotransporters

KCl-cotransporters have a low transport capacity and, in general, play only a secondary role during RVD. In most cell types the cotransporters are responsible for the release of ions initiated by small fluctuations in the osmolarity. Large osmotic differences result in activation of the potassium and chloride channels. (141, 196). In erythrocytes, however, the KCl-cotransporter is the major transporter involved in the RVD (142). There are at least four KCl-cotransport (KCC) genes. Splice isoforms have been reported (196).

The organic osmolyte release pathway

The advantage of organic osmolytes, like polyols, methylamines and amino acids over ions in cell volume regulation is that changes in their concentrations do not significantly affect the membrane potential (20, 93, 345). This is of particular importance for excitable cell types, such as neurons and muscle cells. (20, 93). Figure 1 summarizes the most important osmo-active molecules involved in cell volume regulation.

Uptake of organic osmolytes is facilitated by a number of transporters, which includes the multidrug resistance proteins (MDR), the taurine transporter (TauT), as well as members of the family of organic anion (OAT) and organic cation (OCT) transporters (9, 88, 90, 81, 82, 136, 294, 319).

Unlike the wealth of information available about the uptake of organic osmolytes, as yet little is known about the molecular identity of the organic osmolyte release pathway (see **Chapter 3**).

Aquaporins

A prerequisite for osmotic cell swelling is the uptake of water. The first models proposed that water passes the plasma membrane by diffusion, although the lipid membrane is hydrophobic and only moderately permeable for water. More recently specialized channels for water transport have been identified: the aquaporins (AQPs): for reviews see (1, 7, 137, 209, 289, 325). Expression of these channels is not restricted to organs with a high water transport rate (i.e. the kidneys); several members of the AQP family are ubiquitously expressed. Sofar only AQP0 and AQP2 are related to diseases (272). Mutations in AQP0 lead to cataract (276), whereas mutations in AQP2 lead to autosomal recessive and dominant nephrogenic diabetes insipidus (NDI). Recently it has been proposed that AQPs are involved in active water displacement over cell membranes (7). The osmotic water permeability coefficient (μm/s), which is defined as the net flow of volume across a membrane in response to a hydrostatic or osmotic force, enables quantification of water transport. A value higher than ~30 μm/s is indicative of channel mediated water transport (197). The relative abundancy of AQPs may be a good explanation why some cells expand much faster than others upon hypo-osmotic stimulation.

In addition to water, some members (the aquaglyceroporins) of the aquaporin family (AQP 3, 7, 8 and 9) are also able to conduct small neutral solutes like glycerol, sugars, urea and polyols. Furthermore, Yasui et al. (1999) demonstrated that AQP6 can conduct chloride ions (108, 346).

Signal transduction

This chapter deals with the signalling events triggered by osmotic cell swelling. This will include the putative involvement of an osmo-sensor as well mechanisms leading to activation of the channels and transporters involved in correcting alterations in cell volume.

The osmo-sensor

In the yeast *Saccharomyces cerevisiae*, the compensatory response to osmotic stress is under control of 2 distinct transmembrane osmoceptors: an osmosensor called Sho1p and a "two-component system" consisting of the histidine kinase Sln1p and the response regulator Ssk1p. A transmembrane histidine kinase, the ATHK1 protein (*Arabidopsis thaliana* Histidine Kinase) structurally related to the yeast Sln1p, was recently found in plant (317). Transcription of the ATHK1 gene was found to be upregulated in response to changes in external osmolarity. Expression of ATHK1 gene into yeast lacking functional Sln1p and Sho1p (*sln1Δ /sho1Δ* double mutant) prevented high osmolarity-induced cell death. In mammalian cells, no evidence is available that a related osmosensor is involved in volume sensing; indeed, signalling through “two-component systems” is very rare in vertebrates. Alternative pathways of volume sensing may involve macromolecular crowding and cell membrane or cytoskeletal stretch (21).

Mechanisms of ion channel activation

Activation of ion channels can be achieved by various ways (Fig. 3). Regulation can involve one or more of the following mechanisms: [i] direct activation by membrane stretch (224); [ii] signaling via receptor activation and/or a cascade of enzymatic reactions (25, 329); [iii] regulation through interaction with the cytoskeleton; and [iv] recruitment through vesicle (endosome) fusion.

Mechano-sensitive channels are expressed by many different cell types (189); examples are the *Escherichia coli* non-selective cation channel MscL (239) as well as the recently cloned OTPRC4 (288). In addition to direct activation by membrane stretch (i.e. reduced lateral pressure), enfoldment of the cell membrane as a consequence of cell swelling may expose channels and regulate their activity. Because mammalian cells have a large membrane area with numerous ruffles and wrinkels, this mode of activation may be more likely than regulation by a change in lateral pressure.

As has been discussed above, a specific osmoceptor, leading to activation of a map kinase cascade, triggers the compensatory responses in yeast and plants (317). In mammalian cells, osmotic cell swelling rapidly activate JNK-, p38- and Erk-type

MAP kinases. In line with this, inhibition of VRAC was observed in astrocytes treated with the Erk-inhibitor PD098059 (38). In Intestine 407 cells, however, as well as in IMCD3 renal cells, inhibition of MAP kinases did not affect the RVD (306, 308, 322), arguing against a direct role for these kinases.

Direct activation of ion channels by binding to (components of) the actin cytoskeleton has been reported for a Na^+ channel expressed in toad kidney cells (27). In addition, in melanoma cells lacking actin-binding protein (ABP) the activation of a volume-sensitive K^+ channel was markedly reduced but could be restored by transfecting the cells with the wild type ABP gene (27).

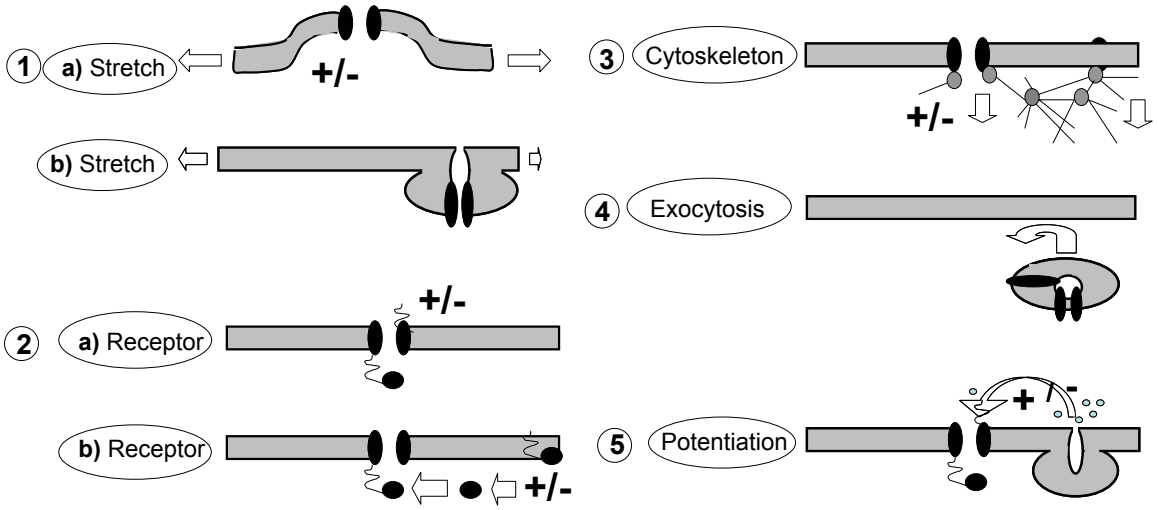

Figure 3. Putative mechanisms of VRAC activation.
1) (**a**) VRAC is activated by mechanical stretch in the lipid bilayer or (**b**) indirectly, by exposing the caveoli. **2)** (**a**) VRAC is activated by an internal domain which functions as a receptor (an osmosensor) or (**b**) a separate receptor signals to the VRAC channels. **3)** Dissociation of a channel from the cytoskeletal proteins activates VRAC. **4)** VRAC is not present in the plasma membrane but VRAC channels are recruited by fusion of VRAC containing vesicles. **5)** A combination of the previous stated mechanisms (stretch together with receptor activation) could be involved as illustrated by a combination of receptor activated and stretch induced exocytosis (2a and 4). This model is an easy explanation for the potentiating effects of several compounds, for example ATP.

Intracellular signalling

Tyrosine kinases

A rapid and transient increase in tyrosine phosphorylation of a number of distinct proteins has been observed after hypo-osmotic stimulation of Intestine 407 cells. These include the focal adhesion kinase p125FAK and members of the MAP (mitogen-activated protein) kinase family Erk-1/2 and p38 (306, 321, 322). Treatment of the cells with tyrosine kinase inhibitors like herbimycin or genistein largely reduced the cell-swelling-induced anion efflux and, vice versa, promoting tyrosine phosphorylation by (per)vanadate-mediated inhibition of phosphotyrosine phosphatases potentiated the anion efflux triggered by non-saturating hypo-osmotic stimulation (310). Taken together, these data demonstrate that protein tyrosine phosphorylation is a prerequisite for eliciting the RVD

response in Intestine 407 cells. Involvement of protein tyrosine phosphorylation in the activation of VRACs has now been established in multiple other cell types, including cardiomyocytes, endothelial cells and lymphocytes (38, 148, 214, 282, 330). The identity of the tyrosine kinase(s) required for the activation of VRACs in Intestine 407 cells has not yet been established. For Jurkat T lymphocytes, however, strong evidence exists that the Src-like $p56^{lck}$ tyrosine kinase is a key regulatory enzyme in the activation of VRAC (148, 295, 310). Activation of volume-sensitive anion channels through a mechanism involving protein tyrosine phosphorylation is not universally observed in all cell models studied: both in ROS 17/2.8 osteoblasts and in CPAE cells tyrosine kinase inhibitors were found ineffective (74, 296).

Small G proteins and the cytoskeleton

Intracellular administration of GTPγS, and thereby activation of guanine nucleotide-binding regulatory proteins (G proteins), was found to activate anion-selective currents in several cell types, including human HT29cl19A colonocytes, and this activation could be inhibited by GDPβS (30, 78, 344). Using *Clostridium botulinum* exoenzyme C3, as a tool to ADP-ribosylate and inactivate $p21^{Rho}$, we have demonstrated that this small G protein is involved in activation of the osmosensitive anion efflux in Intestine 407 cells (224). Involvement of Rho is not restricted to this cell type; comparable results were recently obtained with bovine endothelial cells (49). Rho belongs to the family of small G proteins, of which more than 100 members have now been identified in eukaryotes (13, 84, 297). The 14 mammalian Rho family members are divided into 3 groups (Rho, Rac and Cdc42Rho proteins) with different actions on the actin cytoskeleton (Fig. 4). Whereas Rac and CDC42 primarily regulate the formation of respectively lamellipodia and filopodia (184, 266, 298), Rho plays a role in the formation of actin stress fibers (8, 55, 168, 139, 306). Indeed, hypo-osmotic swelling of Intestine 407 cells rapidly resulted in disruption of the apical actin cytoskeleton and in the formation of stress fibers. To date, several downstream targets of Rho have been identified including citron, PKN, rhophilin, rhotekin, p170mdia, phospholipase D and the Rho kinase p160ROCK, a regulator of myosin light chain phosphatase (for review see (234)). Inhibition of ROCK, using a specific antagonist (Y-27632), prevented the activation of the volume-sensitive Cl^- conductance in bovine endothelial cells, suggesting a role for myosin light chain kinase (MLCK) in channel activation (49). In Intestine 407 cells, however, the ROCK/MLCK pathway is not likely to be involved because treatment of the cells with Y-27632 or KT5926, an MLCK inhibitor, did not affect the development of a volume-sensitive anion efflux (e.g. see **Chapter 5**).

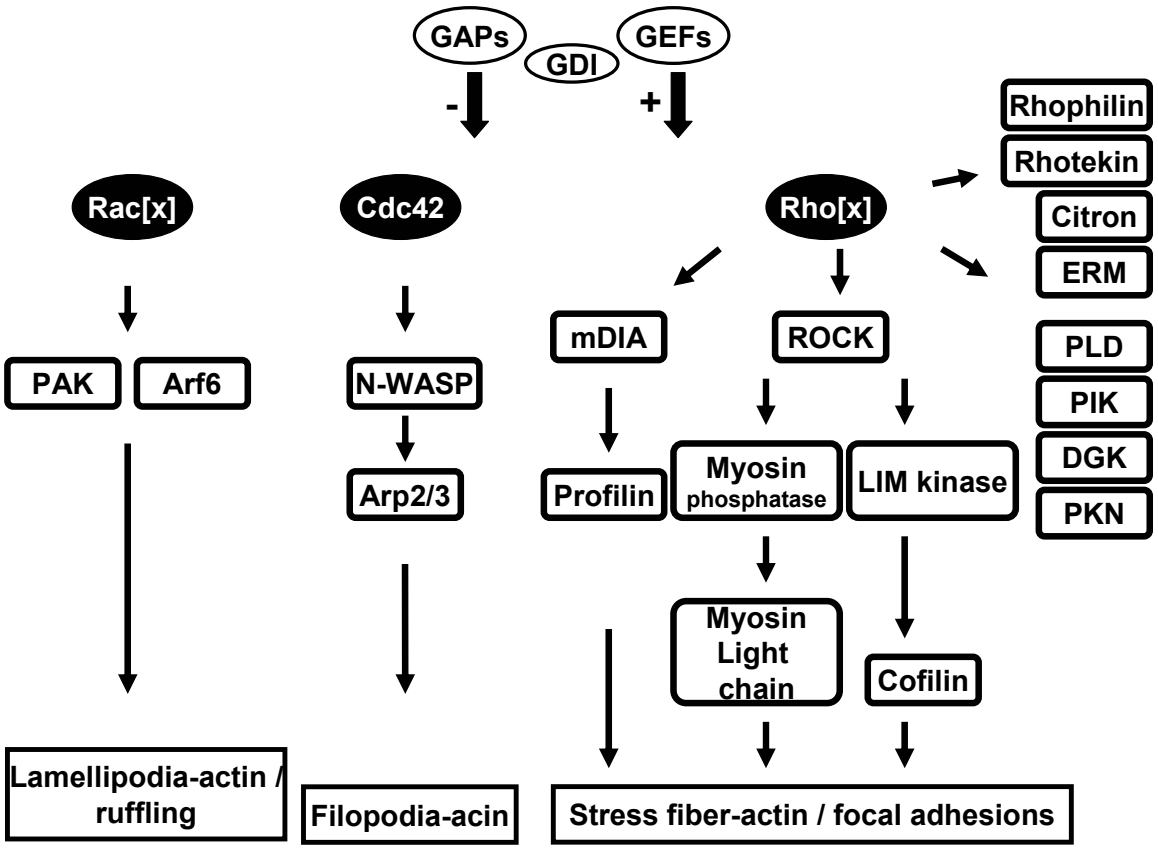

Figure 4. Activation of Rho family proteins and their downstream effectors. Many of these are involved in regulation of the cytoskeleton.

Calcium

In many different cell systems, hypo-osmotic cell swelling leads to an increase in the intracellular free Ca^{2+} concentration (reviewed in (231)). In a number of cell models, a rise in $[Ca^{2+}]_i$ was found to be critically involved in the activation of volume-sensitive Cl^- currents. In Intestine 407 cells, however, as well as in several other cell types, a rise in $[Ca^{2+}]_i$ was not essential for the activation of swelling-induced Cl^- channels (45, 131). Whereas Ca^{2+} not always acts as a direct activator of VRAC, it may play an important role in regulating the magnitude of the compensatory response. Stimulation of Intestine 407 cells with Ca^{2+}-mobilizing (neuro-) hormones resulted in a potentiation of the cell-swelling-induced Cl^- efflux, which was absent in BAPTA-AM loaded cultures (12, 170).

In addition, involvement of Ca^{2+}/calmodulin in rearrangements of the cytoskeleton and ion channnel activation has been reported (36, 169, 188).

Several reports suggest that cytoskeleton reorganization is involved in the hypotonicity-induced increase in intracellular Ca^{2+} levels (84, 269), which, as discussed above, plays an important role in regulating the RVD response in selected cell models. However, Cornet et al. (36) reported opposite results; they found that the increase in $[Ca^{2+}]_i$, was responsible for the reorganization of F-actin through calmodulin and Ca^{2+}-sensitive K^+-channels (36).

Other signaling pathways

In addition to protein tyrosine phosphorylation, cytoskeleton remodelling and Ca^{2+} mobilization, several other messengers have been implicated in activation of the RVD. These include map kinases, protein kinase C, phospholipases (e.g. see **Chapter 5** on phospholipase D involvement), phosphatidylinositol-3-kinase and the formation of arachidonic acid and/or its metabolites (for reviews see (45, 105, 176, 283)). Of particular interest is the role of auto- and paracrine factors. Hypo-osmotic stimulation, like many other forms of cellular stress, may lead to the release of ATP into the extracellular fluid (12). In addition, extracellular ATP, acting through $P2Y_2$-type purinoceptors, has been implicated in activation of the osmosensitive Cl^- current in a number of cell types (257). A more detailed discussion on the mode of ATP release and its role during the RVD response can be found in **Chapters 2** and **4**.

The cell volume alterations are accompanied by changes in nuclear chromatin and phosphorylation of histones and histone-like proteins (267) and consequently changes in cell growth and transformation (100). To date, two cell volume sensitive response elements have been identified: the Fos serum response element and the osmotic response element of Jun (265). In addition, most of the signaling cascades triggered by osmotic cell swelling activate transcription factors. For instance, the Rho GTPase (RhoA, Rac1, CDC42H (100)) is coupled to activation of the transcription factor NFκB (190, 191, 292). Cell volume-induced changes in the activity of MAP kinases may affect gene expression through activation of downstream transcription factors (fig. 5). Ras activates the Raf-Mek1,2-Erk1,2-(RSK) pathway and also Elk-1, Fos and CREB on the response elements SRE, AP-1 and CRE (214).

Osmolarity changes and other types of stress like heat, uv and arsenite initiates the JNK (c-Jun amino-terminal kinase; a stress activated protein kinase or SAPK) and the p38 MAPK phosphorylation cascades (214, 308). Their downstream targets involve Jun, Elk1, ATF2, MAPKAP-2, ATF2 and Max. Recently, the enhancer of the inositol transporter has been found: the "tonicity-responsive enhancer element" or TonE (23).

Additionally, various enzymes are induced by cell volume changes, and many of them are involved in the synthesis of matrix and cytoskeletal proteins (63, 94, 260, 303). During the RVI, increased synthesis of organic osmolytes is also seen, e.g. taurine, betaine, inositol and other amino acid analogs (24, 140). In addition, the expression of several kinases is also regulated; for instance, transcription of the serum and glucocorticoid dependent kinase (sgk) is upregulated during shrinkage (163), resulting in a stimulation of the activity of sodium co-transporters (4), potassium channels and ATPase-pumps (275, 335).

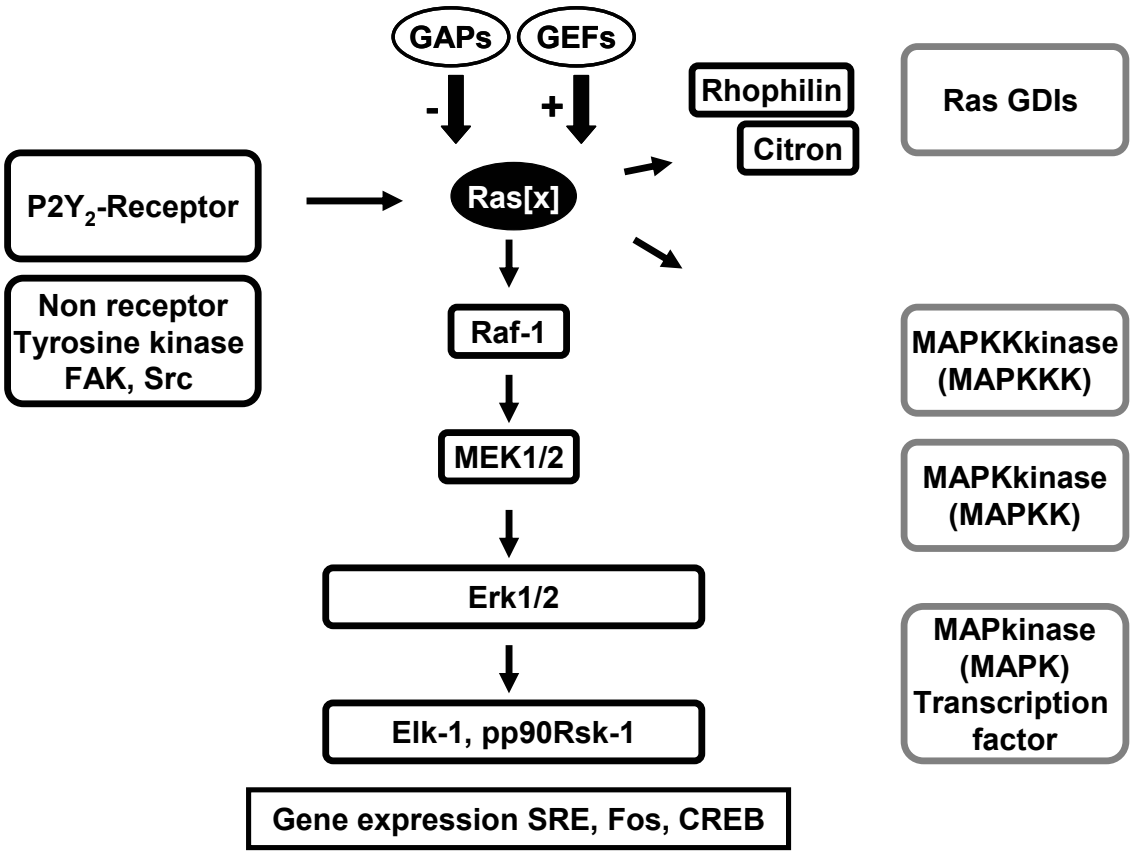

Figure 5. The Extracellular signal Regulated Kinases (ERKs), also known as Mitogen Activated Protein (MAP) Kinases, are activated by diverse extracellular stimuli such as growth factors, cytokines, heat shock, UV and changes in osmolarity. Non-receptor tyrosine kinases such as focal adhesion kinase (FAK) or Src-type kinases modulate this pathway. ERK1 and ERK2 translocate to the nucleus and induce Fos gene expression. Interestingly, it has been proposed that FAK and integrins are involved in mechanosensing (110).

Cell volume regulation and pathophysiology

Most cells experience a constant osmotic stress (242) as a result of: (i) the uptake or release of osmolytes, e.g. nutrients such as sugars and amino acids; (ii) the formation or degradation of macromolecules (proteins, glycogen); or (iii) changes in the osmolarity of the surrounding fluid, as experienced by epithelial cells (kidney, gastro-intestinal tract) or by blood cells traversing the kidney. In addition, osmotic stress can also have a pathological origin (214, 216, 219, 220, 224, 287, 327, 342). For example, as a result of metabolic disturbances, hypoxia or ischemia and lactocidosis, metabolites will accumulate intracellularly, resulting in a tendency to increase cell volume. Alternatively, alterations in plasma osmolarity, due to hyper- or hyponatremia or hyperglycemia, may also impose osmotic stress. For example stroke, cranial trauma, diabetes insipidus, excessive diarrhea and renal failure may limit free water access (145). In addition, a decreased plasma osmolarity due to hyponatremia and decreased water clearance may be the result of congestive heart failure, hepatic cirrhosis, chronic renal failure or the inappropriate secretion of antidiuretic hormone (SIADH) (285).

Aim and scope of this thesis

Activation of volume sensitive chloride channels (VRACs) in Intestine 407 cells was previously found to depend on protein tyrosine phosphorylation as well as on the activity of the small G protein $p21^{Rho}$ and was accompanied by a rapid and transient extracellular release of ATP (321). The aim of this project was to further unravel the signal transduction pathway(s) activated by osmotic cell swelling and their putative role(s) during the RVD (**Chapter 3**). The mechanism responsible for the release of ATP was investigated (**Chapter 4**) as was the role of the Rho effectors p160-Rock, p140-Dia (**Chapter 6**) and phospholipase D (**Chapter 5**). In addition, the contribution of organic osmolyte release in the RVD response was assessed and its regulation was compared with VRAC activation. Finally, based upon the observations that both vesicle cycling and VRAC activation are inhibited by 1-butanol, indicating the involvement of PLD in both responses (**Chapter 5**), and also based on our rather surprising observation that mercury ions dramatically inhibit VRAC activation, a study into the role of water channels or aquaporins was initiated (**Chapter 7**).

Cell volume regulation in intestinal epithelial cells

Sebastian F.B.Tomassen, Hugo R. de Jonge and Ben C. Tilly

Introduction

Most cells have to perform their physiological functions under 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) or changes in the osmolarity of the surrounding fluid. As a consequence of the high permeability of the plasma membrane for water, an osmotic imbalance will immediately lead to a redistribution of intracellular water and, subsequently, to a rapid change in cell volume. Because alterations in cell size are potentially deleterious and may result in a loss of function, almost all cell types have developed compensatory mechanisms. In general, compensation 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 intracellular substances. Whereas the RVI involves the uptake of NaCl through stimulation of the Na^+/H^+- and Cl^-/HCO_3^{2-} exchangers or by activation of Na^+-K^+-$2Cl^-$- and Na^+-Cl^--symporters, the RVD largely depends on the release of KCl, either through specific K^+ and Cl^- selective ion channels or by the activation of K^+-Cl^--symporters (for reviews, see (189, 228, 337)). In addition, osmotic cell swelling is often accompanied by an efflux of small organic osmolytes, such as taurine and betaine, through a release pathway whose molecular identity has not yet been elucidated (for reviews, see (122, 232)).

Because the osmolarity of the interstitial fluid in mammals is carefully regulated, osmotic stress almost always originates from (hormone-induced) alterations in cell metabolism (93). Notable exceptions are the intestinal and renal epithelia that experience alterations in the osmolarity of the luminal fluid, and cells in the circulation, which periodically traverse the osmotic gradients present in the kidneys. To study the RVD in intestinal epithelia, we used monolayers of cultured Intestine 407 cells, an epithelial cell line with stem cell-like properties derived from human foetal jejunum (99), as a model. This cell line is particularly suitable for studying cell swelling-regulated Cl^- channels because no other anion channels, i.e., Ca^{2+}-, voltage-activated or cAMP/protein kinase A-activated CFTR Cl^- channels, are expressed (310). In this review, we will discuss the cellular responses triggered by osmotic cell swelling of Intestine 407 cells, which include the activation of

compensatory osmolyte fluxes and the release of ATP by exocytosis, as well as the signalling pathways involved.

Regulatory Volume Decrease in Intestine 407 cells

Upon hypo-osmotic stimulation, Intestine 407 cells immediately respond with an increase in cell height, indicative of an increase in volume (Fig.1). Rapidly, compensatory mechanisms are activated and within 1 – 2 min the RVD is completed, resulting in an almost full recovery of the original cell volume. Underlying the RVD response is the activation of specific Cl^-- and K^+-selective ion channels. Whereas the K^+ conductance involvement has been identified by the Okada group (333) as a Ca^{2+}-dependent intermediate K^+ channel (I_K) in these cells, the molecular identity of the anion channel has not yet been elucidated. Several potential candidates have been proposed, including MDR-1/P-glycoprotein, ClC-2 and 3 and ICln, none of them, however, have *all* the electrical and pharmacological properties of the Volume Regulated Anion Channel or VRAC (for reviews, see (119, 211)).

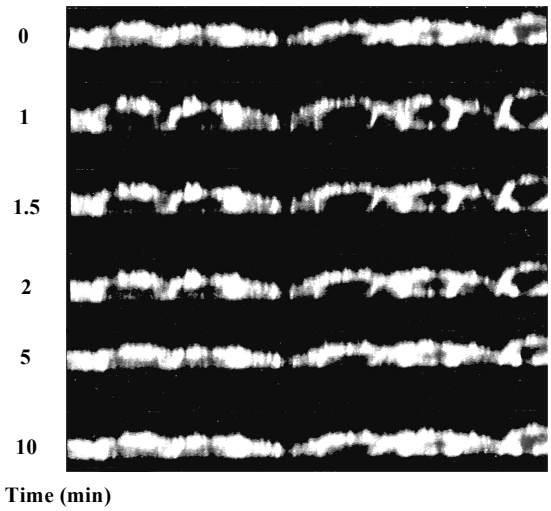

Figure 1. Hypotonicity-induced increase in cell volume.
Cells were loaded with DII-C14 and changes in height were quantitated by constructing optical sections perpendicular to the substratum at the indicated times after changing to a hypo-osmotic medium.

Properties of Volume Regulated Anion Conductance

Although the molecular identity of VRAC remains to be established, the volume-sensitive anion conductance has been studied in numerous different cell types. The electrical characteristics of the conductance are very similar in all models studied and include: 1) a strong outward rectification, 2) a marked inactivation at depolarising potentials, and 3) a permeability sequence that corresponds to the Eisenman's sequence I (SCN^- > I^-> NO_3^- > Br^- > Cl^- > F^- > gluconate$^-$ (119, 211). Activation of VRAC can de inhibited by common Cl^--channel blockers like 4-

acetamido-4'-isothiocyanostilbene (SITS), 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB) and diphenylamine-2-carboxylate (DPC). In addition, extracellularly applied nucleotides, e.g., ATP and UTP in millimolar concentrations as well as the purinoceptor antagonists suramin and Reactive Blue, inhibit the cell-swelling-induced anion conductance (321). This inhibition is most prominent at depolarising membrane potentials and apparently does not involve purinoceptor activation.

Previously, we demonstrated that protein tyrosine phosphorylation is required for the activation of the cell swelling-induced anion conductance (310). Treatment of the cells with tyrosine kinase inhibitors like herbimycin A or genistein largely reduced the cell-swelling-induced anion efflux and, vice versa, promoting tyrosine phosphorylation by (per)vanadate-mediated inhibition of phosphotyrosine phosphatases potentiated the anion efflux triggered by non-saturating hypo-osmotic stimulation (310). Involvement of protein tyrosine phosphorylation in the regulation of VRAC has now been established in several other, but not all, cell types. To date, the identity of the kinase(s) and phosphatase(s) leading to channel regulation has not yet been established in most models studied. For Jurkat T lymphocytes however, strong evidence exists that the Src-like $p56^{lck}$ tyrosine kinase is both essential and adequate for the activation of volume-sensitive anion channels (148). This notion is supported by our observations that in Intestine 407 cells, the hypotonicity-provoked anion efflux is largely reduced after treating the cells with damnacanthal, an inhibitor of $p56^{lck}$ (B.C.Tilly, unpublished results).

Intracellular administration of GTPγS, and thereby activation of G proteins, was found to activate anion-selective conductance in several cell types, including human HT29cl19A colonocytes (309), and this activation could be inhibited by GDPβS (46, 330). Using *Clostridium botulinum* exoenzyme C3 as a tool to ADP-ribosylate and inactivate $p21^{Rho}$, we have demonstrated that this G protein, but not the related $p21^{Ras}$ or $p21^{Rac}$, is involved in the activation of the osmo-sensitive anion efflux in Intestine 407 cells (306). Although a functional Rho pathway is required for the opening of osmo-sensitive anion channels, recent studies in bovine endothelial cells, however, have shown that Rho activation alone is not adequate (29). Most plausibly, Rho exerts its function through the induction of cytoskeletal remodelling. Indeed, immediately after hypo-osmotic stimulation, a rapid and transient remodeling of the actin cytoskeleton has been observed in Intestine 407 cells as well as in other cell types (28, 237, 306). It therefore seems likely that a reorganization of the actin cytoskeleton is necessary but not sufficient to activate VRAC. This notion is supported by the observation that hormones and growth factors capable of activating Rho and inducing cytoskeletal rearrangements are able to potentiate but not to activate the anion conductance

(307). Linking these signalling pathways to an independent, but yet unidentified 'volume sensor' has two important physiological consequences. First, activation of VRAC occurs only during cell swelling thereby preserving the specificity of the response. Second, by coupling the volume response to pathways activated upon hormonal stimulation, small changes in cell volume which may occasionally take place during hormone-induced changes in cellular metabolism (93) are more adequately corrected.

Release of organic osmolytes

In a number of tissues the release of small organic osmolytes contribute significantly to the RVD response. In several cell types, these molecules were even found to be the major determinants of the volume correction mechanism involved (203, 233). In Intestine 407 cells, osmotic cell swelling also leads to a stimulation of an organic anion release pathway, readily permeable to taurine and phosphocholine (311). 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. The hypotonicity-induced release of organic osmolytes was not sensitive to tyrosine kinase or phosphatase inhibition and did not require $p21^{Rho}$ or PtdIns-3-kinase activity (311) indicating that the efflux is regulated independently of VRAC. As compared to the Cl^- efflux, the threshold for activation of taurine release was reached only at a relatively strong hypotonic stimulation (311), suggesting that, in Intestine 407 cells, the release of organic osmolytes acts a second line of defence or may facilitate the re-uptake of ions and the restoration of the membrane potential.

Activation of vesicle cycling and the release of ATP

In addition to the activation of osmolyte release pathways and like many other forms of mechanical stress, osmotic cell swelling promotes the release of ATP, an auto- or paracrinic factor acting through plasma membrane purinoceptors (98, 302, 321, 334). Extracellular ATP has been shown to regulate the RVD in a number of different cell types (153, 302, 334, 255), either by stimulating Ca^{2+} -dependent K^+ efflux (153) or by activating VRAC (255, 334). In Intestine 407 cells, extracellular ATP was not able to directly activate volume-sensitive Cl- channels nor did addition of purinoceptor antagonists or the ATP hydrolase apyrase prevent the development of the conductance (321). However, (sub)micromolar concentrations of ATP were able to potentiate the hypotonicity-provoked Cl^- efflux in a Ca^{2+}-dependent manner (321).

Osmotically-induced ATP release was found to be critically involved in the activation of extracellular signal-regulated protein kinase-1/2 (ERK-1/2) in Intestine 407 cells (321). Although activation of ERK-1/2 as well as other members

of the MAP kinase family (p38 and JNK) has been observed in most cell models studied, their function during the RVD response has not yet been fully understood.

Several mechanisms have been proposed to underlie the release of ATP including (1) leakage due to (local) membrane damage, (2) activation of specific channel(s) or transporter(s), and (3) exocytotic events. Unlike activation of VRAC, ATP release from Intestine 407 cells was abolished after treatment of the cells with 1,2-bis-(2-aminophenoxy)- ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) acting as a buffer of intracellular free Ca^{2+} levels, or with cytochalasin B (321). Therefore, it was concluded that ATP does not permeate through volume-sensitive anion channels. This notion was supported by our observations that the cell-swelling-induced ATP release developed rather slowly and continued for at least 15 min. (321). Notably, the RVD response under these conditions is completed within approx. 2 ~ 3 min (310).

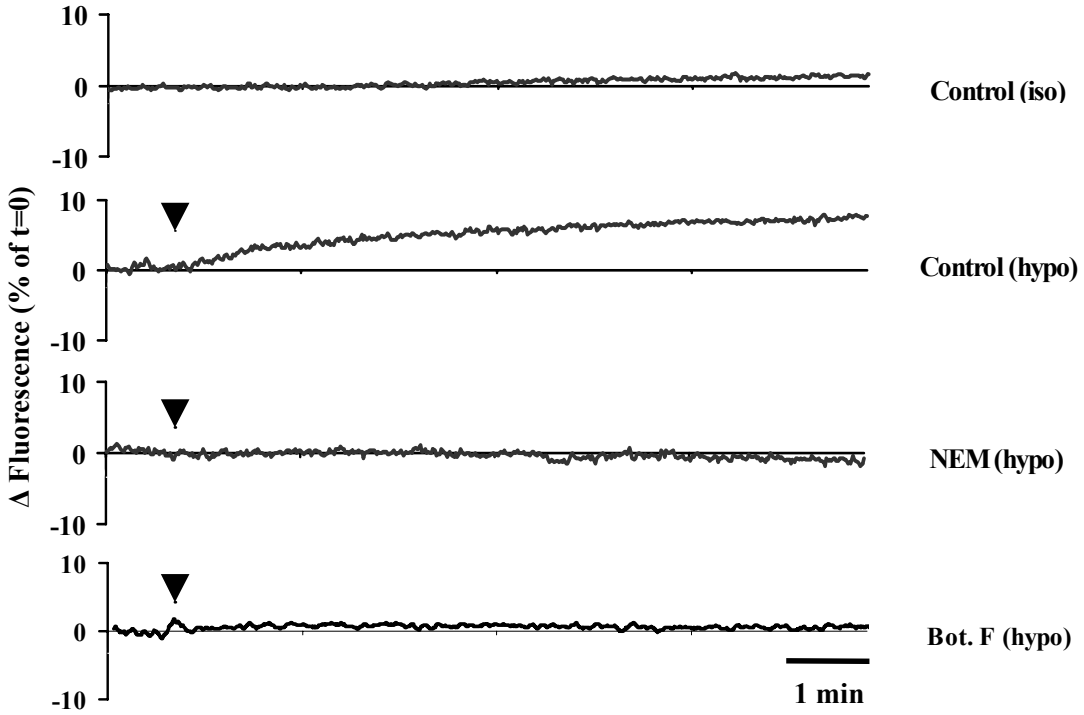

Figure 2. Hypotonicity-provoked change in FM 1 – 43 fluorescence.
Cells grown on coverslips were treated with N-ethylmaleimide (NEM, 1 mM) or Chariot™-conjugated C. botulinum toxin F for respectively 15 min or 2 h. Thereafter, the coverslips were placed in a cuvette with isotonic medium containing 1 µM FM 1-43. Arrow indicates a shift to a hypotonic medium (for experimental details see (324)).

Osmotic swelling of Intestine 407 cells leads to an immediate increase in cell surface membrane area as determined using the fluorescent membrane dye FM 1-43 (Fig. 2). Like the release of ATP, the increase in FM 1-43 fluorescence was abolished after loading the cells with BAPTA-AM or after cytochalasin B treatment, indicating that the increase in surface labelling is dependent on intracellular Ca^{2+} and on an intact actin cytoskeleton (324). To investigate whether

exocytosis is involved in the increase in fluorescence, cells were treated with N-ethylmaleimide (NEM) to inactivate SNAP-25, one of the SNARE (soluble-NEM-sensitive factor–attachment protein receptor) proteins involved in vesicle docking and fusion. Alternatively, *Clostridium botulinum* toxin F was introduced into the cells using the Chariot™ protein delivery kit to inactivate the vesicle associated SNARE protein VAMP2 by proteolytic cleavage (324) As shown in Fig. 2, both NEM treatment and intracellular delivery of *C. botulinum* toxin F almost completely abolished the cell-swelling-induced increase in fluorescence, suggesting that exocytosis occurs rapidly upon osmotic cell swelling.

Because exocytosis is often accompanied by subsequent internalisation of membrane fragments, we studied endocytosis in Intestine 407 cells by quantitating intracellular accumulation of TRITC-dextran. After a distinct lag time of 2-3 minutes, a robust (> 100 fold) increase in rate of endocytosis was observed in hypo-osmolarity-stimulated cells that lasted for approx. 10-15 minutes (250). The delayed onset of endocytosis may suggest that it is triggered by the prior increase in exocytosis. In Intestine 407 cells, however, we found that exo- and endocytosis are regulated independently, because cytochalasin B treatment and BAPTA-AM loading, both strong inhibitors of exocytosis, did not affect the cell swelling-induced endocytosis. In contrast, our observation that the hypotonicity-provoked endocytosis is inhibited by inactivation of VRAC and promoted by K^+ channel blockers and high extracellular [K^+] (Table 1) suggests that membrane depolarisation, a known consequence of VRAC activation (97), may promote or trigger endocytosis.

Table 1. Effects of Cl^- and K^+ channel inhibition on the hypotonicity-induced TRITC-dextran uptake (% of hypotonic control).

		Isotonic	Hypotonic
Control		9 ± 9	100 ± 39
Cl^- channel	SITS	8 ± 7	11 ± 45
	Suramin	5 ± 5	7 ± 5
	ATP (5 mM)	1 ± 1	22 ± 10
K^+ channel	Quinidine	26 ± 22	545 ± 159
	Gd^{3+}	2726 ± 914	5443 ± 1685
	High $[K^+]_{out}$ (50mM)	7 ± 4	229 ± 107

Using *C. botulinum* toxin F treated cultures, a putative role for exocytosis in the release of ATP was investigated. To summarise the results, both basal and hypotonicity-induced ATP release was strongly reduced in toxin treated cultures, supporting the notion that the efflux of ATP is mediated by exocytosis. Furthermore, we found that inhibition of ERK-1/2 activation or apyrase-catalysed

removal of extracellular ATP strongly reduced the rate of exocytosis, suggesting that purinergic activation of ERK-1/2 plays a role in a positive feedback loop that may contribute to the release of ATP from hypo-osmotically stimulated cells (324).

Regulation of VRAC by lipid rafts

Recently, a putative role for lipid rafts, cholesterol-rich domains of the plasma membrane, in the regulation of cell-swelling activated Cl^- channels has been indicated (150, 313, 314). Trouet et al. (314) demonstrated that the volume-sensitive anion conductance was limited in caveolin-1 deficient cell types, but could be enhanced robustly by overexpression of this protein. In addition, they found that transfection of calf pulmonary artery endothelial (CPAE) cells with mutant caveolin-1, thereby disturbing the formation of caveolea, markedly reduced the hypotonicity-induced anion current (313), suggesting that intact caveolea play an important role in VRAC regulation. However, disruption of caveolea in Intestine 407 cells, brought about by extracting plasma membrane cholesterol using 2-hydroxypropyl-β-cyclodextrin in the presence of acceptor lipid vesicles, did not reduce the volume-sensitive anion conductance but was found instead to potentiate the response. This potentiation was also observed in caveolin-1 deficient CaCo-2 colonocytes as well as in sphingomyelinase-treated cells, indicating that these cholesterol-rich micro domains do not play a crucial role in VRAC activation in this cell type.

Alternative targets for the action of cyclodextrin/lipid vesicles are the rapidly recycling endosomes or retrosomes involved in cholesterol transport between intracellular compartments and the plasma membrane (109). Treatment of the cells with cyclodextrin may accelerate endosome cycling in an attempt to replenish plasma membrane cholesterol. Vesicle cycling during the RVD response is not unprecedented, because, as described above, osmotic swelling of Intestine 407 cells is accompanied by exo- and endocytosis. Treatment of the cells with *Clostridium botulinum* toxin F, to abolish exocytosis (c.f. see Section 5), partly inhibited the volume sensitive anion efflux, indicating insertion of additional channels by vesicle fusion with the plasma membrane. Together, these results support a model in which the recruitment of volume-sensitive anion channels to the plasma membrane acts as an important step in the mechanism of VRAC activation and suggest a role for rapidly recycling endosomes in the response to osmotic cell swelling.

Conclusions

A plethora of signalling cascades are activated upon hypo-osmotic stimulation of mammalian cells. In Intestine 407 cells, $p21^{Rho}$ as well as tyrosine kinase(s) and PtdIns-3-kinase were found to be a prerequisite for VRAC activation, whereas

protein kinase C is likely to be involved in the regulation of a distinct organic osmolyte release pathway. In addition, a number of cellular responses are able to modulate the volume-sensitive anion conductance, including Ca^{2+} mobilization, cytoskeletal re-arrangements and vesicle cycling. The molecular mechanism(s) by which these signalling molecules affect ion channel opening is still fragmentary and detailed investigations into the mode of activation, including reconstitution studies, are hampered by the current lack of information about the molecular identity of the channel(s) involved. Our observation that at least part of the anion channel activation occurs through their recruitement from intracellular compartments to the plasma membrane now adds intracellular Cl^- channels to the list of potential VRAC candidates.

Other signalling pathways, like the stress kinases p38 and Jnk as well as the ATP-provoked activation of Erk-1/2, are apparently not involved in channel regulation. Although their physiological role in the RVD response remains to be elucidated, it is tempting to speculate that they may have a function in restoring cellular homeostasis and in maintaining cell viability.

Osmotic swelling-provoked release of organic osmolytes in human intestinal epithelial cells

Relation to chloride channel activation and regulation by PKC

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Abstract

Human Intestine 407 cells respond to osmotic cell swelling by the activation of Cl^-- and K^+-selective ionic channels, as well as by stimulating an organic osmolyte release pathway readily permeable to taurine and phosphocholine. Unlike the activation of volume-regulated anion channels (VRAC), activation of the organic osmolyte release pathway shows a lag time of ~30–60 s, and its activity persists for at least 8–12 min. In contrast to VRAC activation, stimulation of organic osmolyte release did not require protein tyrosine phosphorylation, active $p21^{rho}$, or phosphatidylinositol 3-kinase activity and was insensitive to Cl^- channel blockers. Treatment of the cells with putative organic anion transporter inhibitors reduced the release of taurine only partially or was found to be ineffective. The efflux was blocked by a subclass of organic cation transporter (OCT) inhibitors (cyanine-863 and decynium-22) but not by other OCT inhibitors (cimetidine, quinine, and verapamil). Brief treatment of the cells with phorbol esters potentiated the cell swelling induced taurine efflux, whereas addition of the protein kinase C (PKC) inhibitor GF-109203X largely inhibited the response, suggesting that PKC is involved. Increasing the level of intracellular Ca^{2+} by using A-23187- or Ca^{2+}-mobilizing hormones, however, did not affect the magnitude of the response. Taken together, the results indicate that the hypotonicity-induced efflux of organic osmolytes is independent of VRAC and involves a PKC-dependent step.

Introduction

Because of the water permeability of the plasma membrane, perturbations of the osmotic equilibrium between the cell and its surrounding medium result in an

immediate change in cell volume. To avoid the potential deleterious consequences of cell shrinkage or swelling, most eukaryotic cells have developed compensatory mechanisms that involve the activation of ion channels and/or transporters in the plasma membrane (for review, see Refs. (140, 147, 286, 287)). Hyposmotic stimulation of human Intestine 407 epithelial cells leads to a rapid, transient increase in cell volume accompanied by the activation of distinct K^+- and Cl^--selective ion channels [i.e., regulatory volume decrease (RVD)] (307). The cell swelling-induced activation of the anion channels involved was found to require protein tyrosine phosphorylation and to depend on active $p21^{rho}$ (213, 310). Similar results were obtained with several other cell models, including vascular endothelial (29, 220) and neuronal cells (52, 53), as well as plant cells (29).

In a number of tissues, the release of small molecules such as taurine, betaine, and sorbitol contributes significantly to RVD. In excitable cells, these metabolites were found to be the major osmolytes released (203, 233). Taurine especially has been implicated as an osmolyte involved in volume regulation (14, 65, 102).

As is the case for volume-regulated anion channels (VRAC), the molecular identity of the organic osmolyte release pathway has not been elucidated yet (for review, see (212)). In a number of cell types, the efflux of organic osmolytes and anion conductance was regulated similarly, suggesting that a single release pathway is involved [i.e., volume-sensitive organic osmolyte and anion channel (VSOAC)] (89, 115, 116, 117, 195, 263). In other cells, however, distinct pathways and/or transporters have been proposed (195, 238). Indeed, in hippocampal slice preparations, at least two pathways for taurine release have been identified that differ in their kinetics of activation/inactivation and in their sensitivity for inhibitors (67).

Using Intestine 407 cells, we investigated the hypotonicity-provoked release pathway for organic osmolytes and compared its properties with those of VRAC activation. This cell line was particularly suitable for this study because no plasma membrane Cl^- conductances other than VRAC, such as CFTR and voltage-sensitive or Ca^{2+}-activated anion channels, are expressed (98, 307). In this article, we report that osmotic swelling of Intestine 407 cells results in massive release of taurine after a distinct lag period. The results indicate that the efflux of taurine is regulated independently of VRAC and uses a signalling pathway involving protein kinase C (PKC).

Materials and methods

Materials

Radioisotopes ($^{125}I^-$, ^{3}[H]-taurine and ^{3}[H]-choline) were purchased from Amersham Netherlands B.V. (s'Hertogenbosch, The Netherlands). A-23187, GF-109203X and LY-294002 were obtained from Molecular Probes (Eugene, OR), Biomol (Plymouth Meeting, PA) and Biotechnology (Lake Placid, NY), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Intestine 407 cells were routinely grown as a monolayer in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 25 mM Hepes, 10% FCS, 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. Before the experiments, cells were serum-starved overnight.

Radio-isotope efflux assays

Confluent monolayers of Intestine 407 cells were loaded with 5 µCi/ml $^{125}I^-$, 0.1 µCi/ml ^{3}[H]-choline or 0.1 µCi/ml ^{3}[H]-taurine for 2 h in 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 7.40) and washed three times with isotonic buffer (66 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 123 mM mannitol and 20 mM Hepes, pH 7.4). Hypotonic buffers were prepared by adjusting the concentration of mannitol. Full time courses were prepared by replacing the medium at 1- to 2-min intervals. For inhibitor studies, a single fraction of 8 min was collected. Radioactivity in the media was determined by gamma ($^{125}I^-$) or beta (^3H) radiation counting, and expressed as fractional efflux per minute as previously described (318).
The most abundant ^{3}H-labeled osmolyte released after hyposmotic stimulation of ^{3}[H]choline-loaded cells was found to be phosphocholine (~87% of the ^{3}H-radioactivity), as determined by thin layer chromatography (Silica Gel 60 plates; Merck, Darmstadt, Germany) using a methanol-1.2% NaCl-13.3 M NH_4OH (10:10:1 vol/vol) solvent system (305). In addition to phosphocholine low levels of ^{3}[H]choline and ^{3}[H]glycerophosphocholine (~12 and 1% ^{3}H radioactivity, respectively) were found. In contrast to the release of ^{3}[H]phosphocholine which was increased dramatically after hyposmotic stimulation (isotonic: 184 ± 95 dpm, hypotonic: 1778 ±219 dpm) the release of ^{3}[H]choline and ^{3}[H]glycerophospho-choline (isotonic: 147 ± 22 and 14 ± 2 dpm, respectively, hypotonic: 253 ± 46 and 27 ± 3 dpm, respectively) was only moderately affected by hypotonicity.

Amino acid analysis

Media and lysate fractions of 250 µl were collected corresponding to 1- to 2-min stimuli. Taurine and β-alanine contents of cell lysates and incubation media were analyzed by reverse-phase HPLC after pre-column derivatization with o-phthaldi-aldehyde as previously described (60).

Results

Osmotic cell swelling-induced release of organic osmolytes

Hypo-osmotic stimulation of Intestine 407 cells not only triggers the activation of K^+ and Cl^- selective ionic channels, but also activates an organic osmolyte release pathway as evidenced by the efflux of ^{3}H-taurine from isotope-loaded cells (Figs. 1, 2) and by quantitating the taurine content of the incubation media (Fig. 1). The release of taurine started after a lag period of approx. 30 – 60 s and lasted for at least 8 – 12 minutes, thereafter a slightly elevated efflux remained (Fig. 1). During this period, the cellular content of taurine is reduced by 70% (Table 1). In contrast, the $^{125}I^-$ efflux displayed no apparent lag-period (Fig 1). As compared to the activation of volume-sensitive anion channels, the release of organic osmolytes developed considerably slower and lasted for a longer period (Fig. 1). The organic osmolyte release pathway is not exclusively selective for taurine but is also able to conduct phosphocholine with similar kinetics, as determined using ^{3}H-choline-loaded cells (Fig 1), as well as β-alanine, a precursor of taurine (Table 1).

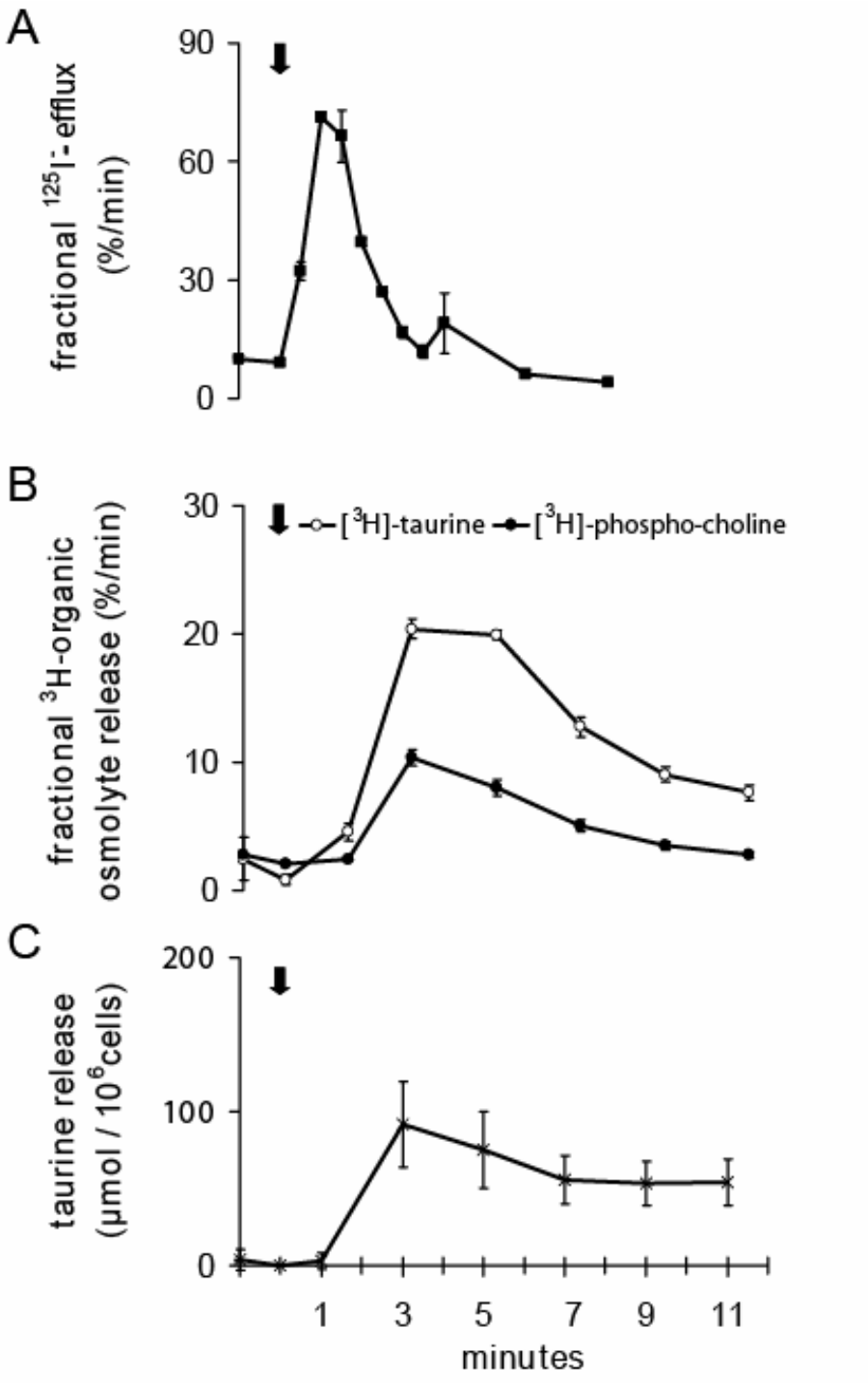

Figure 1. Comparison of osmotic swelling-induced anion- and taurine efflux.
Cultures of Intestine 407 cells were loaded with $^{125}I^-$ (A), ^{3}[H]taurine, or ^{3}[H]choline (B), and fractional isotope efflux (%/min) induced by osmotic cell swelling (30% hypotonicity) was determined as described in 'Materials and Methods'. C: release of taurine into the incubation medium (µmol/10^6 cells). Data are expressed as means ± SD for n = 3. Arrows indicate shift to a hypotonic medium.

		Amino acid release (µmol/10^6cells) taurine	β-alanine
Isotonic			
	medium	19 ± 3.9	< 8
	lysate	471 ± 7.4	44.8 ± 0.26
Hypotonic			
	medium	386 ± 39.3	34.4 ± 0.5
	lysate	157 ± 11.8	21.9 ± 0.49

Table 1. Hypotonicity-induced release of taurine and β-alanine into the medium.
Amount of taurine and β-alanine (µmol/10^6 cells) in cell lysates and bathing solution (8 min incubation) under isotonic and during hypo-osmotic (40%) shock. Data are expressed as mean ± SD for n=3.

To determine the threshold for activation, cultures of cells were treated with buffers of different osmolarity. As shown in Fig. 2, the release of taurine required a reduction in osmolarity of the external medium to ±225 mosmol/l, whereas saturation of the response was observed at an extracellular osmolarity of 100 mosmol/l. In contrast, hypotonicity-induced $^{125}I^-$ efflux was already observed at 290 mosmol/l (i.e., a 10% reduction in osmolarity) and was maximal at 190–225 mosmol/l (Fig. 2). Taken together, the results indicate that the hypotonicityprovoked efflux of organic osmolytes acts as a relatively slow volume-correcting mechanism that starts only when the Cl^- conductance has reached its maximum.

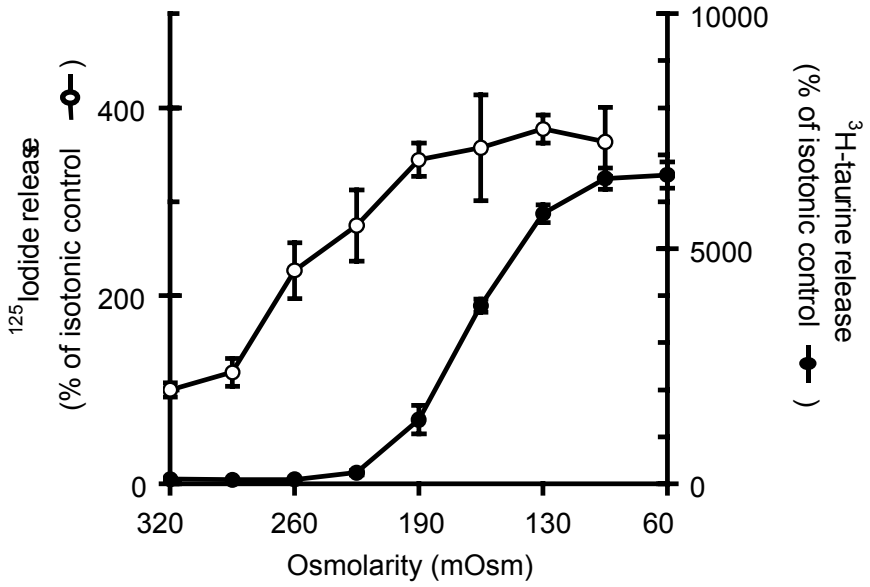

Figure 2. Iodide and taurine efflux from Intestine 407 cells in reponse to a hypotonic challenge.
^{3}[H]taurine (•;closed symbols) and $^{125}I^-$ (o; open symbols) efflux from Intestine 407 cells was determined as described under "Materials and Methods". Data are presented as the increase in osmolyte efflux (peak value) relative to the unstimulated control (mean ± S.D. for n=3).

The organic release pathway does not involve VRAC

To date, the molecular nature of the organic anion release pathway has not been elucidated. In a number of cell models, the release of both Cl^- and organic osmolytes seems to be mediated by the same channel protein; in other models, however, the release of Cl^- and the release of taurine are apparently distinct processes. In an attempt to further discriminate between the release pathways for Cl^- and taurine in Intestine 407 cells, we also studied the effects of pharmacological inhibitors on anion conductance and the release of taurine.

Treating the cells with the purinoceptor antagonist and VRAC inhibitor suramin completely prevented the hypotonicity-induced release of taurine (Fig. 3), suggesting that the same channel and/or transporter is involved. In contrast, however, DIDS and millimolar concentrations of extracellular ATP, which efficiently inhibit VRAC activation (321), were unable to block the taurine efflux (Fig. 3).

Previously, we (307) and others (148) demonstrated that activation of a tyrosine kinase, or inhibition of a phosphotyrosine phosphatase, is a prerequisite for the activation of volumesensitive anion channels. To investigate putative involvement of protein tyrosine phosphorylation in the regulation of the cell swelling-induced taurine release, we treated cultures of Intestine 407 cells with the tyrosine kinase inhibitor genistein and with vanadate, an inhibitor of phosphotyrosine phosphatases. Unlike the activation of VRAC, the release of taurine and phosphocholine was neither increased by vanadate nor reduced after genistein treatment (Fig. 3).

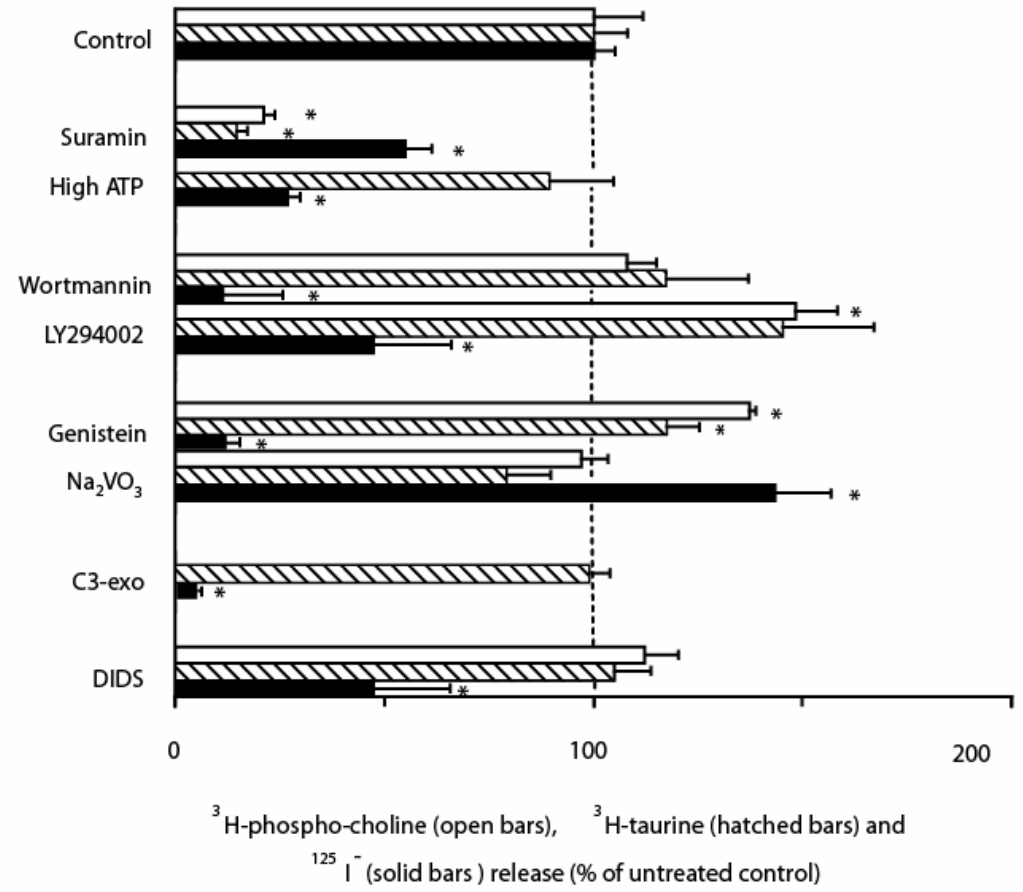

Figure 3. Effects of VRAC inhibitors on the release of taurine and phosphocholine.
Effects of volume-regulated anion channel inhibitors on the release of taurine and phosphocholine. ^{3}[H]taurine (had bars)-, ^{3}[H]choline (open bars)-, and $^{125}I^-$loaded (closed bars) cells were exposed to a hypotonic medium, and fractional isotope efflux was determined as described in "Materials and Methods". Cultures were treated with suramin (100 µM, 5 min), ATP (5 mM, 5 min), wortmannin (10 nM, 1 h), LY-294002 (60 µM, 1 h), genistein (200 µM, 1 h), Na_2VO_3 (100 µM, 5 min), *Clostridium botulinum* C3 exoenzyme (C3-exo; 50µg/ml, overnight incubation), and DIDS (50 µM, 1 h). Data are expressed as the percent change in osmolyte efflux relative to the hypotonic control (mean ± SD for n = 3). *P < 0.05, significant difference from control.

In contrast, a small but significant increase of the efflux of taurine was observed in genistein-treated cells (Fig. 3). These results suggest a pronounced difference in regulation between volume-sensitive Cl^- channels and organic osmolyte release pathways. To further substantiate this notion, phosphatidylinositol 3-kinase (PtdIns 3-kinase) and $p21^{rho}$, both of which are critically involved in VRAC activation, were inhibited using respectively wortmannin or LY-294001 and Clostridium botulinum C3 exoenzyme. Again, wortmannin and LY-294001, as well as C3 exoenzyme treatment, did not affect the cell swellinginduced taurine efflux (Fig. 3). Taken together, these results clearly indicate that the volume-sensitive taurine efflux in Intestine 407 cells involves a different transport protein or channel and is regulated by a pathway independent of tyrosine kinases, PtdIns 3-kinase, and $p21^{rho}$.

Role of organic anion or cation transporters

Movement of taurine and other organic osmolytes across the plasma membrane is mediated by at least four different transport proteins (OATs 1–3 and Taut, (81, 136, 294)). To investigate whether these transporters contribute to the swelling-induced release of taurine and phosphocholine, cells were treated with established inhibitors of each of these transport systems. As shown in Table 2, inhibitors of the OAT family of transporters, such as probenecid, methothrexate, fenobarbital, and tunicamycin, which lead to an intracellular retention of OATs, did not inhibit or only partially inhibited (20–40% reduction) the hypotonicity-induced taurine efflux (104). In contrast, decynium-22, cyanine-863, and quinidine, inhibitors of organic cation transporters (OCTs) (77, 138, 340), strongly diminished the efflux of both taurine and phosphocholine (Table 2).

Treatment	Taurine efflux, (% of control)
Control	100 ± 7
OCT inhibitors	
Cyanine-863 (30 μM)	32 ± 1 *
Decynium-22 (200 μM)	46 ± 8 *
Quinidine (1 mM)	10 ± 3 *
Quinine (80 μM)	109 ± 6
Cimetidine (1 mM)	94 ± 12
Corticosterone (10 mM)	38 ± 17 *
Verapamil (200 μM)	106 ± 12
OAT inhibitors	
Probenecid (1 mM)	73 ± 9 *
Methotrexate (50 μM)	63 ± 11 *
Fenobarbital (200 μM)	79 ± 10
Tunicamycin (5 μg/ml)	80 ± 6

Table 2, ^{3}H-taurine efflux from cultures treated with OCT and OAT inhibitors.
Cultures were loaded with ^{3}[H]taurine, and the fractional efflux was determined as described in 'Material and Methods' the cells were treated with the indicated inhibitors for 30 min, except for corticosterone and tunicamycin, which were added 16 h before the start of the experiment. Data are presented as percentages of the hypotonic control (mean ± SD, n ± 3). *P < 0.05 indicates significant difference from control. OCT, organic cation transporter; OAT, organic anion transporter.

However, several OCT inhibitors (i.e., cimetidine, quinine, and verapamil) were found to be ineffective (Table 2). These combined pharmacological data argue against the concept of a single, "classical" organic osmolyte transporter (OAT or OCT) accounting for the cell swelling-induced release pathway.

PKC activation regulates the release of taurine

Among the cell models studied, considerable differences are observed in the regulation of taurine transport. Signaling pathways including PtdIns 3-kinase, calmodulin-dependent kinase, tyrosine kinases, and PKC have been reported. As described above, the release of taurine from hypotonicity-provoked Intestine 407 cells did not require tyrosine kinase or PtdIns 3-kinase activity, nor did it depend on functional $p21^{rho}$. Loading cultures with BAPTA-AM or treating them with extracellular EGTA hardly affected the magnitude of the response (EGTA: 102 ± 8%, BAPTA-AM: 116 ± 13% of untreated control). In addition, raising the intracellular concentration of calcium ($[Ca^{2+}]_i$) by adding the Ca^{2+} ionophore A-23187 or by stimulating the cells with bradykinin, histamine, or EGF did not potentiate the response to a submaximal hypotonic medium (fractional efflux: 126 ± 8%, 111 ± 9%, 80 ± 22%, and 76 ± 16% of untreated control, respectively).

In the presence of the specific PKC inhibitor GF-109203X, marked inhibition of the cell swelling-induced taurine efflux was observed, and vice versa, activation of PKC by brief treatment of the cells with the phorbol ester PMA increased the response approximately twofold (Fig. 4).

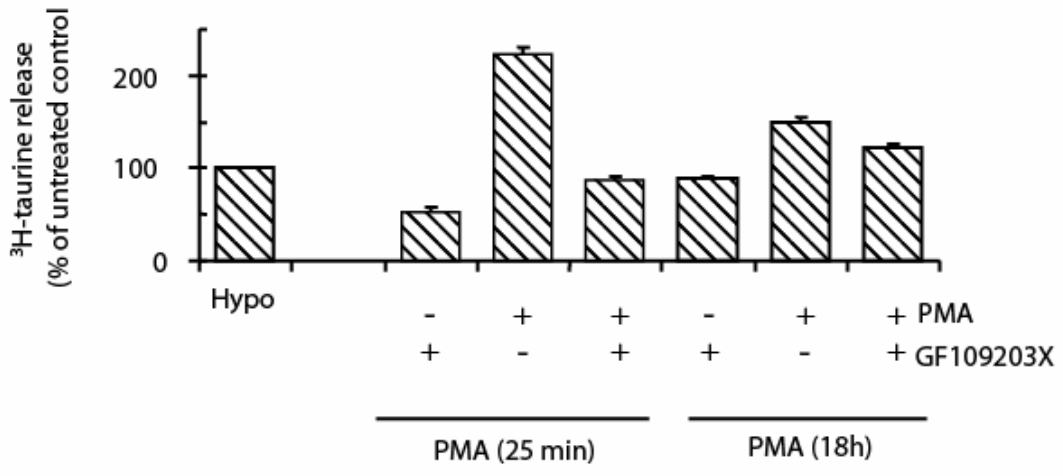

Figure 4. Protein kinase C is involved in the regulation of osmotically-induced taurine release.
^{3}[H]taurine-loaded cultures of Intestine 407 cells were treated with the phorbol ester PMA (200 nM, 25 min or 18 h) and/or GF-109203X (1 µM, 15 min) as indicated. 3-H-taurine release was determined as described under "Materials and Methods". Data are expressed as taurine release relative to the untreated isotonic control (means ± SD, n = 3).

The potentiation by PMA was fully reversed in the presence of GF-109203X, indicating that PKC activation is involved. Notably, PMA did not increase basal taurine efflux (basal: 8 ± 2%, PMA: 2 ± 4% of hypotonic control). Prolonged treatment of the cultures with PMA in an attempt to downregulate PKC resulted in reduced basal efflux but only slightly affected the hypotonicity-induced efflux. Notably, the volume-sensitive anion efflux was not affected by PKC activation or inactivation (306). Taken together, the results suggest the involvement of a Ca^{2+}-

insensitive PKC isoenzyme in the regulation of the cell swelling induced release of organic osmolytes.

Discussion

Most animal cells regulate their volume to avoid irreversible cellular damage or even cell death due to imbalances between intra- and extracellular osmolarity. We report a release pathway for organic osmolytes in Intestine 407 epithelial cells, distinct from the compensatory anion efflux reported previously (307, 310, 321) and regulated by PKC. Hyposmotic release of organic osmolytes such as taurine, betaine, and inositol has been observed in many different cell models (19, 22, 72, 93, 127, 233, 238). Unlike chloride ions, which can have additional effects on enzyme activity, membrane potential, and, in muscle and nerve cells, the generation of action potentials, these organic osmolytes do not affect other cellular functions, making them especially suitable for volume regulation (19, 79). Stimulation of Intestine 407 cells with mild hypotonic solutions (70–90% tonicity) was found to promote anion efflux in the absence of effects on taurine efflux, whereas the efflux of taurine and phosphocholine was observed only after a more severe hyposmotic challenge (<70% tonicity; cf. Fig. 1). These results suggest that, in this cell type, activation of Cl^- conductance is the first line of defense against osmotic cell swelling and that slower, more prolonged release of organic osmolytes serves as a last way out to prevent further cell swelling.

To date, at least two different mechanisms have been reported to be responsible for the release of organic osmolytes: a polyspecific one transporting both taurine and GABA and one selective for amino acids (127). The latter involves VSOAC (25), a pathway facilitating the release of both Cl^- and organic osmolytes (127, 132) and VSOC, a transporter of organic osmolytes only (67). The molecular identity of these pathways remains to be established. Our experiments clearly showed a difference in time course as well as in the threshold for activation between the release of $^{125}I^-$ and [^{3}H]taurine, suggesting that different pathways are involved. A differential sensitivity to DIDS of the Cl^- and taurine release from HeLa cells has been reported, also pointing to the existence of separate pathways (290). In contrast, in calf pulmonary endothelial (CPAE) cells (175) the efflux of taurine was inhibited by chloride channel blockers, including DIDS and millimolar concentrations of ATP. In Intestine 407 cells, however, DIDS treatment did not affect hypotonicity-induced taurine efflux (Fig. 3). Recently, two different release pathways for taurine, a “fast” one and a “slow” one, have been observed in hippocampal brain slices, with the slow one being sensitive to DIDS (67). It is therefore likely that only the fast release pathway is present in Intestine 407 cells.

In an attempt to identify the transporters involved, cells were treated with a variety of OAT and OCT inhibitors. None of the OAT inhibitors tested were found

to inhibit the release of taurine dramatically; however, a moderate (20–40%) reduction in efflux was observed with some of the inhibitors tested. In contrast, taurine release was inhibited strongly by several OCT inhibitors but not by others. Because the taurine efflux was abolished almost completely after quinidine treatment, whereas the related and potent OCT inhibitor quinine (136) was ineffective, we think that a transport system other than the OCT is involved. This notion is supported by our observation that cimetidine and verapamil, inhibitors of all three OCT subtypes (339), did not reduce the hypotonicity-induced osmolyte efflux. However, we cannot completely rule out the possibility that a subset of transporters with different sensitivities for the inhibitors used may contribute to the observed organic osmolyte efflux.

In several selected cell models, the hypotonicity-provoked taurine release was found to be sensitive to inhibitors of protein tyrosine phosphorylation as well as to wortmannin, an inhibitor of PtdIns 3-kinase (67, 192). In Intestine 407 cells, however, in clear contrast to the activation of VRAC (310, 307), the release of taurine was found to be independent of tyrosine kinases and PtdIns 3-kinase and did not involve $p21^{rho}$. A similar low sensitivity to genistein has been reported for isolated rat supraoptic astrocytes (43). Taken together, these results indicate not only that the release of taurine does not involve VRAC in Intestine 407 cells but also that distinct signaling pathways are involved in the regulation of Cl^- and taurine efflux (306).

An increase in intracellular free Ca^{2+} has been reported to be involved in the activation of taurine efflux for several cell types, including erythroleukemia cells (106), cultured rat astrocytes (151), and rat cerebral cortex (143). In astrocytes and cerebral granule cells, however, the hypotonicity-provoked taurine efflux was independent of $[Ca^{2+}]_i$ or required basal Ca^{2+} levels (188, 193, 195). With the use of Intestine 407 cells, modulation of $[Ca^{2+}]_i$ by BAPTA-AM loading, extracellular EGTA, Ca^{2+}-mobilizing hormones, the Ca^{2+} channel blocker verapamil, or the Ca^{2+} ionophore A-23187 did not affect the volume-sensitive taurine efflux. However, we cannot exclude the possibility that a minimal basal $[Ca^{2+}]_i$ is required, as observed previously for astrocytes (188).

Brief treatment of Intestine 407 cells with the phorbol ester PMA potentiated the cell swelling-induced taurine efflux, whereas addition of the PKC inhibitor GF-109203X largely inhibited the response, suggesting a major role for PKC as an activator of this process. The PKC family of serine kinases consists of a large group of isoenzymes playing crucial roles in cellular signaling and involved in a variety of biological processes (for review, see Ref. 183). The group can be divided into subgroups on behalf of their behavior and their protein sequence: the classical (α, β, γ), novel (δ,ε,η,θ), and atypical (ι,ζ) PKCs and the PKC-related kinases (PRK1, PRK2, PRK3). Whereas activation of PKC-α, -β, and -γ requires binding of

phosphatidylserine, both classical and novel PKCs are activated by the phorbol ester PMA. Because binding of phosphatidylserine occurs in a Ca^{2+}-dependent manner, the permissive role of Ca^{2+} (188) in the activation of hyposmotically triggered taurine efflux might be explained by the activation of these isoforms of PKC. In CPAE cells, however, the release of organic osmolytes was found to be independent of PKC (175).

To summarize, the results of this study indicate that in Intestine 407 cells, the hypotonicity-induced efflux of organic osmolytes is independent of VRAC and involves an activation pathway that includes PKC. These observations are not generally applicable, however, because in several other model systems, a close relationship between Cl^- conductance and release of organic osmolytes has been established (89, 115, 116, 117, 195, 263). Therefore, a definite answer to the question of whether the release pathways are the same or different awaits the molecular identification of the transporters involved.

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Increased vesicle recycling in response to osmotic cell swelling

cause and consequence of hypotonicity-provoked ATP release

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Abstract

Osmotic swelling of Intestine 407 cells leads to an immediate increase in cell surface membrane area as determined using the fluorescent membrane dye FM 1-43. In addition, as measured by TRITC-dextran uptake, a robust (> 100 fold) increase in rate of endocytosis was observed, starting after a discrete lag time of 2 – 3 minutes and lasting for approx. 10 – 15 minutes. The hypotonicity-induced increase in membrane surface area, like the cell-swelling-induced release of ATP (321), was diminished after BAPTA-AM loading or cytochalasin B treatment. Uptake of TRITC-dextrans however, was not affected. Treatment of the cells with v-SNARE specific protease *Clostridium botulinum* toxin F, not only nearly eliminated the hypotonicity-induced increase in membrane surface area but also strongly diminished the release of ATP, indicating the involvement of regulated exocytosis. Both the ATP hydrolase apyrase and the MEK inhibitor PD-098059 diminished the osmotic swelling-induced increase in membrane surface area as well as the subsequent uptake of TRITC-dextrans. Taken together, the results indicate that extracellular ATP is required for the hypotonicity-induced vesicle recycling and suggest that a positive feedback loop, involving purinergic activation of the Erk-1/2 pathway, may contribute to the release of ATP from hypo-osmotically stimulated cells.

Introduction

In mammalian cells, hypotonic cell-swelling leads to the activation of cell volume regulatory processes, which in general involves a transient increase in the K^+ and Cl^- conductances (for reviews see: (93, 118, 228)). As a result, KCl leaves the cell and cellular volume is rapidly restored (Regulatory Volume Decrease, or RVD). In addition to ion channel activation, osmotic swelling, like many other forms of mechanical stress, is known to promote the release of ATP, a potentially auto- or

paracrinic factor acting through plasma membrane purinoceptors (98, 302, 321, 334). Extracellular ATP has been shown to regulate to the RVD response in a number of different cell types (153, 255, 302, 334), either through the stimulation of a Ca^{2+}-dependent K^+ efflux (153), or by the activation of the volume-sensitive Cl^--channels (255, 334). In Intestine 407 cells, extracellular ATP is not required for the direct activation of volume-sensitive Cl^--channels (321). However, (sub-)micromolar concentrations of extracellular ATP were able to potentiate the hypotonicity provoked Cl^- efflux in a Ca^{2+}-dependent manner (321). In addition, osmotically-induced ATP release was found to be critically involved in the activation of extracellular-signal-regulated protein kinase (Erk)-1/2 in Intestine 407 cells (321). Although the role of Erk-1/2 activation in the RVD response remains to be elucidated, activation of these MAP kinases by cell-swelling has been observed in all cell models studied sofar (38, 41, 87, 221, 265, 270, 271, 279, 310, 322, 350).

To date, several potential mechanisms have been proposed to explain cellular release of ATP. These include; (1) leakage due to (local) membrane damage; (2) activation of specific channel(s) or transporter(s) and (3) exocytotic events. Previously, members of the ABC-superfamily of transporters were suggested to permeate ATP (for reviews see (26, 3)). Intestine 407 cells, however, lack CFTR expression and, in the subclone we use, P-glycoprotein expression was not detected (310, 321) arguing against a role for ABC transporters in the ATP release. Because the cell-swelling-induced ATP release differs from the activation of osmo-sensitive Cl^--channels in both the time-scale of activation/inactivation and in its sensitivity to inhibitors (98, 321), it was concluded that ATP does not permeate through volume-sensitive anion channels. In contrast, in Intestine 407 cells, the ATP release was found to depend largely on $[Ca^{2+}]_i$ as well as on an intact cytoskeleton (321).

In this study, we pursued the hypothesis that regulated exocytosis might be involved in the release of ATP. In a number of cell models it was found that disruption of SNARE (soluble-*N*-ethyl-maleimide-sensitive factor–attachment protein receptor) complex assembly, by *Clostridium botulinum* toxins or N-ethylmaleimide, resulted in a specific inhibition of exocytotic events (for review see (107)). Our results clearly show that in *C. botulinum* toxin F- or NEM-treated cells, the increase in exposed membrane surface as well as the release of ATP is markedly reduced, suggesting the involvement of exocytosis. Furthermore, we found that extracellular ATP is required for both exocytosis and endocytosis to occur. Taken together, the results not only indicate that the hypotonicity-induced release of ATP is mediated by exocytosis, but also suggest the involvement of purinoceptors and ERK-1/2 activation in autoregulation of the ATP release.

Materials and methods

Materials

Luciferin/luciferase reagent was obtained from Promega Corporation (Medison, WI). FM 1-43 and A23187 were from Molecular Probes (Eugene, OR) and Boehringer (Mannheim, Germany) respectively. PD098059 and *Clostridium botulinum* toxin F were purchased from Calbiochem (La Jolla, CA, U.S.A.) respectively. Chariot™ protein delivery kit was obtained from Active Motif (Rixensart, Belgium). Other chemicals were purchased from Sigma (St. Louis, MO).

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-ethylpiperazine-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% O_2 and 5% CO_2 at 37°C. Prior to the experiments, cells were serum-starved overnight. *C. botulinum* toxin F was introduced intracellularly by using the Chariot™ protein delivery kit according to the manufacturer's instructions. 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).

Extracellular release of ATP

Cells were seeded at a concentration of 10,000/cm^2 and incubated under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Thereafter, cells were washed four times with isotonic buffer (80 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM Hepes, pH = 7.4). The cultures were stimulated with isotonic or hypotonic (60% tonicity) media for 15 min. Media were collected, centrifugated for 5 min at 300 × g and the supernatants were transferred to fresh vials. ATP content was determined using a luciferin-luciferase luminiscence kit Promega Corporation (Medison, WI) and a Topcount.NXT luminometer (Packard, Meriden, CT).

Membrane staining with FM 1- 43

Loading experiments were performed with cells grown on coverslips, at approx. 50% confluency. During exposure to 1 µM FM 1-43, changes in fluorescence intensity were measured online (excitation wavelength = 479 nm; emission wavelength = 598 nm) in a fluorescence spectrophotometer (Hitachi F4500, Tiel, Holland) at 37°C. Experiments were started after at least 5 min of exposure to the FM 1-43 to reach an equilibration of FM 1-43 dye partitioning into the plasma-membrane.

TRITC-dextran uptake

Cells on coverslips at 80-90% confluency were incubated with 0.5 mg/ml TRITC-dextran (MW=10,000 Da) in iso- or hypotonic buffer (80 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM Hepes, pH 7.4; diluted in case of hypotonic stimulation). After incubation, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and fixed with in 2% formaldehyde (20 min incubation). Confocal images of 512x512 pixels were constructed by summation of 15 optical sections parallel to the substratum and each 1 µm apart, using a 63x oil immersion objective (Axiovert 135M, Zeiss, Oberkochen, Germany). Endocytosis was quantified using KS400 software (Zeiss, Oberkichen Germany) by counting the number of fluorescent spots with a size between 3-75 pixels, after substraction of background fluorescence. Background fluorescence was determined by measuring the mean pixel intensity and adding 5 times its standard deviation of a circle drawn in the background.

Results

Osmotic cell swelling leads to an increase in cell surface labelling

The effects of hypo-osmotic stimulation on the cell surface membrane area was studied using the styryl dye FM 1-43, a water soluble probe that becomes intensely fluorescent at the lipid-liquid interface and does not pass the membrane (280). An increase in membrane surface area, due to exocytosis or to unfolding of FM 1-43 inaccessible plasma membrane, results in an increased binding of the dye and, consequently, in augmented fluorescence. As shown in Figure 1, a shift from an

isotonic to a hypotonic medium led to a rapid increase in fluorescence above control values. Like the release of ATP (321), the hypotonicity-provoked increase in FM 1-43 fluorescence was inhibited after loading the cells with BAPTA-AM or by treatment with cytochalasin B (Fig. 1A), implying that the increase in surface labelling is Ca^{2+} dependent and requires an intact actin cytoskeleton. Treating the cells with N-ethylmaleimide (NEM), an inhibitor of SNARE proteins involved in vesicle docking and fusion, almost completely abolished the cell-swelling-induced increase in fluorescence, suggesting that exocytosis plays a crucial role. This notion was strengthened further by the finding that intracellular delivered C. botulinum toxin F, resulting in proteolytic cleavage of the vesicle associated SNARE protein VAMP2 almost completely prevented the increase in FM 1-43 fluorescence (Fig.1B). Taken together, these results strongly indicate that osmotic swelling in this epithelial cell type is paralleled by rapid exocytosis.

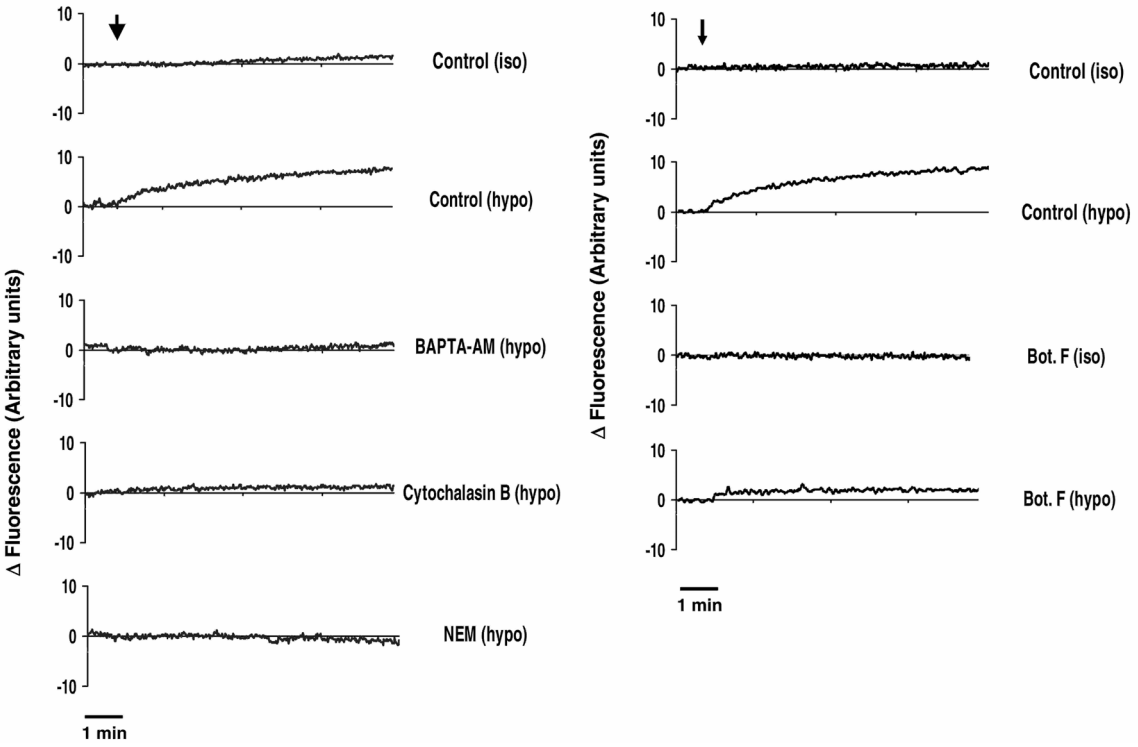

Figure 1. Hypotonicity-provoked change in FM 1 – 43 fluorescence.
Left) Inhibition by BAPTA-AM loading and cytochalasin B or N-ethyl-maleimide treatment Intestine 407 cells, grown on coverslips were incubated with isotonic medium containing BAPTA-AM (25 μM for 30 min), cytochalasin B (50 μM for 30 min), BAPTA-AM (25 μM, 1h) or N-ethylmaleimide (1 mM for 15 min) prior to the experiments. Thereafter, the coverslips were placed in a cuvette with isotonic medium containing 1 μM FM 1-43. After approx. 5 min equilibrium of labelling was achieved. Subsequently, the cells were exposed to either isotonic or hypotonic medium (60 % tonicity) containing 1 μM FM 1 – 43; arrow indicates a shift in medium. Traces are representative of at least 3 experiments. **Right)** FM 1–43 fluorescence in *C. botulinum* toxin F treated cells. Cells grown on coverslips were treated with Chariot™-conjugated *C. botulinum* toxin F for 2 h. The toxin F containing medium was replaced for DMEM and the cells were allowed to recover for another 2 h. Thereafter, the hypotonicity-induced changes in FM 1–43 fluorescence was determined as described above. Traces are representative for three experiments. Arrow indicates change of medium.

Hypotonic cell swelling induces endocytosis

In addition to exocytosis, hypotonic cell-swelling also induced an increase in the uptake of TRITC-dextran, a marker for endocytosis. Figure 2 shows confocal images of Intestine 407 monolayers incubated for 5 minutes in isotonic or hypotonic (80% or 60% tonicity) medium containing TRITC-dextran. A time course of TRITC-dextran uptake is shown in Figure 3. The cell-swelling-induced increase in the rate of endocytosis showed an apparent lag phase of 2-3 min and lasted for approx. 10-15 min. The hypotonicity-induced uptake of TRITC dextran could not be mimicked by an increase in $[Ca^{2+}]_i$ and, unlike the increase in membrane surface area, was found to be insensitive to BAPTA-AM loading or cytochalasin B treatment (Table 1). Treating the cells with jasplakinolide however, leading to actin stabilization and polymerisation (103), partly inhibited the uptake of dextran (Table 1), suggesting that actin remodelling is required.

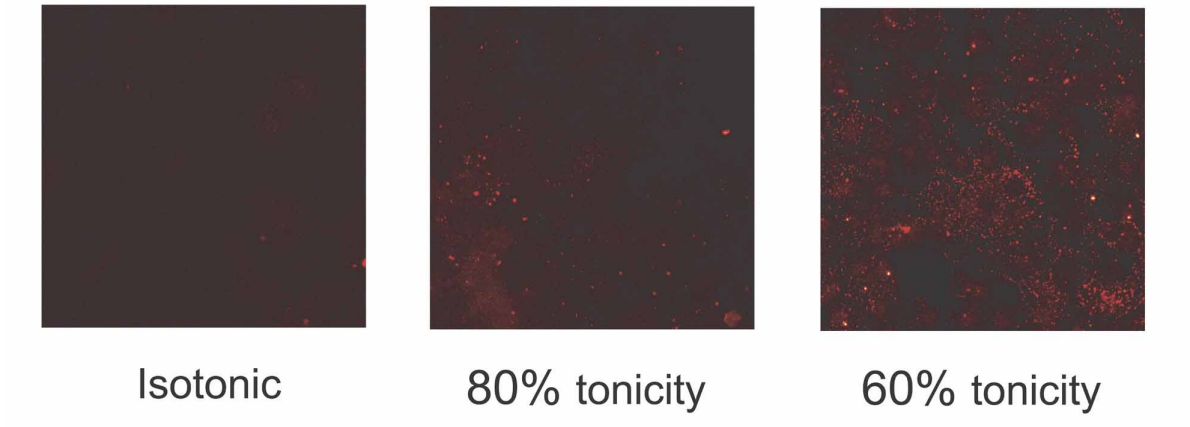

Figure 2. Uptake of TRITC-dextran in control and hypo-osmotically stimulated cells.
Cells grown on coverslips were incubated for 5 min with TRITC-dextran (0.5 mg/ml) containing isotonic or hypotonic (80 or 60% tonicity) medium. Thereafter, cells were washed, fixated and images were constructed as described under 'Materials and Methods' using a CSLM.

	TRITC-dextran uptake (% of hypotonic control)	
Experimental condition	Isotonic	Hypotonic
Control	6 ± 2 (10)	100 ± 6 (10)
BAPTA-AM	3 ± 1 (7)	86 ± 19 (7)
A23187	2 ± 1 (5)	91 ± 26 (5)
Cytochalasin B	12 ± 3 (5)	109 ± 20 (5)
Jasplakinolide	6 ± 2 (5)	32 ± 7 (5)

Table 1. Role of $[Ca^{2+}]$I and F-actin in the regulation of osmotic cell swelling- induced endocytosis.
Control, BAPTA-AM (25 μM for 1 h) loaded and A23187 (5μM), cytochalasin B (50μM for 30 minutes) and Jasplakinolide (100 nM for 1 h) treated Intestine 407 cells were incubated with 0.5 mg/ml TRITC dextran in either isotonic or hypotonic medium for 10 minutes. Endocytosis was quantified as described under 'Materials and Methods'. Data are expressed as Mean ± S.E.M. Number of experiments is given in parentheses. An asterisk indicates a significant difference from the control ($p < 0.05$; Student t-test).

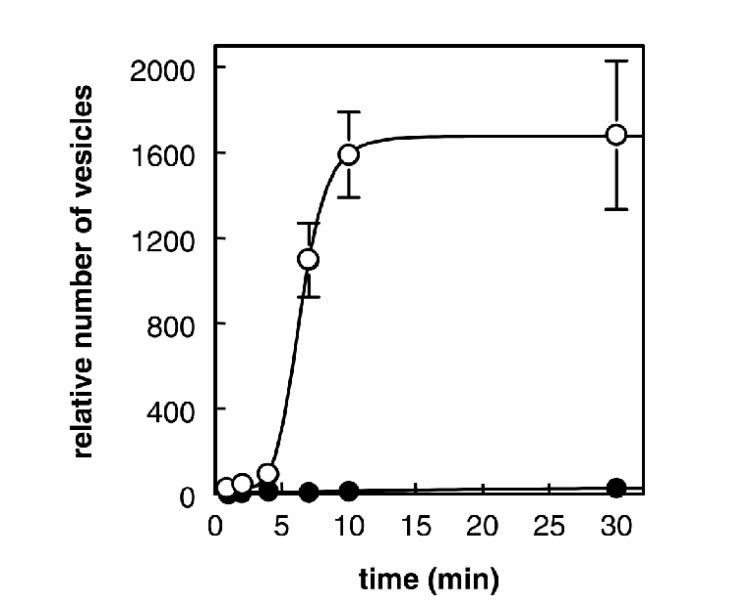

Figure 3. Time course of hypotonicity-induced TRITC-dextran uptake.
Cells grown on coverslips were incubated for the times indicated with Isotonic (closed symbols) or hypotonic medium (60% tonicity; open symbols) containing 0.5 mg/ml TRITC-dextran. After fixation, images were constructed as indicated in Materials and Methods using CSLM. The number of vesicles is determined as described 'Materials and Methods'. Data are given as mean ± S.E.M. (n=5).

Hypotonicity-induced ATP release

To investigate a putative role for exocytosis in the accumulation of extracellular ATP, cells were treated with *C. botulinum* toxin F. As shown in Fig. 4, both basal and hypotonicity-induced ATP release was strongly reduced after treatment with the toxin, supporting the notion that the efflux of ATP is mediated by exocytosis.
Incubation of the cells with the ATP hydrolase apyrase, not only completely inhibited the cell swelling-induced increase in plasma membrane surface area (Fig. 5), but also largely abolished the hypotonicity-induced increase in TRITC-dextran uptake (Table 2), suggesting that extracellular ATP plays an important role during both exocytosis and endocytosis. Notably, apyrase treatment did not reduce the intracellular ATP concentration as determined after cell lysis (results not shown).

A similar reduction in endocytotic activity was observed upon exposure of the cells to the purinoceptor antagonist suramin (Table 2), suggesting that purinoceptor activation is involved. Previously, we reported that the release of endogenous ATP leads to a stimulation of the MAP kinase Erk-1/2 through the activation of $P2Y_2$ purinoceptors (321). To investigate a putative role for Erk-1/2 type MAP kinases in the regulation of cell swelling-induced vesicle recycling, cells were treated with the tyrosine kinase inhibitor genistein as well as with the MAP kinase kinase (MEK) inhibitor PD098059. As shown in Fig. 5 and Table 2, treatment of the cells with genistein or PD098059 completely prevented the cell

swelling-induced increase in FM 1-43 fluorescence (Fig. 5) but only partly prevented the hypotonicity-provoked increase in the rate of endocytosis (Table 2).

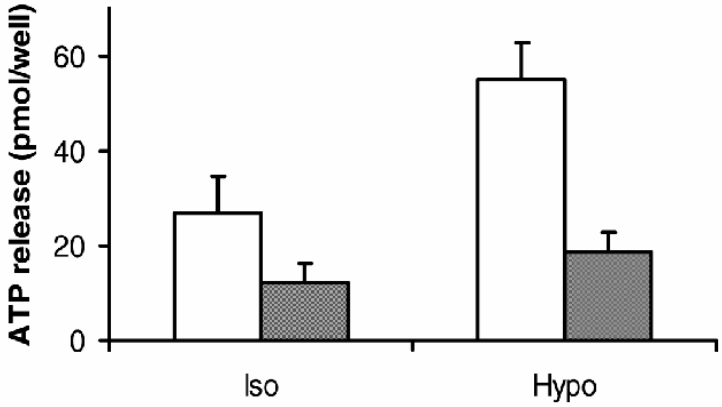

Figure 4. Hypotonicity-induced ATP release from control and *C. botulinum* toxin treated cells.
Control and *C. botulinum* treated cultures were stimulated with isotonic (open bars) or hypotonic (60% tonicity; hatched bars) medium for 15 min. Media were collected and ATP content was determined as described under 'Materials and Methods'. Data are expressed as mean ± S.E.M. (n=6).

These results suggest that the release of ATP plays an important role in the regulation of vesicle recycling by promoting exocytosis and endocytosis through a pathway involving Erk-1/2.

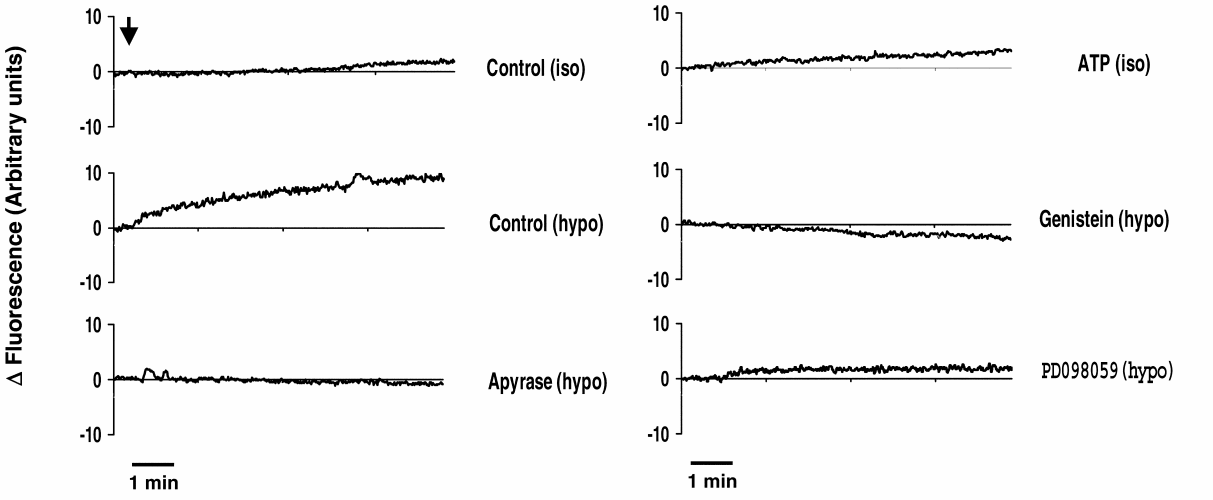

Figure 5. Apyrase and the MEK inhibitor PD059098 prevented a hypotonicity-induced increase in FM 1– 43 fluorescence.
Intestine 407 cells grown on coverslips were treated with apyrase (3 I.U./ml for 5 min), ATP (10 μM for 5 min), genistein (200 μM, 2h) or PD098059 (50 μM, 2h) prior to the experiments. Thereafter, the coverslips were placed in a cuvette containing 1 μM FM 1-43 and fluorescence was monitored continuously as described under 'Materials and Methods'. Arrow indicates shift in medium. Traces are representative of at least 3 experiments.

Importantly, under isotonic conditions, extracellular ATP, at concentrations leading to full purinoceptor activation (321), did neither result in an increase in membrane surface fluorescence (Fig. 5), nor did it promote the uptake of TRITC-dextran (Table 2).

	TRITC-dextran uptake (% of hypotonic control)	
Experimental condition	Isotonic	Hypotonic
Control	5 ± 1 (6)	100 ± 3 (13)
Apyrase	1 ± 1 (5)	11 ± 3* (7)
Suramin	5 ± 5 (3)	7 ± 5* (3)
ATP	7 ± 2 (5)	135 ± 20 (5)
Genistein	3 ± 2 (5)	54 ± 11 (5)
PD0989059	4 ± 1 (5)	45 ± 10 (6)

Table 2. Involvement of ATP-provoked Erk-1/2 activation in the hypotonicity-induced endocytosis. Control, apyrase (3 I.U./ml), ATP (10 μm), genistein (200 μM) and PD098059 (50 μM) treated Intestine 407 cells were incubated in isotonic or 40% hypotonic medium containing 0.5 mg/ml TRITC-dextran for 10 minutes. Genistein and PD098059 were added 2 h prior to the experiments. Thereafter, endocytosis was quantitated as described under 'Materials and Methods'. Data are expressed as Mean ± S.E.M. Number of experiments are given in parentheses. An asterisk indicates a significant difference from the control ($p < 0.05$; Student t-test).

Discussion

In many different cell models, mechanical stress, such as caused by osmotic cell swelling, leads to the release of ATP into the surrounding medium (98, 302, 321, 334). Several potential mechanisms have been proposed to be involved, including anion channel activation (i.e. CFTR and/or VRAC), utilization of specific (ABC-type of) transporters and exocytosis (54, 75). In Intestine 407 cells, results from previous studies argued against the involvement of anion channels because 1) these cells do not express CFTR and 2) there is a clear difference in the regulation of osmo-sensitive anion channels and the release of ATP (95, 98, 310, 321). In Intestine 407 cells, the hypotonicity-provoked release of ATP was found to depend critically on an increase in $[Ca^{2+}]_i$ and on an intact actin cytoskeleton (321), suggesting the involvement of exocytosis. This notion is supported by the observation that an increase in $[Ca^{2+}]_i$ alone is able to trigger the release of ATP (199, 321).

Upon hypo-osmotic stimulation, a rapid increase in plasma membrane surface area was observed, as evidenced by an increased FM 1-43 fluorescence. This increase was abolished after treating the cultures with N-ethylmaleimide or *C. botulinum* toxin F, indicating that SNARE complex formation and subsequent exocytosis is involved. Like the hypotonicity-induced ATP release, the increase in total membrane area depends on an intact actin cytoskeleton and is regulated by intracellular free calcium. Activation of exocytosis by osmotic cell swelling is not unprecedented. An increase in membrane capacitance has been reported after hypo-osmotic stimulation of Intestine 407 cells (225). In addition, morphometric analysis of electron micrograph images of rat hepatocytes revealed a marked

enlargement of the membrane surface area within 5 min of hypotonic exposure (240). Furthermore, Bruck et al. (18) observed an increase in the release of horse radish peroxidase (HRP) after hypotonic stimulation from an HRP loaded perfused liver. Because the HRP release was found to be sensitive to colchicine, the involvement of exocytosis was suggested (18). In line with this concept, reducing the osmolarity of the surrounding medium promoted the release of FITC-coupled dextran (MW 72000 Da) from preloaded inner medullary collecting duct kidney cells (40).

Subsequent to enhanced membrane surface labelling, an increased rate of endocytosis was observed after osmotic cell swelling, as evidenced by the robust uptake of TRITC-labeled dextran. Uptake of dextran started after a discrete lag time of 2-3 min, reached maximal activity after approx. 5 min. and lasted for 10-15 min. The delayed onset of endocytosis in response to cell-swelling may suggest that endocytosis is triggered by an increase in exocytosis. Smith and Betz (280) observed a similar lag time between the onset of Ca^{2+}-provoked exocytosis and the occurrence of endocytosis in adrenal chromaffine cells. They suggested that endocytosis starts only when the increase in cell size due to exocytosis reaches a certain threshold and limits a further increase (280). In addition, a negative correlation has been proposed between membrane tension and membrane expansion (249), implying that membrane expansion through exocytosis will result in an increased rate of endocytosis. In Intestine 407 cells, however, we found that exo- and endocytosis are regulated independently, because cytochalasin B treatment and BAPTA-AM loading, both strong inhibitors of exocytosis, did not affect the cell swelling-induced endocytosis. In contrast, our observation that the hypotonicity-provoked endocytosis is inhibited by inactivation of VRAC and promoted by K^+ channel blockers and high extracellular [K^+] [T. van der Wijk, unpublished results] suggests that membrane depolarization, a known consequence of VRAC activation (97), may promote or trigger endocytosis.

The ATP hydrolase apyrase completely inhibited the hypo-shock induced FM 1-43 fluorescence as well as the uptake of TRITC-dextrans, suggesting that extracellular ATP is required for both the cell swelling-induced exocytosis and endocytosis. Extracellularly added ATP however, does not induce an increase in either one of these parameters by itself, indicating that ATP acts as a permissive and propulsive factor and that an additional trigger, perhaps an increase in membrane tension is needed. Both an increase as well as a decrease in the rate of exocytosis has been reported after purinergic activation, the effects being attributed principally to its effect on $[Ca^{2+}]_i$ (76, 80, 146, 154, 222, 243, 244, 315). In addition, regulation of exocytosis by purinoceptor activation through Ca^{2+}-independent and G protein dependent pathways have been demonstrated (146, 154, 243). Therefore, although

extracellular ATP leads to an increase in $[Ca^{2+}]_i$ in our cell system (321), the effect of ATP on exocytosis may be modulated by other, Ca^{2+}-independent pathways.

Extracellular ATP not only affects exocytosis, but is also involved in the regulation of endocytosis. Nakashima et al. (205) reported that binding of EGF and insulin to their receptors lead to the endocytosis of the receptor-ligand complex which is mediated by the activation of the small G protein $p21^{Ras}$. Osmotic cell swelling rapidly activates the Erk-1/2 MAP kinases through a mechanism involving the Ras/Raf pathway, using extracellular ATP as an autocrinic factor (321, 322). Therefore, it is tempting to speculate that ATP promotes endocytosis through the activation of $p21^{Ras}$. This notion is supported by our observation that inhibition of the Erk-1/2 signaling cascade, using the MEK inhibitor PD098059, inhibited osmotic cell swelling-induced endocytosis.

Although the hypotonicity-induced endocytosis was completely inhibited in the presence of apyrase or suramin, inhibition of exocytosis by cytochalasin B or BAPTA-AM treatment did not significantly affect the uptake of TRITC-dextran (cf. Table 1). However, as has been found for several other cell models (144), even under isotonic conditions, low levels of extracellular ATP are present. In addition, under hypotonic conditions, a small but significant increase in extracellular ATP levels was observed in cytochalasin B or BAPTA-AM-treated cells (321). Because complete removal of ATP by apyrase or the inhibition of purinoceptors by suramin completely blocked both exo- and endocytosis, it is tempting to speculate that extracellular ATP, acting through purinoceptor activation, serves two functions. First, basal concentrations of ATP are a prerequisite for vesicle recycling to occur and second, elevated levels promote but do not trigger endocytosis through a pathway involving Erk-1/2.

To conclude, osmotic swelling of Intestine 407 cells rapidly leads to an increase in the rate of exo- and endocytosis. Subsequently to the exocytotic release of ATP, by using a signaling pathway that involves purinoceptor activation, Erk-1/2 activation enhances the vesicle recycling and promotes the additional release of ATP. Extracellular ATP may then play an important role in autocrine potentiation of volume-sensitive Cl^- channels, as well as in cell-to-cell communication through activation of purinergic receptors in neighboring cells or tissues.

Acknowledgments

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Activation of phospholipase D by osmotic cell swelling

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Abstract

In response to osmotic cell swelling, Intestine 407 cells react with a rapid and transient activation of phospholipase D (PLD). To investigate the role of PLD during the regulatory volume decrease, cells were treated with 1-butanol resulting in a depletion of PLD substrates. Activation of volume-regulated anion channels, but not the cell swelling-induced release of taurine, was largely inhibited in the presence of low concentrations of 1-butanol. In addition, hypotonicity-induced exocytosis, ATP release and subsequent endocytosis were found to be largely abrogated. The results support a model of cell volume regulation in which PLD plays an essential role in the cell swelling-induced vesicle cycling and in the activation of volume-sensitive anion channels.

Introduction

As a consequence of the relatively high water permeability of the plasma membrane, an osmotic imbalance between cells and their surrounding fluids will immediately lead to a change in cellular volume. Because volume alterations are potentially deleterious, almost all mammalian cell types have developed compensatory mechanisms that keep the cell volume within a narrow range. Upon osmotic cell swelling, Cl^-- and K^+-selective ion channels are rapidly activated, leading to a net efflux of KCl followed by water (regulatory volume decrease, RVD). In addition, a release of organic osmolytes (taurine and β-alanine) has been demonstrated in a variety of different cell types (for review see (233)).
Previously, we reported that in Intestine 407 epithelial cells, the activation of volume-regulated anion channels (VRACs) depends on protein tyrosine phosphorylation and on the activity of the $p21^{Rho}$ G protein (306, 307). In addition, we demonstrated that osmotic cell swelling promotes a robust increase in vesicle cycling, responsible for the concomitant release of ATP (323, 324). Because RhoA and ARF type GTPases have been implicated in the regulation of phospholipase D (PLD) (57, 158, 245) and a potential role of PLD in membrane traffic has been

emerged from previous studies (17, 120), we hypothesized that this enzyme may act as a key regulator of the RVD response.

In a variety of cell models, activation of PLD has been observed upon stimulation with growth factors, hormones as well as cytokines (for review see: (10, 57, 158)). In addition, activation of PLD by hypo-osmolarity has been demonstrated in the protozoan *Leishmania donovani* and in skate erythrocytes (68, 201). To date, only two PLD genes have been cloned from mammalian cells, PLD1 and PLD2, each being expressed as two splice variants (293, 284). Whereas PLD1 appears to be localized primarily on intracellular membranes (31, 157), including the Golgi apparatus, PLD2 seems to be confined mainly to theplasma membrane (245, 157, 31). Like many other esterases, PLD can utilize short chain primary alcohols (like ethanol and 1- butanol) as phosphatidyl group acceptors, resulting in the formation of metabolically stable phosphatidyl alcohols (10, 34, 158, 178, 277). This property can be used not only to detect PLD activation, but, due to depletion of cellular PtdOH levels, can also serve as a tool to evaluate the physiological role of PLD (158).

In this study, we have used 1-butanol-treated cultures of Intestine 407 epithelial cells as a model to investigate the role of PLD during the RVD response. The results indicate that the compensatory efflux of organic osmolytes is rather insensitive to 1-butanol treatment. In contrast however, the hypotonicityprovoked cycling of membrane vesicles as well as the activation of volume-sensitive anion channels were largely inhibited.

Materials and methods

Materials

Radioisotopes ($^{125}I^-$, ^{32}[P]orthophosphate and ^{3}[H]taurine) were purchased from Amersham Netherlands B.V. (‘s Hertogenbosch, The Netherlands). FM 1–43 was obtained from Molecular Probes (Eugene, OR). All other reagents were from Sigma–Aldrich (St. Louis, MO).

Cell culture

Intestine 407 cells were grown as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 25 mM HEPES, 10% FCS, 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. Prior to the experiments, the cells were serum-starved overnight.

Efflux assay

Monolayers of Intestinal 407 cells were loaded for 2 h with 5 μC_i $^{125}I^-$ and washed three 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-min intervals. Hypotonic buffers were prepared by reducing the concentration of mannitol. Radioactivity in the media was determined by c-radiation counting and expressed as fractional efflux per minute as previously described (307).

Phospholipase D activity

Cells were labelled overnight with ^{32}[P]-orthophosphate (100 µCi/ml). 1-Butanol was added 15 min before the start of the experiments. Incubations were terminated by replacing the medium by ice-cold 0.1 M HCl. Culture plates were then stored at -20 ºC for 30 min. Lipids were isolated by a modified acid Bligh and

Dyer extraction [21]. Briefly, after thawing, cells were scraped and transferred to 2 ml vials and centrifuged at 10 000×g for 60 s. Supernatants were discarded and the pellets were resuspended in 1 ml $CHCl_3$:MeOH:HCl (50:100:1 v/v). Phase separation was achieved by adding 750 µl chloroform and 300 µl of 2 N HCl. The lower phase was collected, washed twice with $CHCl_3$:MeOH:1 M HCl (3:48:47 v/v), dried and dissolved in chloroform. Phospholipids were separated on Silica-60 HPTLC plates (Merck Darmstadt, Germany) using the upper phase of an ethyl-acetate: iso-octane:formic-acid:H_2O (130:20:30:100 v/v) solvent system (198).

Exocytosis

FM 1–43 loading experiments were performed with cells grown on coverslips, at approx. 50% confluency. During exposure to 1 µM FM 1–43, changes in fluorescence intensity were measured online (excitation wavelength=479 nm; emission wavelength=598 nm) in a fluorescence spectrophotometer (Hitachi F4500, Tiel, Holland) at 37 ºC, as described previously (324).

ATP release

Cultures of Intestine 407 cells were washed four times with isotonic buffer prior to osmotic stimulation. Thereafter, media were collected, centrifuged for 5 min at 300×g and the supernatants were transferred to fresh vials. ATP content was determined using a luciferin–luciferase luminescence kit (Promega Corporation, Medison, WI) and a Topcount. NXT luminometer (Packard, Meriden, CT) as described (323).

TRITC-dextran uptake

Cells grown on coverslips (90% confluent) were incubated with 0.5 mg/ml TRITC-dextran (MW=10 000 Da) in iso- or hypotonic buffer. After incubation, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and fixed with in 2% formaldehyde in PBS (20 min incubation). Confocal images of 512×512 pixels were constructed by summation of 15 optical sections parallel to the substratum and each 1 lm apart, using a 63× oil immersion objective (Axiovert 135 M, Zeiss, Oberkochen, Germany). Endocytosis was quantified using KS400 software (Zeiss, Oberkochen, Germany) (324).

Results and discussion

Osmotic cell swelling-provoked activation of PLD

Using 1-butanol-treated cultures of Intestine 407 cells, we studied the activation of PLD during the RVD response. As shown in Fig. 1A and B, reducing the osmolarity of the medium from isotonic to 70% tonicity rapidly activates PLD, as evidenced by the accumulation of PtdBut. A steep increase in PtdBut was observed during the first 2 min of hypo-osmotic stimulation, thereafter the amount slowly declined, reaching control levels after 10–15 min (Fig. 1B). Although PtdBut is generally believed to be relatively stable (277), a considerable degradation of PtdBut was apparent in this intestinal epithelial cell line, possibly leading to an underestimation of the extent of the PLD activation during the first 5–10 min of the RVD response. Notably, osmotic swelling of Intestine 407 cells did not activate phospholipase C, as determined by quantitating inositol phosphate formation (Fig. 1C).

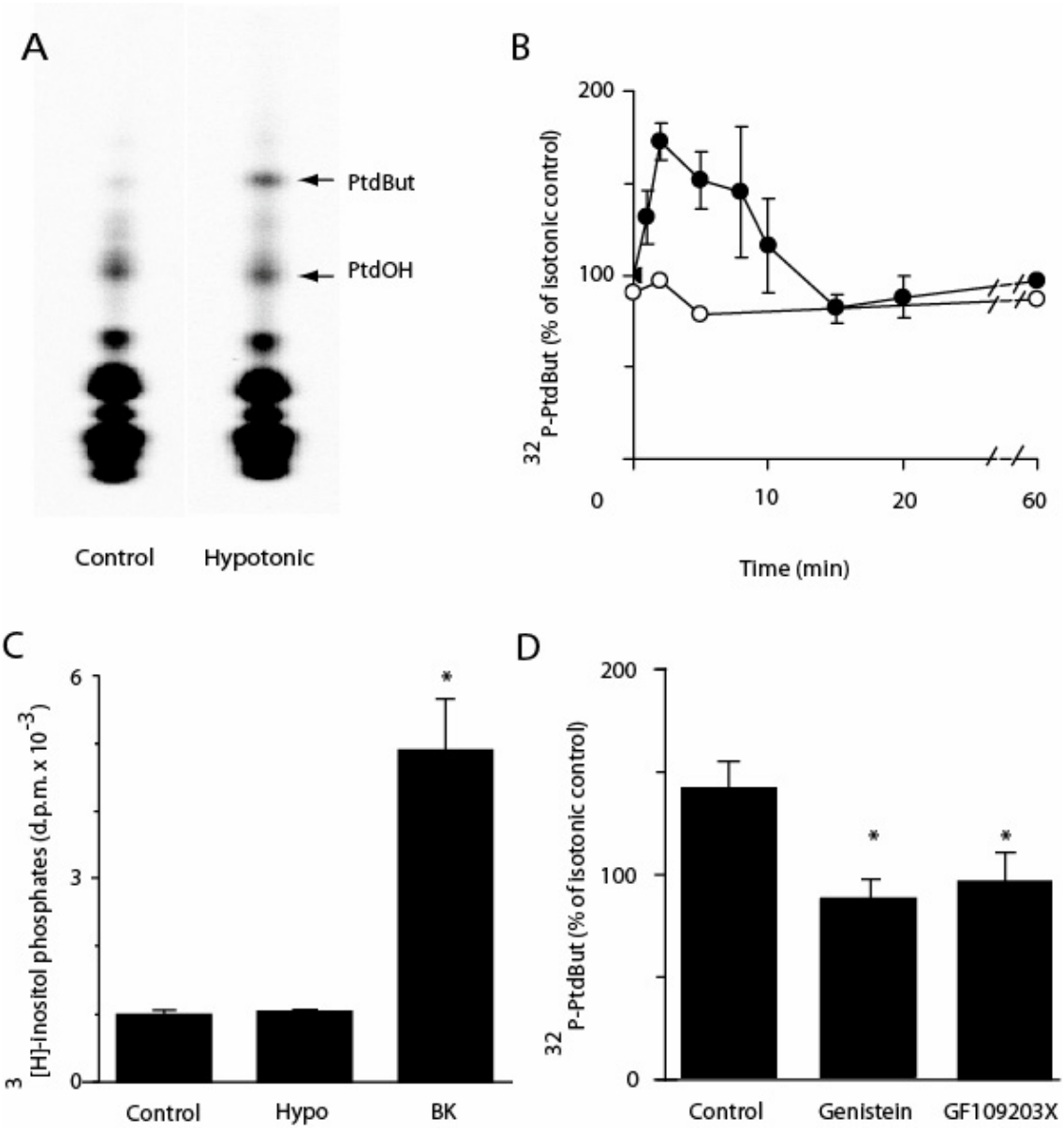

Figure 1. Activation of PLD by osmotic cell swelling.
Intestine 407 cells were labelled with $^{32}PO_4^3$ and PLD activity was subsequently determined by quantitating the amount of ^{32}[P]PtdBut formed. (A) Autoradiogram of ^{32}P-labelled phospholipids in control and hypo-osmotically (70% tonicity) stimulated cells. Phospholipids were separated by HPTLC, and the positions of the PtdBut and PtdOH were indicated by arrows. (B) Time dependency of the PtdBut formation in control (open symbols) and hypotonicity-treated cells (closed symbols). (C) Accumulation of inositol phosphates in control (Con), hypo-osmotically (Hypo) and bradykinin (1 µM, 10 min, BK) stimulated 3[H]inositol loaded (2 µM, 18 h) cultures. Inositol phosphates were quantitated by Dowex AG 1 X 8 anion-exchange chromatography as described [32]. (D) Inhibition of the osmotic cell swelling-induced PtdBut formation in genistein (200 µM, 30 min) and GF-109203X (1 µM, 15 min) treated cells. Data are expressed as percentage relative to the isotonic control (means ± S.E.M. for n = 3).

Protein tyrosine phosphorylation plays an important role during the RVD response. In Intestine 407 cells, as well as in a number of other cell models (148, 282, 330), tyrosine kinase inhibition resulted in a reduced volume-sensitive anion efflux (307) and a diminished rate of vesicle cycling (324). In contrast however, in Intestine 407 cells, the cell swelling-induced release of organic osmolytes like taurine and betaine was not affected by tyrosine kinase inhibition (311). To investigate the role of tyrosine kinases in the activation of the hypotonicity-

induced PLD activation, cells were treated with genistein, a broad specificity tyrosine kinase inhibitor. As shown in Fig. 1D, genistein treatment completely prevented the cell swelling-induced activation of PLD, indicating the involvement of (a) tyrosine kinase(s) in the mechanism of osmotic activation of the enzyme. In addition, the cell swellinginduced activation of PLD was inhibited after treatment of the cells with the broad range protein kinase C inhibitor GF-109203X, indicating that active protein kinase C is required for its activation (Fig. 1D).

Possible role of PLD at the activation of compensatory osmolyte fluxes

To investigate a putative role for PLD in the regulation of the cell swelling-induced anion and taurine release, 1-butanol treated cells are used to prevent the activation of PLD. Low concentrations of 1-butanol (1%) largely reduced the cell swelling-provoked $^{125}I^-$ efflux from isotope-loaded cells but did not appreciably affect the release of ^{3}[H]taurine (Fig. 2).

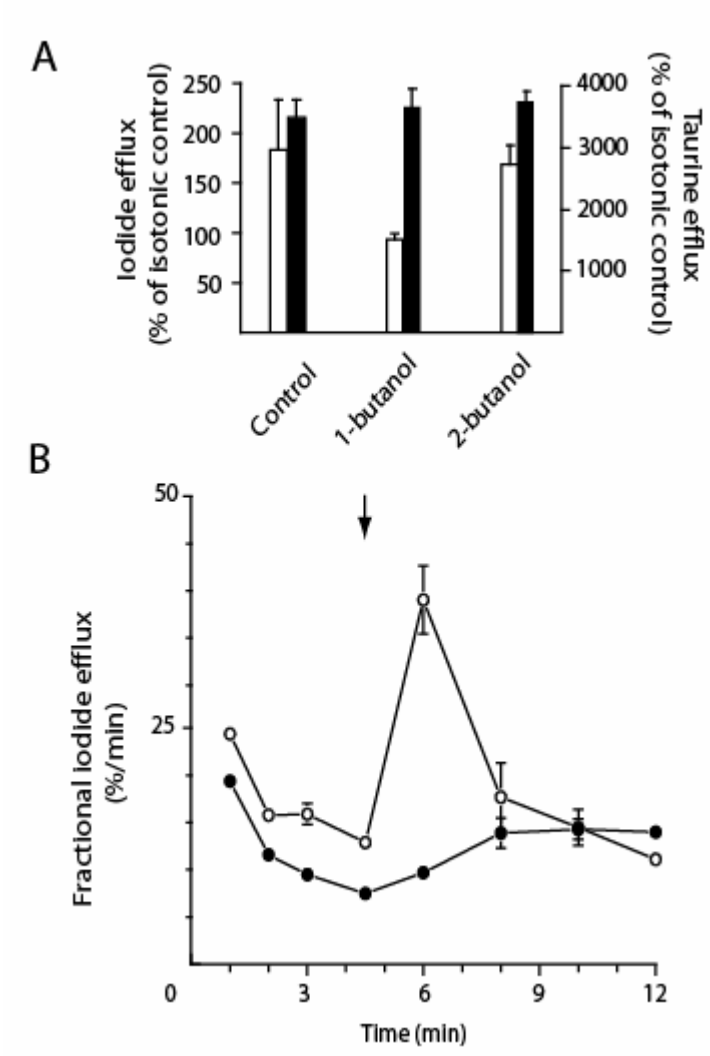

Figure 2. (**A**) ^{125}Iodide (open bars) and ^{3}[H]taurine (closed bars) release from osmotically stimulated (70% tonicity) control, 1-butanol- (1%, 60 min) and 2-butanol-treated (2%, 60 min) Intestine 407 cells. (**B**) Time course of the hypotonicity-induced (70% tonicity) ^{125}Iodide efflux in the control (open symbols) and 1-butanol-treated (1%, 60 min, closed symbols) cultures. Arrow marks the shift to a hypotonic medium. Data are expressed as mean ± S.E.M. for n = 3. Asterisk indicates a significant difference from the control ($P < 0.01$ Student's t-test).

The insensitivity of the ^{3}[H]taurine release to 1-butanol was observed at all tonicities tested (30–50% hypotonicity, results not shown). Notably, comparable concentrations of 2-butanol (Fig. 2) or tert-butanol (not shown) were not able to diminish the release of osmolytes. Because in most cell models studied, 1% 1-butanol or less resulted in a complete inhibition of PLD and its downstream targets (32, 227), the insensitivity of the taurine release to 1-butanol treatment suggests that PLD is not likely to be involved in its activation pathway.

Involvement of PLD in the cell swelling-induced vesicle cycling

Osmotic cell swelling is accompanied by exo- and endocytosis as well as by the release of ATP (324, 321). Because PLD activation was found to be an essential step in the mechanism of exocytosis in several cell models (321), the role of this lipase in the hypotonicity-provoked vesicle cycling was studied. Both the osmotic cell swelling-induced increase in total cell surface area, as determined by FM 1–43 fluorescence, as well as the release of ATP were completely abolished in 1-butanol (1%) treated cells (Fig. 3A and B). In addition, a considerable reduction in the uptake of TRITC-dextran was observed (Fig. 3C). Taken together, these results strongly suggest that PLD activation is critically involved in the activation of cell swelling-induced vesicle cycling and in the release of ATP.

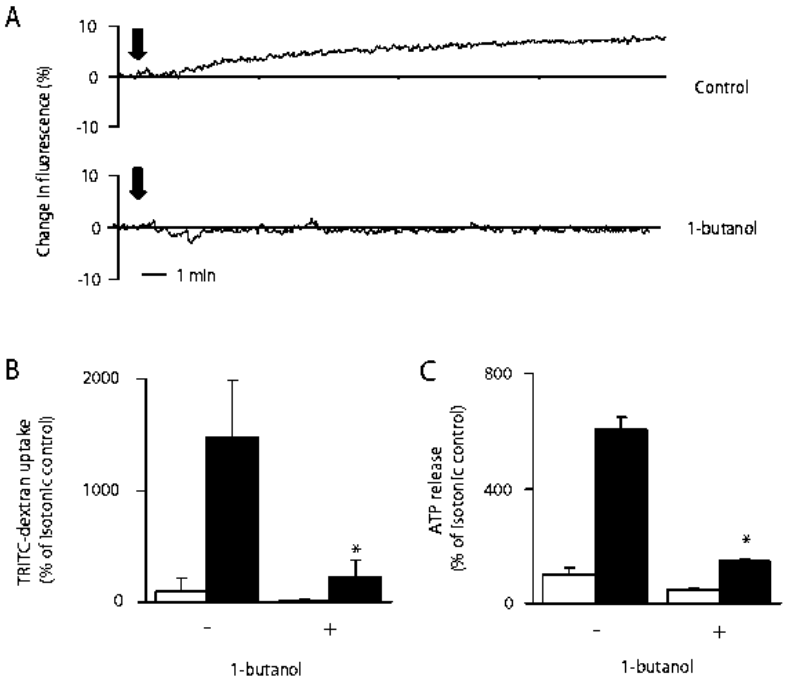

Figure 3. 1-Butanol (1%, 30 min) treated cultures of Intestine 407 cells. (**A)** Time course of the hypotonic shock-induced increase in FM 1–43 fluorescence. Arrow indicates a shift to a hypo-osmotic medium (70% tonicity). Traces are representative of at least three experiments. (B,C) Uptake of TRITC-dextran (**B)** and the release of ATP (C) under isotonic (open bars) and hypotonic (70% tonicity, hatched bars) conditions. Data are expressed as mean ± S.E.M. for n = 3. Asterisk indicates a significant difference from the control ($P < 0.01$, Student's t-test).

Phorbol ester induced potentiation of the taurine efflux involves PLD

Previously we have shown that, unlike the activation of VRAC (307), the volume-sensitive efflux of taurine is potentiated in the presence of the phorbolester PMA (311). Because it is now well established that PMA may act as a strong activator of PLD either directly or through stimulation of PKC (10, 86, 133), its role in the regulation of taurine efflux was further explored. As shown in Fig. 4, low concentrations of 1-butanol (1%) did not affect the hypotonicity-induced taurine efflux under control conditions (cf. see also Fig. 2B). Potentiation of the response by PMA, however, was completely abolished in 1-butanol- as well as in GF-109203X-treated cultures, suggesting that the potentiation of the taurine efflux by PMA involves protein kinase C activation of PLD.

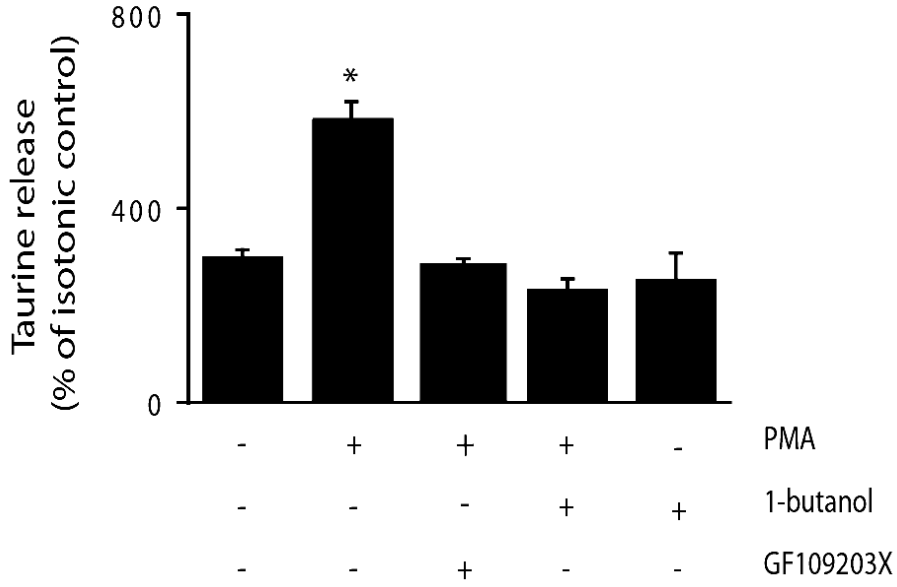

Figure 4. ^{3}[H]Taurine release from osmotically (70% tonicity)-provoked control, PMA (200 nM, 30 min), GF-109203X (1 µM, 30 min) and 1- butanol-treated (1%, 30 min) Intestine 407 cells. Data are expressed as mean ± S.E.M. for n = 3. Asterisk indicates a significant difference from the control ($P < 0.01$, Student's t-test).

Conclusions

Hypo-osmotic cell swelling leads to the activation of PLD through a mechanism involving tyrosine kinase(s). Unlike the hypotonicity-provoked taurine efflux, the cell swelling-triggered increase in anion conductance, exo- and endocytosis as well as the release of ATP were sensitive to low concentrations of 1- butanol. Because 1-butanol induced depletion of PtdOH levels is considered as a highly specific tool to inhibit PLD activity in intact cells, the results suggest that PLD plays an important role in both the activation of osmotic cell swelling-induced activation of compensatory Cl^- channels as well as in the release of the auto- or paracrinic factor ATP. Surprisingly, potentiation of the taurine release by PMA was equally sensitive to 1-butanol treatment. It is therefore tempting to suggest that PMA

potentiation of the taurine efflux involves the recruitment of an additional pool of organic osmolytes transporters to the plasma membrane through a mechanism that includes PLD activation. This phenomenon may account for the observation in many cell types that Ca^{2+}-mobilizing hormones and growth factors coupled to protein kinase C increase the release of organic osmolytes from osmotically challenged cells (159).

Acknowledgements

We wish to express our gratitude to Dr. T. Munnik, Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, for his indispensable help with the PLA activity assays.

Role of Rho and its effectors ROCK and mDia1 in activation of Volume-Regulated Anion Channels

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Abstract

In most cell types, osmotic cell swelling evokes a robust outwardly rectifying anion current. Using *Clostridium botulinum* C3-exoenzyme treated cells, we have previously demonstrated that a Rho GTPase is involved in regulation of these channels in Intestine 407 cells (306). To further unravel the relationship between Rho-mediated signalling and the activation of Volume-Regulated Anion Channels (VRACs), the roles of ROCK and Dia, both downstream targets of Rho, were investigated. Pharmacological inhibition of ROCK, using Y-27632, did not reduce the volume-sensitive anion conductance. However, in Intestine 407 cells, upon expression of a constitutively active (ROCK-Δ3) or inactive (ROCK-KDIA) kinase, VRAC activation was found to be partially inhibited. A similar inhibition of the response was observed in cells expressing the constitutive active mDia1-ΔN3 mutant. Taken together, the results not only support a model in which active Rho is a prerequisite for VRAC, but also suggests a crucial role in the hypotonicity-induced remodelling of the actin cytoskeleton.

Introduction

Using the human Intestine 407 cell line as a model, we have previously demonstrated that protein tyrosine phosphorylation is a prerequisite for the activation of volume-sensitive anion channels (310), a notion supported by our observations that several proteins, including the focal adhesion kinase p125FAK and the p38 and Erk-1/2 members of the family of MAP kinases are phosphorylated and activated upon hypo-osmotic stimulation (247, 308, 310). Although activation of MAP kinases is part of the initial signaling events after osmotic cell swelling, our recent results argue against a causative role in the regulation of osmo-sensitive anion channels (247, 308, 310). Instead, activation of the small G protein $p21^{Rho}$ (RhoA), but not $p21^{Ras}$ or $p21^{Rac}$, was found to be a key regulator of volume sensitive anion channels (308).

Hypo-osmotic cell swelling is accompanied by a massive remodeling of the actin cytoskeleton (308), a process likely to be regulated by $p21^{Rho}$. Several target proteins of $p21^{Rho}$ have now been recognized and cloned, a number of them being involved in the regulation of the cytoskeleton (207). These include the kinases ROCK and PKN as well as several proteins (Dia, rhofilin, rhotekin and citron (172)) lacking known catalytic domains. A splice variant of citron containing a kinase domain has been found in HeLa cells (171).

A role for ROCK in the activation of VRAC has been proposed previously for bovine endothelial cells (220). In addition, Dia, a Rho target implied in a hereditary form of deafness, might be another candidate, because in the inner ear cells, this cytoskeletal protein is involved in the coupling of sound-induced stereocilium movement to ion channel activation and subsequent neurotransmitter release (164, 171). In this study we investigated the role of these two Rho effectors, which are both crucially involved in F-actin dynamics, in regulating VRAC. The results not only confirm our previous notion that Rho is involved in the regulation of VRAC, but also suggest that the hypotonicity-induced actin remodelling, rather than direct activation of VRAC by ROCK or mDia, plays a important role in development of the RVD response.

Materials and Methods

Materials

Radioisotope ($^{125}I^-$) was purchased from Amersham Netherlands B.V. ('s Hertogenbosch, The Netherlands). Y-27632 was a gift from the Welfide Corporation (formerly Yoshitomi Pharmaceutical Industries). KT5926 was obtained from Calbiochem (La Jolla, CA). *Clostridium botulinum* C_3 ADP-ribosyltransferase was donated by Prof. S. Narumiya (Kyoto University Faculty of Medicine, Kyoto, Japan). All other reagents were from Sigma Aldrich (St Louis, MO, U.S.A.).

Constructs and transfections

The plasmids pFL-Dia with mDia1-ΔN3, pCAG-myc-tagged (pCAG-myc) with ROCK-Δ3, ROCK(I)-KDIA and RhoA, were provided by Prof. S. Narumiya (Kyoto University Faculty of Medicine, Kyoto, Japan). Transfections were performed in a 6 wells plate according to the protocol provided by the manufacture (Invitrogen, Groningen, the Netherlands) using Lipofectamin 2000 added 48 hours prior to the experiments. GFP containg pIRES-plasmids were used to asses transfection efficiency.

Cell culture

Intestine 407 cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 25 mM Hepes, 10% FCS, 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. Prior to the experiments, the cells were serum-starved overnight.

Efflux assay

Monolayers of Intestine 407 cells were loaded for 2 h with 5 µCi $^{125}I^-$ 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 minutes 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 previously described (310).

Results and Discussion

Active Rho is required for VRAC activation

Previously we have shown that in Intestine 407 cells the Ras-related GTPases Ras and Rac are not involved in the activation of the volume-sensitive anion conductance (308). In contrast, a role for another member of the Ras family, $p21^{Rho}$, was suggested. To further establish a potential role for Rho, cultures of Intestine 407 cells were treated with the specific Rho inhibitor *C. botulinum* C3 exoenzyme or were transfected with a dominant inactive Rho mutant (RhoN19). Figure 1 shows an almost complete inhibition of the cell swelling-induced anion efflux in C3-exoenzyme treated cultures. In contrast however, only a partial reduction (35 – 40%) of the efflux was observed in RhoN19 transfected cells (Fig. 1).

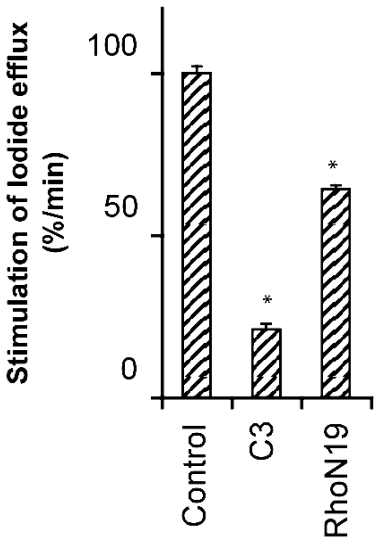

Figure 1. ^{125}Iodide efflux from C3-exoenzyme treated and RhoN19-transfected osmotically stimulated (70% tonicity) Intestine 407 cells.
C. botulinum C3-exo-enzyme (50 µg/ml) was added for 18 h prior to the experiment. Transfection was performed with RhoN19 as described under "Materials & Methods". Data are expressed as a percentage of the untreated control (mean ± SD for n = 3). Asterisks indicate a significant difference from the control ($p < 0.05$).

The incomplete inhibition of the response in RhoN19 transfected cells, as compared to the almost complete inhibition of the efflux in C3-exo-enzyme treated cultures is most plausibly explained by our failure to fully inhibit native Rho protein as the consequence of a rather modest transfection efficiency. This notion is supported by the observation that in GFP expressing cells a mosaic pattern of fluorescence was found corresponding with a transfection efficiency of approximately 40% after 48 h (results not shown). Notably, transfection of Intestine 407 cells with a GFP expression vector did not affect VRAC activation to a significant extent (e.g. see Fig. 3).

Role of ROCK and mDia1 in the activation of VRAC

Rho GTPases have been implicated in F-actin dependent processes such as cell motility, platelet aggregation, lymphocyte adhesion and cytokinesis (348). In addition, Rho proteins, together with Rab GTPases, are involved in regulating endosome movement and distribution (71, 306). Among the most studied downstream effectors of Rho are Dia and ROCK, both involved in initiating actin stress fiber formation (204, 254, 336). The Rho effector ROCK, also known as ROKα or Rho-kinase (112, 149, 180) both phosphorylates myosin light chains and inactivates myosin light chain phosphatase (128). To investigate the involvement of ROCK and its downstream target, the myosine light chain (MLC), cells were treated with the specific ROCK inhibitor Y27632 or with KT5926, an inhibitor of myosin light chain kinase (MLCK, 202). As shown in Figure 2, pharmacological inhibition of ROCK, as well as prevention of MLC phosphorylation by MLCK inhibition did not significantly affect $^{125}I^-$efflux, arguing against a role of ROCK or MLCK-mediated MLC phosphorylation in the activation of VRAC. Similar results were obtained in human umbilical vein endothelial cells (HUVECs; B.C.Tilly, unpublished results). In contrast however, inhibition of VRAC activation by ROCK and MLCK inhibitors has been reported for calf pulmonary endothelial (CPAE) cells (29).

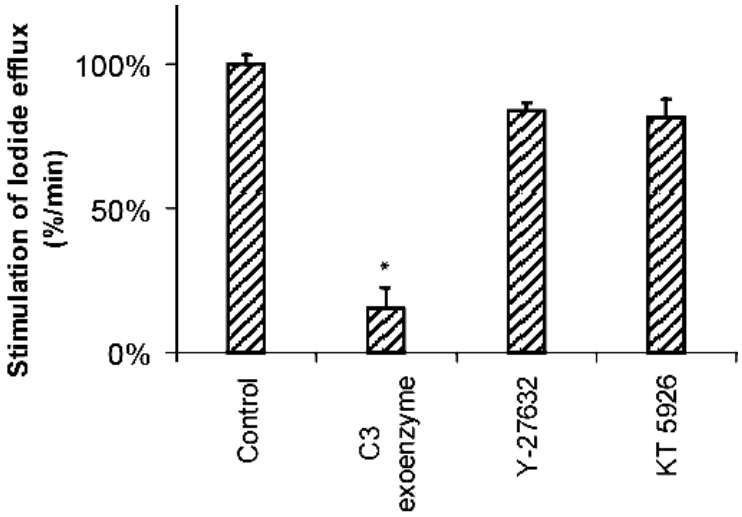

Figure 2. Inhibition of Rho, ROCK or MLCK.
Nearly confluent cultures of Intestine 407 cells were treated overnight with *C. botulinum* C3-exo-enzyme (50 µg/ml) or with either the specific ROCK inhibitor Y-27632 (100 µM) or the MLCK inhibitor KT 5926 (10 µM) 30 minutes prior the efflux assay. VRAC activation by hypo-osmotic stimulation (70% tonicity) was determined by quantitating the anion efflux from ^{125}I-loaded cells as described under "Materials & Methods". Data are expressed as a percentage of the untreated control (mean ± SD for n = 3). Asterisk indicates a significant difference from the control ($p < 0.05$).

As an alternative approach to investigate the potential role for ROCK, cultures of Intestine 407 cells were transfected with either wildtype ROCK, ROCK-Δ3 or

ROCK-KDIA. ROCK-Δ3 is a C terminal truncated and constitutively active mutant lacking the Rho-binding Domain (RBD), a PH domain and the Cys-rich region (113). ROCK-KDIA is a dominant negative mutant which was found to inhibit formation of stress fibers and focal adhesions in HeLa cells (204). As shown in Figure 3, overexpression of wild-type ROCK partially decreased the hypotonicity-induced $^{125}I^-$-efflux by approx. 50%, suggesting that Rho-activated ROCK blocks anion channel activation. Surprisingly, however, expression of either the constitutively active ROCK-Δ3 or the inactive ROCK-KDIA inhibited the anion conductance to a similar extent. Importantly, transfection of the cells with a GFP expression vector did not significantly affect the response. Addition of the ROCK inhibitor Y27632, as expected, did not further diminish the inhibition of the anion efflux in ROCK-Δ3 expressing cells (Fig. 3). Equal expression of WT and mutant proteins was confirmed by Western blotting (results not shown).

Taken together, these data indicate that (over)expression of wild type, constitutively active as well as inactive ROCK prevented the activation of

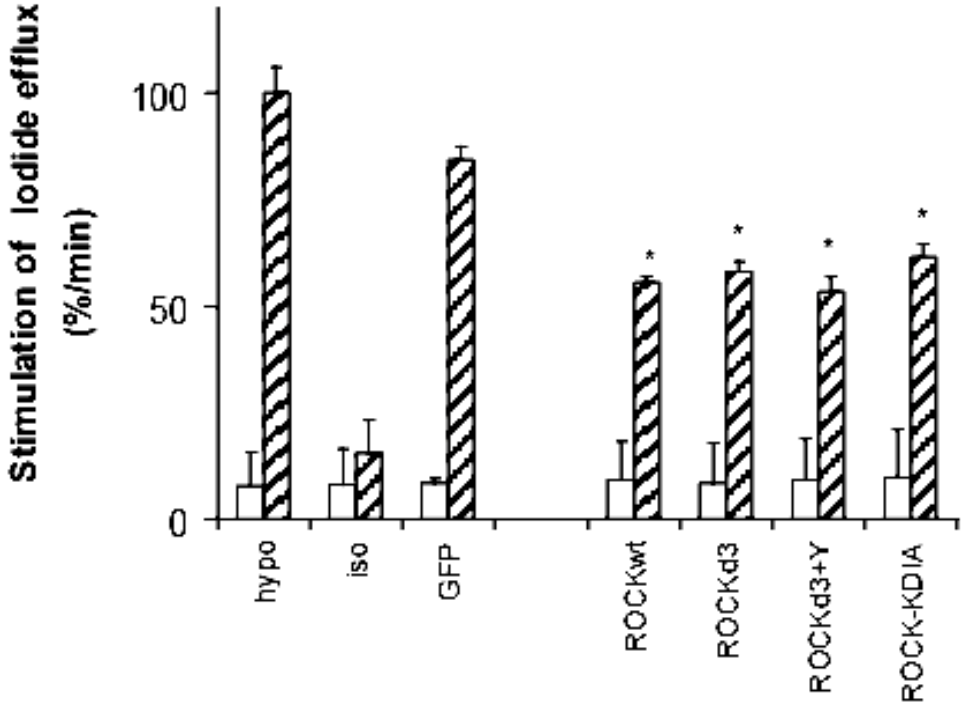

Figure 3. ROCKwt, ROCK-Δ3 and ROCK-KDIA transfected Intestine 407 cells.
Intestine 407 cells were transfected with wild-type ROCK, constitutively active ROCK-Δ3 and inactive ROCK-KDIA 48 h prior to ^{125}I-loading and hypo-osmotic stimulation (70% hypotonicity). Where indicated, Y-27632 (100 μM); Y-27632 was added 30 min prior to the start of the experiments. Data are expressed as a percentage of the untreated control (mean ± SD for n = 3).

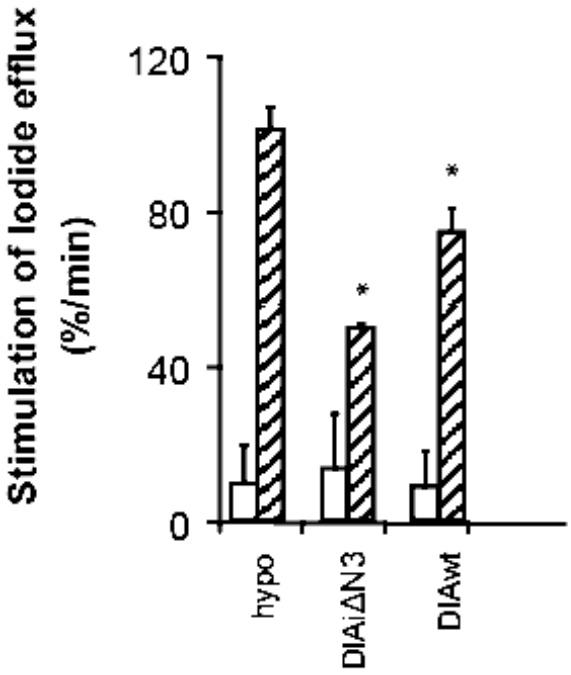

Figure 4. mDia1(wt) and mDia1-ΔN3 transfected cells.
Anion efflux from hypo-osmotically-stimulated (70% tonicity) ^{125}I-loaded Intestine 407 cells transfected with wild-type mDia1 or constitutive active mDia1-ΔN3. Data are expressed as a percentage of the untreated control (mean ± SD for n = 3).

volume-sensitive anion channels. Moreover, pharmacological inhibition of ROCK (Y-27632) did not result in a reduction of hypotonicity-provoked anion efflux. This indicates that the kinase domain that is inhibited by Y-27632 is not involved in VRAC activation. However this does not exclude the involvement of other domains of the protein i.e. a pleckstrin homology domain or a Cys-rich zinc finger-like motif (69).

Following a similar approach, the involvement of Dia in the activation of a compensatory anion conductance was investigated by transfecting the cells with wild-type Dia and a constitutively active mutant, mDia1-ΔN3. Like in ROCK transfected cells, the hypo-osmotically triggered $^{125}I^-$ efflux was markedly inhibited in mDia1 overexpressing cells, the active mutant mDia1-ΔN3 being more potent than the wild-type kinase (Fig. 4).

Because ROCK and mDia1 have profound effects on the F-actin cytoskeleton, it remains possible that these Rho targets are not directly involved in VRAC regulation but, due to their effects on the organization of the actin cytoskeleton, may prevent or affect the hypotonicity-induced actin remodelling in transfected cells.

Conclusions

In this study, we further explored the role of Rho and its downstream effectors ROCK and mDia1 in regulation of VRAC. The results indicate that active Rho is essential for a proper activation of the cell swelling-induced anion channel.

Unlike, treatment of cells with the ROCK inhibitor Y-27632, transfection of the cells with ROCK (wild-type, constitutively active and kinase-dead) or mDIA constructs (wild-type and constitutively active), resulted in inhibition rather than activation of VRAC, arguing against a role of the kinase domain of ROCK in the activation of VRAC and suggesting indicating that another domain of ROCK protein inhibits VRAC. In addition, activated mDIA has an increased inhibitory activity as compared with the transfection of wild type mDIA indicating that a suppressing sequence of mDIA could be involved in the inhibition of VRAC.

The mechanism of inhibition is apparently independent of kinase activity and may involve effects on the organization of the actin cytoskeleton. Due to overexpression and their increased and prolonged association of the constitutively active forms with cytoskeletal elements, the F-actin cytoskeleton might be dramatically altered and shifted into a "frozen" or "stiffened" state, thereby hindering VRAC activation. In line with this concept, treatment of Intestine 407 cells with cytochalasin B was found to potentiate the hypotonicity induced $^{125}I^-$ efflux (306). Furthermore, a rapid and transient reduction in the apical actin cytoskeleton was observed in Intestine 407 cells, starting immediately after hypo-osmotic stimulation (306). Permanent changes in the morphology of the cytoskeleton due to the transfection of regulatory proteins are not unprecedented: a biphasic effect of RhoA on the actin cytoskeleton has been reported (281). At low levels of expression, RhoA stimulated the assembly of actin stress fibers without affecting cell growth. At high levels, however, there was a paradoxical disruption of the actin cytoskeleton accompanied by growth arrest (281).

Alternatively, over-expression of the ROCK and Dia may also lead to competition with other regulatory molecules for essential cofactors or for access to intracellular sites, and thereby, indirectly, diminish the response (204).

Acknowledgements

We are grateful to Dr. Y. Miura (Welfide Corporation, Japan) for his generous gift of the Rho kinase inhibitor Y-27632 and to Prof. S. Narumiya (Kyoto University Faculty of Medicine, Kyoto, Japan) for donating C_3-exoenzym and the pFL-Dia, pFL-mDia-ΔN3, pCAG-myc-ROCKwt, pCAG-myc-ROCK-Δ3 and pCAG-myc-ROCKI-KDIA expression vectors.

Role of aquaporins in cell volume regulation

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Abstract

Treatment of Intestine 407 cells with bivalent mercury ions resulted in an inhibition of the cell swelling-induced $^{125}I^-$ and $^{36}Cl^-$ efflux, but hardly affected the release of ^{3}[H]-taurine. Because Hg^{2+} is a potent inhibitor of aquaporins (AQPs), expression of these water channels in Intestine 407 cells was investigated. RT-PCR analyses revealed expression of several AQPs, aquaporin 3 being most abundantly expressed. In addition, a lower expression of AQP4 and AQP6 was detected. To further study the inhibition by Hg^{2+} during the Regulatory Volume Decrease (RVD) we measured hypotonicity-provoked anion currents by whole cell patch clamping in the absence and presence of micromolar concentrations of Hg^{2+}. The results showed a marked inhibition in the development of the chloride current by NEM and Hg^{2+} treatment.

Introduction

Cells are continuously exposed to volume changes caused by a variety of (patho-) physiological conditions, e.g. changes in metabolism and hormone secretion, alterations in the osmolarity of the extracellular fluid, as well as during cell proliferation and differentiation (140). The defense mechanism against osmotic cell swelling (Regulated Volume Decrease or RVD) leads to a reduction of the intracellular tonicity by a rapid release of (in-)organic osmolytes, often accompanced by the formation of osmotically less active macromolecules (induction of protein or glycogen synthesis) that drives the efflux of water. Transport of water is facilitated by (1) the relative high water permeability of the plasma membrane, (2) ionic channels transporting hydrated ions, or (3) specialized channel proteins, the aquaporins or “water channels” (326).

Salt and water transport is a major function of the gastro-intestinal (GI) tract with more than 9 liters/day of fluid being secreted and, subsequently, absorbed across the epithelia (165). The magnitude of fluid transport in the GI tract is exceeded only by the kidney (in humans ~180 l of fluid per day; 165). Aquaporins are highly expressed in water transporting organs such as the kidney and the GI tract as well as in numerous other tissues and cell types, including the choroid plexus, the eye

lens, endothelium and erythrocytes (165, 179, 328). In the GI tract, aside their function in water transport, a role for aquaporins in fat malabsorption, intrahepatic bile production, pancreatic secretion and saliva production has been proposed (165). At least two AQPs have been associated with diseases in humans, namely AQP0 and AQP2 (272). Mutations in AQP0 lead to a semi-dominant form of cataract (276), whereas mutations in AQP2 lead to nephrogenic diabetes insipidus (NDI).

In mammalian tissues, eleven members of the aquaporin family have been identified (AQP 0-10). Whereas several AQPs are exclusively permeable to water, others (AQP3, 7, 9 and 10) are also able to transport small polar molecules including glycerol and urea (259, 326). In addition, although rather controversial, a role for AQPs in the transport of CO_2 has been proposed (165, 206). Recently, an unexpected property of AQP6 has been reported (108, 346), namely its ability to conduct Cl^-. Interestingly, AQP6 is primarily localized in intracellular vesicles of renal collecting duct cells. There it may serve, together with ClC-5, to maintain electroneutrality during V-type H^+-ATPase activity (346). AQP6 is activated by low pH (4 - 5.5) and, unlike the other AQPs, by mercury ions (96) and is insensitive to DIDS (311). In contrast, most other AQPs are inhibited by Hg^{2+} treatment (for reviews see (165, 209, 326). However, AQP4 facilitated water transport was found not to be affected by Hg^{2+}-ions (166).

Previously, we have studied the mechanism of activation of Volume-Regulated Anion Channels (VRACs) in Intestine 407 cells (311). Despite its ubiquitous expression throughout the animal kingdom and its marked biophysical profile, the molecular identity of this channel has not yet been identified. Over the years, a number of candidates have been proposed, but none of them fulfill all the necessary criteria found in Intestine 407 cells. Here we pursue a putative role for AQPs during the RVD response in Intestine 407 cells.

Materials and Methods

Materials

Radioisotopes (^{3}H-taurine, $^{125}I^-$, $^{36}Cl^-$) were purchased from Amersham Netherlands B.V. (s'Hertogenbosch, The Netherlands). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

Intestine 407 cells were routinely grown as a monolayer in Dulbecco's modified Eagle's Medium supplemented with 25 mM Hepes, 10% FCS, 1% non-essential amino acids, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere with 5% CO_2 at 37°C. Prior to the experiments, cells were serum-starved overnight.

Radio-isotope efflux assays

Confluent monolayers of Intestine 407 cells were loaded with 5 µCi/ml $^{125}I^-$, 50 µCi/ml $^{36}Cl^-$ or 0.1 µCi/ml ^{3}[H]-taurine for 2 h in 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 7.40) and subsequently 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). Full time courses were prepared by replacing the medium at 1 – 2 minute intervals. For inhibitor studies, a single fraction of 8 minutes was collected. $^{125}I^-$ Radioactivity was determined by γ-radiation counting. ^3H- and $^{36}Cl^-$ radioactivity in the media was determined by beta-radiation counting. Data are expressed as fractional efflux per minute as previously described (318).

RNA isolation, amplification, and analysis

Total cellular RNA from Intestine 407 cells, grown on a 150 cm^2 plate, was isolated by the Rneasy Plant minikit (Qiagen, Valencia) using the protocol provided by the manufacturer. Plates were chilled at 4°C before scraping. Synthesis of cDNA from total RNA was performed in a 10 µl reaction mixture containing 1 µg RNA, 500 ng random hexamers of Promega, 1×RT buffer (50 mM Tris (pH 8.3), 3 mM $MgCl^-$, 75 mM KCl, 10 mM DTT), 2 mM dNTP's, 10 units RNAsin and 5 units reverse transcriptase (RT) M-MLV (Promega , Madison, WI). RT reaction was performed during 1 hour at 37°C. The RT-reaction was inactivated by raising the temperature to 70°C for 5 min, followed by the addition of 5 µg RNAse (15 min at 37°C).

Aquaporin PCR primers were designed based on the published sequences from the human gene bank in the National Center for Biotechnology Information (NCBI) by comparing chromosomal DNA with cDNA. Primer sites were selected around intron splice sites. The amplifications were performed with the following forward (f) and reverse (b) primer sets: AQP0 (gi=6912505) f: TGTCTTCTTTGGGCTGGGGTCCTC / b: ATTCCGCCTCTCGTCGTATGT, AQP1 (gi=180500) f: CCCTGGGCTTCAAATACC / b: CGGCCAAGCGAGTTCCCAGTCAG, AQP2 (gi=474058) f: TCCATGAGATCACGCCAGCAGA/ b: GCGAGCGGATTCAGCCATTACC, AQP3 (gi=9257193) f: CCTTTGGCTTTGCTGTCACTCTGG/ b: CTCGGGGGACGGGGTTGTTGTAAG, AQP4 (gi=1072054) f: TTCTGGCCATGCTTATTTTTGTTC/ b:CACTGGGCTGCGATGTAGA, AQP5 (gi=20550620) f: CGGGGTTGCGCTGGACGAC / b: GGCGGCGGAGGAGGTGGATG, AQP6 (gi=1293545) f: ACGGTGGGGGCTGCTCTGCTTTAT/ b: TGCCCCTGTCCCCACCTCTA, AQP7 (gi=2317273) f: CTACACGGCCATTCTCCACTTTTC/ b: ACCCCGATGATGACCACGAGGATG, AQP8 (gi=6606075) f: GTGGGTGGCAGGTGGCGAGTGT/ b:CCCCCGAGCAGCTGTGAGACC, AQP9 (gi=2887406) f: AACTGCTGATCGTGGGAGAAAATG/ b: CAACCAAAGGGCCCACTACAGGAA, and Aq10f: GGTCAAGCTCCCCATTTACATC/ b: GGAGGCCAGCACCAGGAGAGTC (gi=16564852). PCR was performed in a total volume of 10 µl of reaction mix containing 0.4 units of REDTaq DNA polymerase (Sigma, St. Louis, Mi), about 10 ng of total cDNA, 1×REDTaq PCR buffer, 2.5 mM $MgCl_2$, 250 µM dNTPs, and 0.25 µM of each primer. The PCR program consisted of one cycle at 94°C for 1 min followed by 30 cycles of 39 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C and by final extension at 72°C for 10 min. The PCR product then was analyzed on 1.5% ½xTBE-agarose gel containing 0.2 µg/ml EtidiumBromide.

Western blotting

Cells were washed four times and lysed in sample mix. Lysates were subjected to SDS-PAGE and proteins were electrophoretically transferred to nitrocellulose membranes. Expression of aquaporins are determined with monoclonal antibodies against AQP0 (Alamone Labs, Jerusalem, Israel) or AQP3 (SantaCruz Biotechnology, SantaCruz, Ca), using Horseradish peroxidase coupled secondary antibodies (1:10000) and an ECL detection system (Pierce, Rockford, Il), according to the instructions provided by the manufacturers.

Measurement of whole-cell Cl^- currents

Cells were bathed in a solution containing 110 mM CsCl, 5 mM $MgSO_4$, 3.5 mM sodium gluconate, 12 mM Hepes, 8 mM Tris and 100 mM mannitol, pH 7.4. 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) and had a resistance of 1–2 MΩ. 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. For command pulse control, data acquisition and analysis, pCLAMP 6 software (Axon Instruments, Union City, CA) was used. All data were sampled at 5 kHz after being low-pass filtered at 500 Hz.

Results and Discussion

Activation of VRAC and the organic osmolyte release pathway in the presence of bivalent mercury ions

Addition of $HgCl_2$ (30 µM) to $^{125}I^-$-loaded cultures of Intestine 407 cells almost completely abolished the cell swelling activated anion efflux (Fig. 1A). To exclude that the observed reduction of the efflux is due to the formation of a HgI_2

precipitate (although, under the experimental conditions used, the solubility product was not exceeded), the experiments were repeated with $^{36}Cl^-$-loaded cells. Again, as shown in Fig. 1B, addition of Hg^{2+} resulted in a full inhibition of the volume-sensitive efflux. Mercury ions are established inhibitors of most of the aquaporins (167), acting through binding to a single free sulphydryl group near the pore region of the channel (246) (for review see Agre (1)). N-ethylmaleimide, a different sulphydryl reagent unable to block aquaporins but a potent inhibitor of SNARE proteins involved in exocytosis (2), was unable to reduce the hypotonicity-provoked increase in iodide efflux when applied acutely (e.g. only present in the hypotonic solution) but was able to inhibit the volume-sensitive anion efflux completely in pre-treated cultures (1 mM, 20 min; Fig. 1A). These results suggest that exocytosis plays an important role in the regulation of VRAC.

In addition to the activation of volume-sensitive ion channels, osmotic cell swelling also triggers the release of organic osmolytes. Previously, we have reported that both compensatory mechanisms are regulated independently and involve different release pathways (311). To further investigate the specificity of the Hg^{2+}-mediated inhibition of the anion efflux, its effects on the release of taurine was investigated. As shown in Fig. 2, low concentrations of Hg^{2+} (30 μM), shown to fully block the cell swelling-induced $^{125}I^-$ and $^{36}Cl^-$ efflux (see Fig. 1), only slightly affected the hypotonicity-provoked taurine release. Furthermore, even a ten-fold higher concentration of Hg^{2+} ions (300 μM) only reduced the taurine efflux to a minor extent. The basal efflux of taurine, however, is slightly increased in the presence of mercury ions (not shown). Because of the observed insensitivity of the taurine efflux, the results argue against a direct inhibitory effect of Hg^{2+} ions on osmotic cell swelling and consequently, the involvement of mercury-sensitive AQPs during transmembrane water transport. However, transmembrane flow of water may not be carried exclusively by mercury inhibitable aquaporins, but in addition, AQP4, AQP6 or other transporters could also be involved, for example the human erythroid urea transporter (hUT11a) (278, 61). To test for the possible involvement of hUT11a, we used phloretin as an inhibitor. As shown in Fig. 2, the release of taurine was not affected under these conditions, indicating that cell swelling is normal and that this transporter is not involved in swelling-associated water movement.

The release of taurine was partly inhibited by acute treatment of the cells with NEM, but, unlike the hypotonicity-induced anion efflux, a more prolonged treatment of the cells did not resulted in a complete inhibition (Fig. 2).

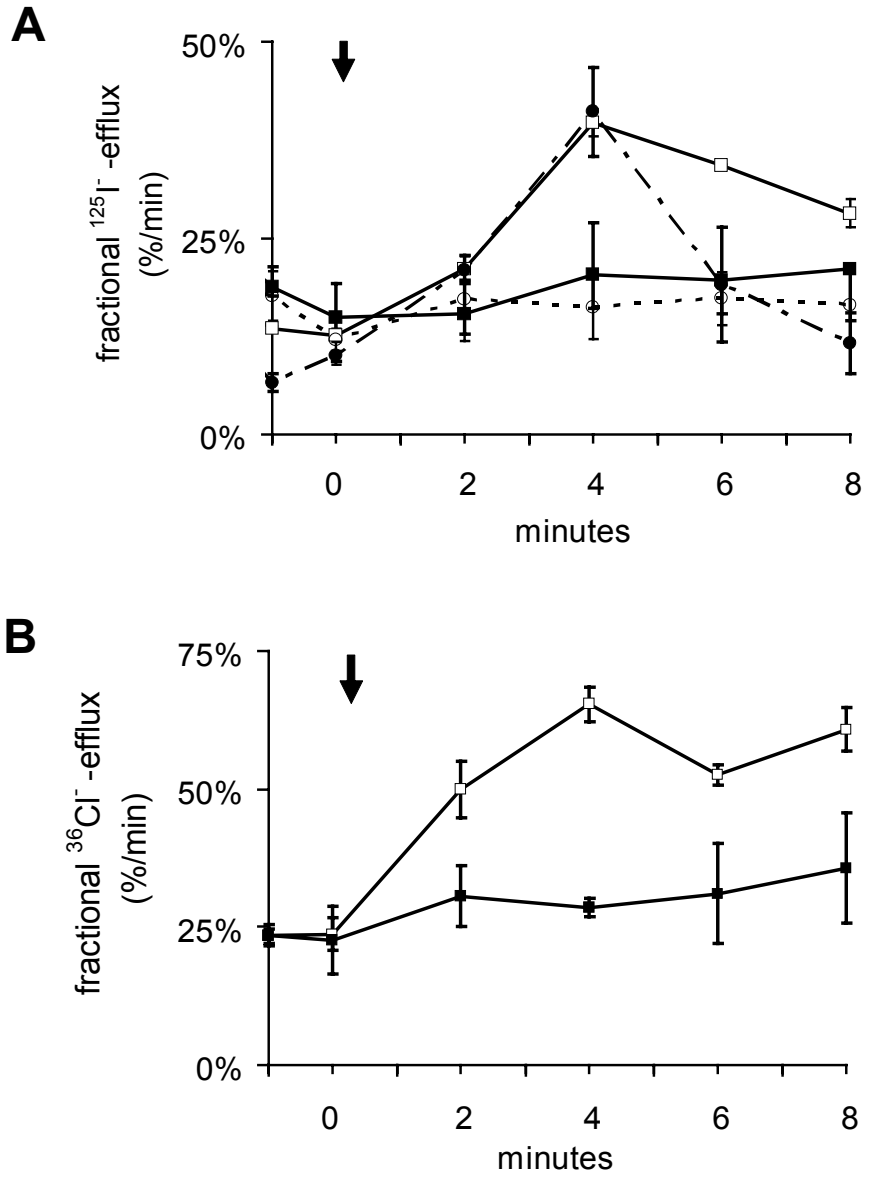

Figure 1. Inhibition of the hypotonicity-induced $^{125}I^-$ and $^{36}Cl^-$ efflux by mercury ions and N-ethylmaleimide (NEM).
(**A**) Cells were loaded with $^{125}I^-$ and exposed to a 40% hypotonic medium as described under "Materials and Methods" in presence of 30 µM $HgCl_2$ (closed boxes; added 20 minutes prior to osmotic stimulation), 1 mM NEM (closed circles; added to the hypotonic medium only) or 1mM NEM (open circles; added 20 minutes prior to osmotic stimulation). Open boxes represent control incubations. (**B**) Hypotonic efflux from $^{36}Cl^-$ loaded cells in the absence (open boxes) or presence of 30 µM $HgCl_2$ (closed boxes) added 20 minutes prior osmotic shock. Arrow indicates shift to hypo-osmotic medium. Data are expressed as fractional efflux (mean +/- SD, n=3) and are representative for 2 other experiments.

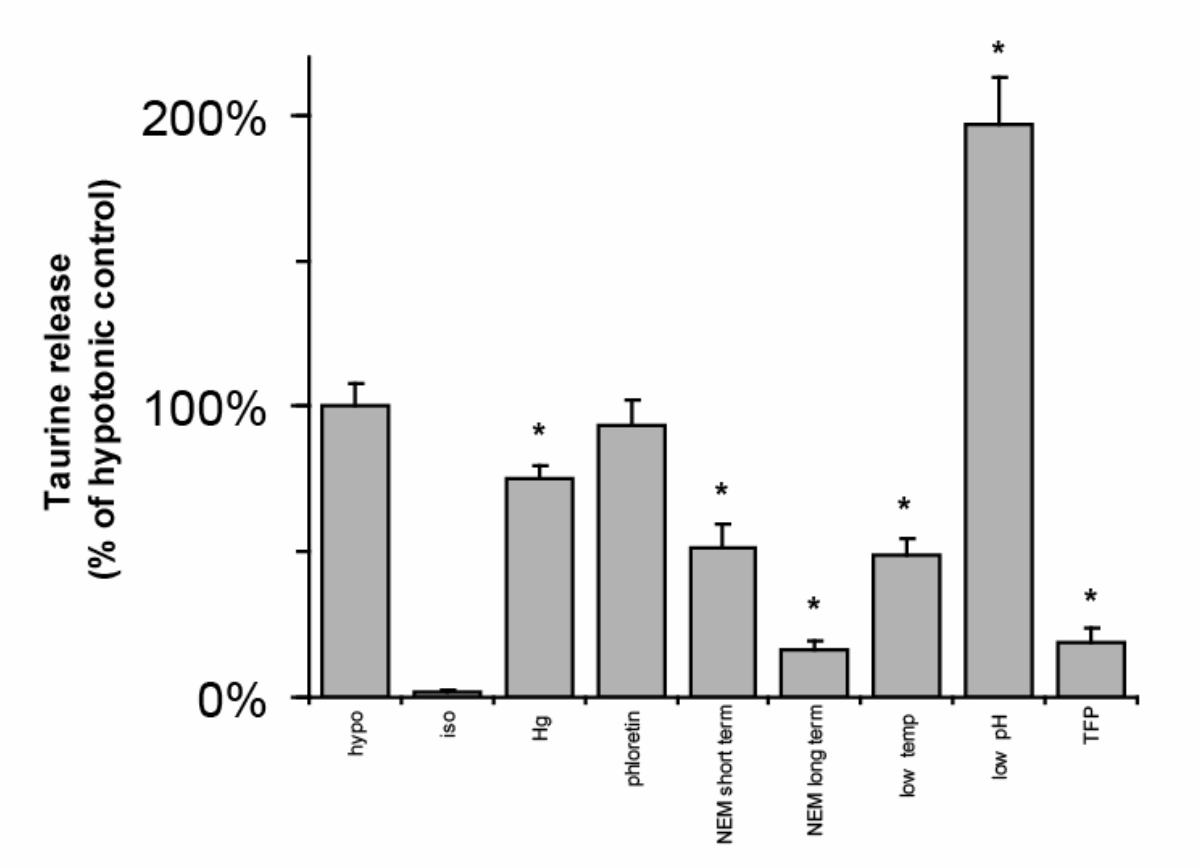

Figure 2. Pharmacological inhibition of the hypotonicity-induced taurine release.
^{3}[H]-taurine-loaded cells were exposed to hypotonic media and the fractional isotope efflux was determined as described under "Materials and Methods". Cultures were treated with Hg^{2+} (30 µM for 20 minutes), phloretin (250 µM, 15 min), low temperature (16°C) or pH (pH5.5), NEM (1 mM, acute or 20 min preincubation) and trifluoperazine (TFP; 25 µM for 15 min). Data are expressed as percentage relative to the hypotonic control (mean ± S.D. for n=3). Asterisks indicate a significant difference from the hypotonic control ($p<0.05$, Student t-test).

Expression of aquaporins in Intestine 407 cells

To investigate transcript levels of aquaporins, RT-PCR was performed for each of the 11 AQPs on cDNA prepared from RNA isolated from Intestine 407 cells. As shown in Fig. 3A, no amplification was observed when primers for AQPs 0, 2, 9 or 10 were used. AQP1 and AQP8 expression was inconclusive because the PCR products are respectively longer or slightly shorter than predicted. The amplified fragments of AQP3, 4, 6, however, have the predicted length, indicating their expression in Intestine 407 cells. The highest expression was observed for AQP3, with lower amounts for AQP 4 and 6 (relative intensities: 53% and 17% respectively as compared to AQP3). PCR amplification of AQP6 fragments revealed an additional shorter fragment.

Levels of RNA expression are not always linear with protein expression because additional factors like mRNA stability, translation efficiency and protein stability are involved (186); we therefore also examined the expression of AQPs at the protein level by the Western blot technique. In line with the PCR data, AQP3 protein could be easily detected in homogenates of Intestine 407 cells as a distinct

31 kDa protein band as well as a 40kDa band most likely representing a glycosylated form (50, Fig, 3B). AQP1, as expected, was not detected (results not shown).

Both AQP3 and AQP6 are regulated by pH. Activation of AQP6, by lowering the pH of the medium to pH 5.5, resulted in an increased efflux of taurine (Fig. 2). A potential role for vesicle cycling and calmodulin-dependent processes in the regulation of the taurine efflux can be concluded from our results obtained at low temperature or NEM and after treatment of the cells with trifluoperazine respectively. None of these conditions however, resulted in a complete inhibition of the organic osmolyte release (Fig 2).

Because the AQPs 4, 6 and 8 are localized primarily in intracellular compartments (51, 51, 92), involvement of these channels in cell volume regulation requires their transient recruitment to the plasma membrane. It is possible therefore, that exocytosis not only plays a role in the activation of VRAC, but also in the activation of water channels, and, to a lesser extent, of the organic osmolyte transporter.

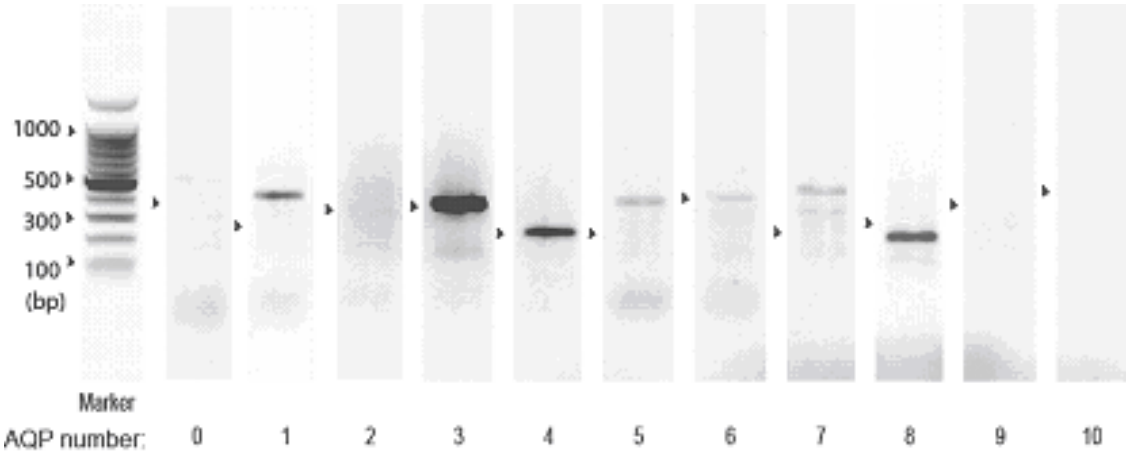

Figure 3A. RNA expression of aquaporins in Intestine 407 cells.
RNA isolated from Intestine 407 cells was used for aquaporin expression analysis by using PCR as described in "Materials and Methods". The expected size of the PCR fragments is indicated by arrow heads. Genomic DNA sequences and immature mRNA will result in larger band(s), due to amplification of the introns and only amplification of mature mRNA results in the predicted band.

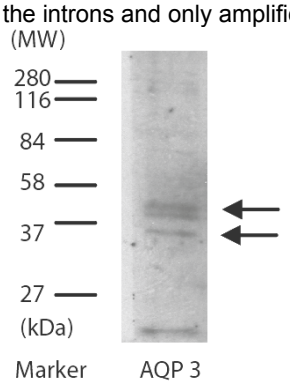

Figure 3B. Aquaporin 3 expression in Intestine 407 cells.
Whole cell lysate of Intestine 407 was analyzed by SDS-PAGE followed by Western blotting with a primary antibody against AQP3. The arrows indicate the positions of AQP3 (31 kDa and 40 kDa).

Role of aquaporins in the development of the cell swelling activated anion current

To investigate the involvement of AQPs in cell volume regulation and to pursue whether AQPs could function as (a) molecular candidate(s) for VRAC, hypotonicity-provoked anion currents were studied by whole-cell patch clamping. Due to the progressive loss of the required high resistance membrane seal in the presence of mercury ions, recordings of the VRAC activity were limited to a 15-25 min. period. As shown in Fig. 4, extracellular addition of Hg^{2+} or NEM resulted in an inhibition of the hypotonicity-induced chloride current. During the experiment, digital images of the attached cell were acquired continuously and its perimeter, taken as an estimate of its volume, was measured. Table 1 shows that, during the first 10 min, the perimeter of Hg^{2+}- and NEM-treated cells did not differ significantly from the untreated controls.

To investigate the biophysical characteristics of the cell swelling-activated anion current, voltage pulses were applied ranging from –100 to +100 mV (25 mV increments). In these experiments, the rate of osmotic swelling was accelerated by increasing the tonicity of the pipette solution to 450 mOsm by adding mannitol. As has been reported previously (321), the volume-sensitive anion conductance displayed an outwardly rectification and a marked voltage-dependent inactivation at depolarizing holding potentials. As shown in Fig. 4B, despite the reduction of the current amplitude, these electrical characteristics are still observed in Hg^{2+}- as well as in NEM-treated cells. Surprisingly, unlike the hypotonicity-provoked isotope efflux (Fig. 1) and the conductance triggered by reducing the osmolarity of the bathing solution (Fig. 4A), the anion currents elicited by an increase in the tonicity of the pipette solution was only partly inhibited by Hg^{2+} and NEM (40-50%, Fig. 4B, Hg^{2+} and NEM in medium). Notably, addition of Hg^{2+} or NEM to the pipette solution did not (NEM) or only slightly (Hg^{2+}) reduces the volume-sensitive anion conductance (Fig 4B).

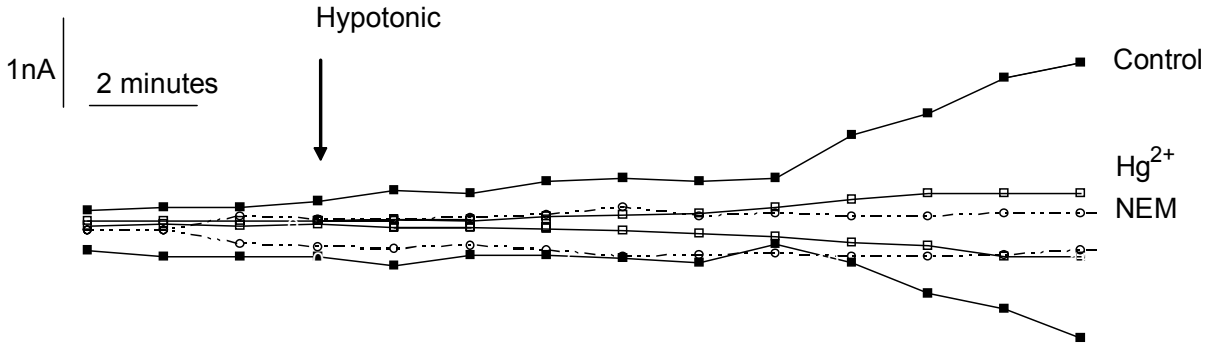

Figure 4A. Cell swelling-activated anion conductance.
Contineous registration of the whole cell Cl- currents in control (solid boxes) and Hg^{2+}- (30μM, 20 minutes prior; open boxes) or NEM-treated (1 mM, 20 minutes prior hypotonic shock; open circles) cells at –100mV and +100mV holding potentials.

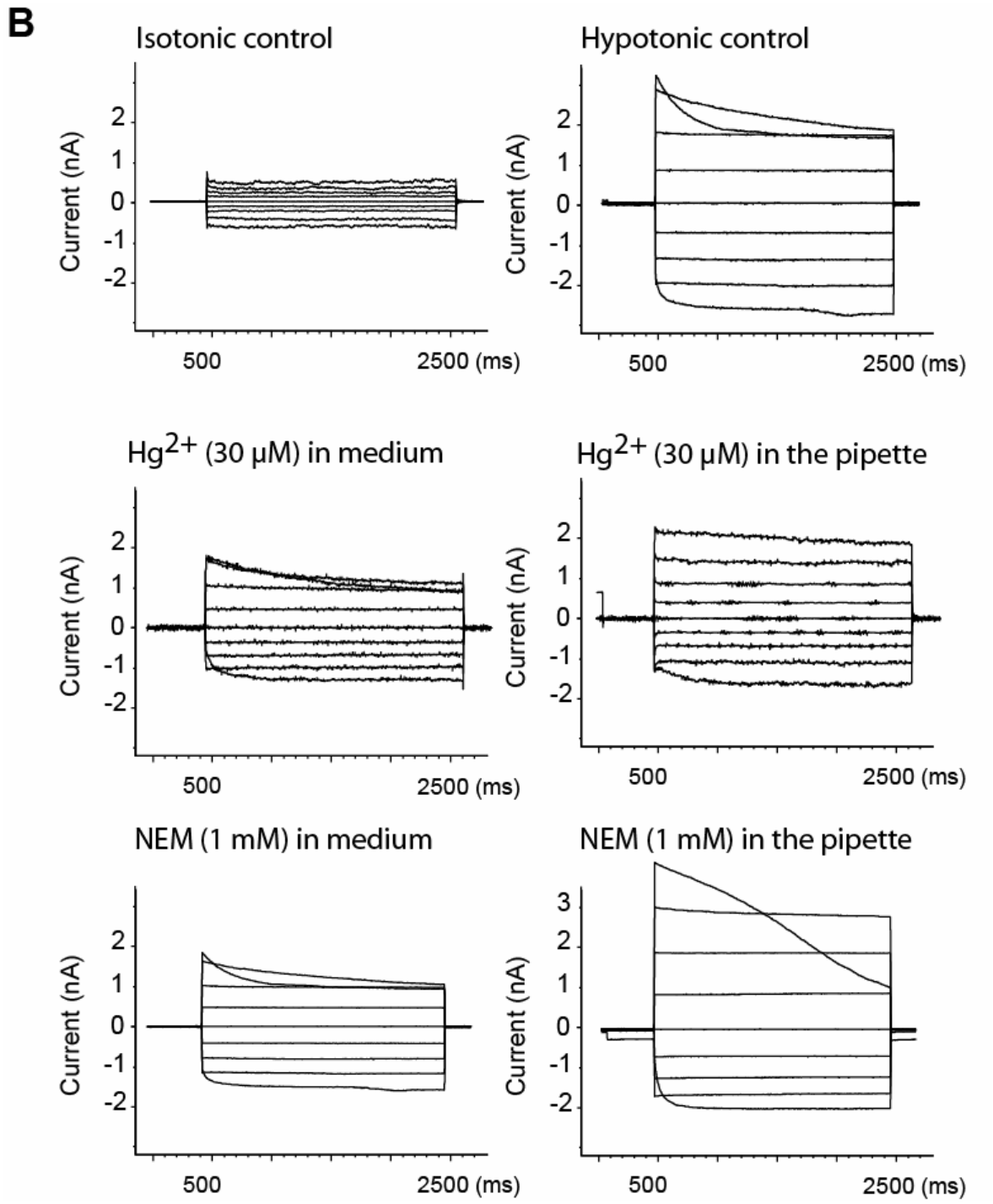

Figure 4B. Current-Voltage relationship of the osmo-sensitive anion conductance.
Anion currents in response to step pulses from -100mV to +100mV (25mV increments) from control cells and cells exposed to extracellular (10 minutes preincubation) or intracellular (no pre-incubation) $HgCl_2$ (30µM) or NEM (1 mM). The anion conductance was provoked by a hyperosmotic (450mOsm) pipette solution. Traces are representative of 5 independent experiments.

	Perimeter (%)	
	Average	SD
Isotonic	101	1
Hypotonic	104	1
Hypo + Hg^{2+}	102	9
Hypo + NEM	102	4

Table 1. Changes in cell volume.
Cell size was quantitated by calculating its perimeter from digital images acquired during whole cell patch clamp experiments. The change in perimeter, after 10 min. of exposure to a hypotonic medium, in control and Hg^{2+} (30 μM) or NEM (1 mM) treated cells is presented as percentage relative to its value under isotonic conditions.

Conclusions

Treatment of Intestine 407 cells with Hg^{2+} resulted in an inhibition of the cell swelling activated efflux of $^{125}I^-$ and $^{36}Cl^-$. Because of the well established sensitivity of AQPs to mercury ions, due to the presence of several cysteine residues in the pore region of the channel (246), a possible involvement of AQPs in the RVD response was investigated. There are at least two potential ways in which AQPs could be involved; firstly, as a water channel, AQPs may facilitate transmembrane water movement, i.e. may be essential for the actual change in volume. Secondly, in addition to water transport, AQPs may conduct osmolytes. Furthermore, these data also rule out the possibility that Hg^{2+}-sensitive AQPs are involved in organic osmolyte efflux in Intestine 407 cells. It has been recently reported that AQP6 is able to conduct Cl^- (346). Although some AQP6 expression was observed in Intestine 407 cells, it is unlikely that the molecular identity of VRAC is represented by AQP6, because, unlike the other AQPs, transport through AQP6 is increased rather than decreased in the presence of Hg^{2+} (96).

Recently, we have reported that, in addition to the activation of K^+- and Cl^--selective ion channels, osmotic cell swelling also resulted in the release of several organic osmolytes, including taurine and phospho-choline (311). We also demonstrated that the release of organic osmolytes serves as a second line of defence, being activated after a distinct lag and only after a more severe hypo-osmotic stimulus (< 70% tonicity); 311). Because, as compared to the anion efflux, the cell swelling provoked release of taurine is much less sensitive to Hg^{2+} treatment it is unlikely that, in intact cells, Hg^{2+} inhibits VRAC activation by preventing osmotic cell swelling.

Like Hg^{2+} treatment, exposure of the cells to NEM resulted also in an inhibition of the osmo-sensitive $^{125}I^-$ efflux and Cl^- conductance. It is now well established that water movement through AQPs is insensitive to NEM (2). Recently, we reported that osmotic cell swelling is accompanied by exocytosis through a mechanism

involving NEM-sensitive SNARE proteins (324; and Chapter 4). It is therefore tempting to suggest that NEM affects the development of the volume-sensitive anion conductance by inhibiting VRAC recruitment to the plasma membrane.

At this stage, it remains unclear why Hg^{2+} and NEM exerted a much less pronounced inhibitory effect on VRAC currents under whole cell patch clamp conditions, in particular when added intracellularly through the pipette, as compared with the conditions of the anion efflux assay in intact cells. A major difference may be the time allowed for Hg^{2+} introduction (i.e. typically 20 minutes for efflux assays but 0 minutes under patch clamp conditions). Alternatively, the Hg^{2+} binding site of AQPs might be less accessible from the cell inside (2). Furthermore, we cannot discriminate between the potential cellular targets involved in the Hg^{2+} inhibition of VRAC. Firstly, Hg^{2+} may act on a mercury-sensitive AQP which is also able to conduct Cl^-. Secondly, a different, yet unidentified, chloride channel might be involved, which is directly inhibited by mercury ions and finally, Hg^{2+} could interfere with the signalling mechanisms leading to recruitment of VRAC. To further unravel the role of AQPs during the RVD response in Intestine 407 cells, additional further experiments, including the generation of AQP 3, 4 and 6 deficient cells, using the siRNA approach, are needed.

Discussion and Perspectives

Introduction

The major goal of the studies described in this thesis has been to unravel mechanisms underlying the RVD response in epithelial cells and to identify proteins involved. In the literature, many different cell types have been used for studying regulation of cell volume. Although the mechanisms underlying the RVD may in part be cell type specific, several steps in the cascade appear to be universal. The hitherto identified universal steps in VRAC activation sofar include the activation of tyrosine kinases and small G proteins (330).

The tyrosine kinase(s) involved probably belong to the p56 Src-family (91, 310, 312). The Src-kinases are non-receptor tyrosine kinases known to be involved in mitogenesis, cytoskeletal organization, differentiation, motility, adhesion and membrane trafficking (73, 300). Recently, $p56^{lck}$ has been shown to be essential in activation of the swelling-induced chloride currents in lymphocytes (148, 295). Involvement of $p56^{lck}$ in Intestine 407 cells is also likely, because treatment with damnacanthal, a potent and selective inhibitor of $p56^{lck}$, largely reduced the volume-sensitive anion efflux (B.C.Tilly, unpublished results).

Release of organic osmolytes (Chapter 3)

In recent years the concept becomes gradually accepted, that not only inorganic osmolytes such as K^+ and Cl^- are used to restore osmotic equilibrium in mammalian cells but that also a redistribution of organic osmolytes is involved (337). Permeation of organic osmolytes (gluconate, glycine, glutamate, aspartate, polyols and ATP) through VRAC has been reported; however, as compared to the efflux of chloride or iodide, their conductance is rather moderate (211, 214). In contrast to the efflux of $^{125}I^-$, the hypotonicity-provoked organic osmolyte release from Intestine 407 cells was activated only after a distinct lag and lasted much longer. Furthermore, we observed that the signaling pathways involved in activation of the efflux of organic osmolytes markedly differ from those involved in regulation of iodide and chloride transport (cf. see Chapter 3 and Fig. 1). We therefore concluded that, at least in our model cell line (Intestine 407), the release of organic osmolytes utilizes a pathway distinct from VRAC. The existence of multiple release pathways has also been observed in hippocampal cells (67) as well as in Hela cells (290). In contrast, in Chinese hamster ovary (CHO) cells (25), VRAC was shown to conduct both chloride ions and organic osmolytes and was therefore named “volume-sensitive organic osmolyte and anion channel” (VSOAC

(116)). Whole cell patch clamp studies have demonstrated that ATP has to be present at the cytoplasmic face of the plasma membrane to be able to activate VRAC by osmotic cell swelling (114, 223). A similar dependence on ATP of the organic osmolyte release was found in rat glial cells (115, 263), skate hepatocytes (114), rat IMCD cells (261) and endothelial cells (223), showing a diminished taurine efflux in response to a decrease in intracellular ATP level.
The swelling induced organic osmolyte efflux (e.g. taurine, phosphocholine, betain) from Intestine 407 cells was found to be potentiated by treatment of the cells with the protein kinase C activator PMA (311). A similar potentiation was also observed in C6 glioma cells (117). As with Intestine 407 cells, activation of the taurine efflux in these cells was also independent of small G proteins and of a rise in intracellular free Ca^{2+} (261). Unlike our observations on Intestine 407 cells however, the organic osmolyte efflux from C6 glioma cells was potentiated by arachidonic acid and by a forskolin-induced increase in intracellular cAMP level (130). Notably, PMA did not affect the hypotonicity-induced chloride efflux from in Intestine 407 cells (322) as well as from human cervical cancer HT-3 cells (33). Because pharmacological inhibition of the phospho-choline and taurine efflux was very similar, these compounds seem to utilize a single release pathway.
In summary, based on kinetic and pharmacological data obtained in Intestine 407 epithelial cells, our results support a model involving a single organic osmolyte release pathway which is distinct from VRAC (fig. 1). We cannot, however, completely rule out a possible operation of two or more related organic osmolyte transporters (135), with different sensitivities for the inhibitors used in our experiments.

Vesicle cycling (Chapter 4)

Hypo-osmotic swelling of Intestine 407 cells resulted in an increased rate of endo- and exocytosis, as determined by measuring the total plasma membrane surface area as well as by quantitating the uptake of fluorescent labeled dextrans (see chapter 4 and fig. 1) (324). Membrane recycling and vesicle trafficking has been extensively studied in several cell types, including neurons (15, 64, 161, 258). The molecular mechanisms are well conserved during evolution and the proteins involved have been identified in yeast as well as in prokaryotes and invertebrates and appear to be highly homologous (156, 161, 258, 298). These studies have demonstrated that in addition to constitutive, calcium- independent exocytosis, a distinct exocytotic fusion machinery exists which depends on the so-called SNARE proteins (soluble-*N*-ethyl-maleimide sensitive factor (NSF) – attachment protein receptor) (155, 291). Plasma membrane-vesicle fusion involves the interaction of the vesicle-associated SNARE-protein cellubrevin (in neurons: synaptobrevin)

with the plasma membrane-associated syntaxin and SNAP-25 in a zipper-like fashion (152), a process sensitive to N-ethylmaleimide (NEM) treatment (341). In our study, both the cell swelling activated anion conductance, as well as the release of organic osmolytes were inhibited in the presence of extracellular NEM (Chapter 7). Intracellular application of NEM however, by addition via the patch pipette, was unable to inhibit VRAC, suggesting that under whole cell patch clamp conditions (i.e. EGTA-buffered low internal calcium), vesicle fusion and channel recruitment does not play an important role.

Intracellular vesicle movement is guided by the microtubular network (85, 58). The insertion of aquaporins, Cl^-/HCO_3^--exchangers and organic-anion transporters in kidney and liver cells has been reported to be facilitated by this network (58, 85, 140). Future experiments on the role of channel recruitment in VRAC activation could encompass studies of the special membrane areas that are high in cholesterol and sphingolipid content; the caveolae and lipid rafts (241). Caveolae are abundantly present in most cell types (83) and serve as "hot spots" containing many proteins that are critically involved in signal transduction (66, 212). Vesicle transport systems in endothelial cells are closely associated with caveolae, which contain the key proteins that mediate the different aspects of docking, fusion and exocytosis as well as of vesicle budding and endocytosis (212, 101, 56). It has been demonstrated that VRAC is associated with these caveolae in bovine endothelial cells through the caveolin-1 protein (314). However, their role in the RVD response in Intestine 407 cells remains to be explored.

Rho effectors (Chapter 6)

We further extended our previous studies on the role of $p21^{Rho}$ and its downstream effectors during the development of the RVD. Phospholipase D (PLD) regulation by Rho has been reported previously (5). We now have demonstrated that PLD indeed has a function in the activation of VRAC but not in the activation of the organic osmolyte release pathway (see Chapter 5 and Fig. 1).

Both $p21^{rho}$ and $p21^{rac}$ play an important role in the organization of the F-actin cytoskeleton, stress-fiber formation, and cell ruffling (6, 8, 251, 252, 253). Involvement of $p21^{Rho}$ in the RVD response has been published previously by our group (323). RhoA has been proposed as a candidate for the small G protein involved in volume regulation in several other model systems (29 220, 29). Several target proteins of $p21^{Rho}$ have now been recognized and cloned, a number of them being involved in regulating the cytoskeleton (8, 207). These include the kinases p160Rock and PKN as well as several proteins (p140DIA , rhofilin, rhotekin and citron (172)) lacking known catalytic domains.

We investigated a putative role for ROCK and mDia1 in the activation of VRAC. To our surprise, not only expression of constitutively active ROCK or mDia1, but, rather unexpectedly, also inactive ROCK resulted in an inhibition rather than activation of the hypotonicity-provoked $^{125}I^-$ efflux. A possible explanation for the observed inhibition could be a disturbed balance between polymerized and soluble actin, due to a prolonged overexpression of these actin associated proteins, thereby arresting the cells in a state where cytoskeletal remodeling is no longer possible. Alternatively, high expression of these proteins may lead to a competition for essential cofactors or for access to the intracellular sites involved in channel regulation (248). It has been demonstrated that the actin binding proteins Actin Depolymerizing Factor (ADF) and cofilin, structurally related proteins essential for the rapid turnover of actin filaments, are co-transported with actin-binding proteins (139, 298, 301). ADF and cofilin activity is regulated through phosphorylation by the so-called LIM kinases, again downstream effectors of the Rho family of GTPases (13, 84, 139, 174, 297, 298). A putative role for LIM kinases in the regulation of the RVD response in Intestine 407 cells however, remains to be established.

A critical role for actin and actin-associated proteins during the RVD response is supported by observations from our lab and by others that osmotic cell swelling is accompanied by a rapid and transient remodeling of the actin cytoskeleton (139). In addition to Intestine 407 cells (308, 324), involvement of actin remodeling has also been observed in epithelial cells, Ehrlich ascites tumor cells and HSG cells (36, 59). Notably, expression of wild type RhoA in the epithelial U2OS-cells had multiple effects on the architecture of the actin cytoskeleton, which was found to depend primarily on the level of expression (281).

To conclude, in Intestine 407 cells, the cytoskeleton may serve two functions during osmotic cell swelling and the subsequent RVD response. First, the actin filaments just below the cell membrane surface (terminal web) may increase the cell rigidity, thereby counteracting the tendency to increase cell volume. In addition, the microtubular network may facilitate vesicle trafficking and the recruitment of channels and transporters to the plasma membrane (see Fig. 1 for a model).

Aquaporins (Chapter 7)

Cell swelling depends on the net influx of water. After swelling, cell volume can be restored by the efflux of water by (i) facilitated diffusion through water channels or aquaporins, (ii) by transport of hydrated of ions and molecules or (iii) by diffusion through the cell membrane. In most cell types, specific aquaporin subtypes fulfill a crucial role in water transport (129, 162, 274, 304).

In Intestine 407 cells AQP3 was most abundantly expressed. In addition, there is strong evidence that other water channels, AQP4 and possibly AQP6 and 8, are also expressed (Chapter 7). Our functional data obtained with the AQP inhibitor Hg^{2+} and NEM suggested that AQPs can potentially play a role during the RVD response in Intestine 407 cells (e.g. see Fig. 1). A role for AQPs in osmotic cell swelling is supported by the observation that the rate of swelling of corneal endothelial cells is reduced in AQP5 knockout mice (304). Moreover, mice lacking AQP4 are partially protected from brain swelling in response to acute hyponatremia and ischemic stroke. Therefore, aquaporins are potential targets for treatment of brain edema (230). The involvement of AQPs in transepithelial water transport has been studied most extensively in the kidneys. Several aquaporins are kidney specific but a number of them are also expressed in many other tissues (39, 111, 165, 179, 209, 230, 304, 328, 325, 349).

The regulation of aquaporin channel function still needs to be fully clarified. In the kidney, AQP2 is recruited to the membrane of the medullar collecting duct by vasopressine/V_2 receptors that activate adenylate cyclase/protein kinase A (PKA) and initiate fusion of AQP containing vesicles with the plasma membrane (124, 134). Some of the serines in AQP2 need to be phosphorylated for this translocation to occur (320). Translocation is critically dependent on the microtubular network as well as on dynein (177, 273). Vanadate, calmodulin and calcium chelation can block this recruitment (for a review see (209)). In addition, PKA indirectly promotes the expression of AQP2 by phosphorylation of a cAMP-response element binding protein (CREB) and Fos (347). Interestingly, a hypertonicity responsive element has been described in the 5'-flanking region of the AQP1 and AQP2 gene (181, 316).

Whether the aquaporins expressed in Intestine 407 cells, in particular AQP 3, fulfill a similar role in water transport during cell volume regulation has not been fully explored yet. In addition to water, several aquaporins can also conduct small organic molecules like glycerol and urea or chloride (91, 108, 182, 268, 259, 326, 346, 346), making AQPs molecular candidates for VRAC and for the organic osmolyte release pathway. To investigate these options a useful approach will be to specifically knock-out each individual AQP, for instance by using the RNA-silencing technique (47, 126, 187, 299), and to evaluate their contribution to each of the processes, i.e. to the efflux of osmolytes, the efflux of chloride ions and the transport of water.

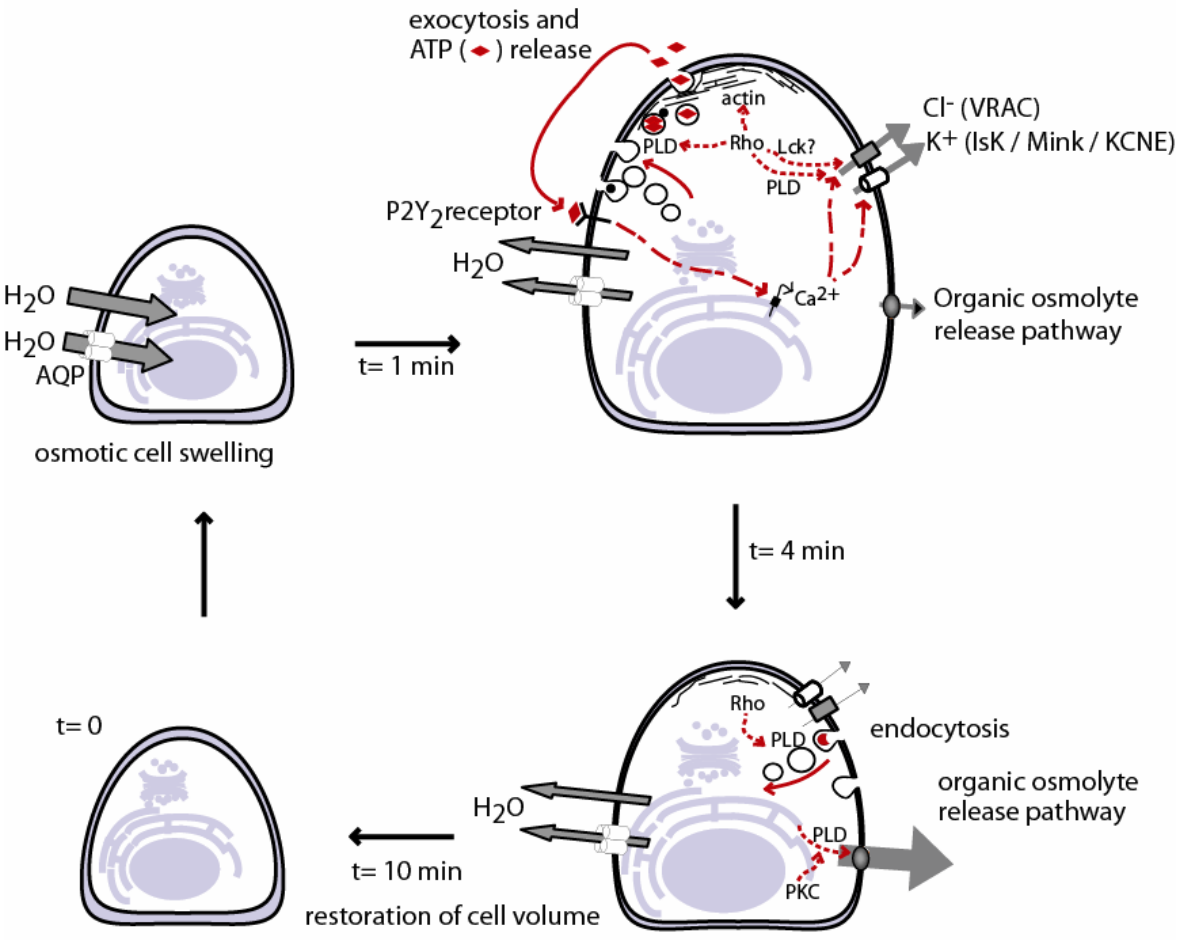

Figure 1: Regulation of compensatory responses activated by osmotic cell swelling.

Stimulation of Intestine 407 cells with a hypotonic solution immediately triggers an intracellularly directed flow of water, either by passive diffusion through the plasma membrane or by utilizing specialized water channels (AQPs; t=0). The relative contribution of these two entry pathways to the rate of cell volume increase, however, remains to be established. Subsequently, Cl^--and K^+-selective ion channels are activated, resulting in a net efflux of KCl and subsequently water (t=1 min). The cation channel (IsK/Mink/KCNE) is triggered by a rise in intracellular free Ca^{2+}, whereas the anion channel requires tyrosine kinase (possibly lck) and PLD activation, as well as active p21Rho and a remodelling of the actin cytoskeleton for activation. In addition, this time point of the RVD response is characterized by massive exocytosis and the extracellular release of ATP. Previously, we have demonstrated that ATP, through $P2Y_2$-type purinergic receptors, potentiate the anion efflux by (further) increasing intracellular Ca^{2+} (Van der Wijk et al., 1999). After a distinct lag period, the efflux of organic osmolytes (taurine, phosphocholine) is initiated, leading to a further decrease in the intracellular tonicity and loss of cellular water (t=4 min). Activation of the organic osmolyte release pathway requires active protein kinase C and could be

potentiated by a mechanism involving PLD. Associated with this phase of the RVD response is a robust PLD-dependent increase in endocytosis. After approx. 10 min, the efflux of osmolytes has returned to pre-stimulatory levels and the initial volume is restored. The major novel findings presented in this thesis are: (1) a role of organic osmolyte release in the RVD response of Intestine 407 cells and its VRAC independent mode of regulation; (2) the recognition that osmotic cell swelling stimulates vesicle cycling at the level of the plasma membrane (exo- and endocytosis) and provokes ATP release by means of exocytosis; (3) the involvement of PLD in vesicle cycling and its dual role regulation of VRAC and PKC-dependent potentiation of the release of organic osmolytes; and (4) the finding that several members of the AQP superfamily are expressed in Intestine 407 cells and may play a role during the RVD, either as water channels or as anion channels or both.

Summary

The volume of a cell is the result of the delicate balance between water efflux and influx across the plasma membrane. The cell can regulate its volume by redistributing osmolytes and thereby driving vectorial water transport. Although volume regulation is based on this simple principle, the exact regulation of these processes and molecular identity of the channels and transporters involved are still not fully understood. **Chapter 1** and **Chapter 2** summarize the state of the art. **Chapter 3** describes the putative role for organic osmolytes such as taurine and phospho-choline during the Regulatory Volume Decrease (RVD) in Intestine 407 cells. Our results demonstrate that in this epithelial cell line the release of organic osmolytes serves as a second line of defense. As compared to Volume-Regulated Anion Channels (VRACs), the release of organic osmolytes started after a distinct lag time and its threshold for activation was reached only by a more severe osmotic stress. Our data indicate that the efflux pathway is distinct from VRAC and is regulated independently. In **Chapter 4** we report a rapid increase in the rate of exo- and endocytosis triggered by osmotic cell swelling. The hypotonicity-provoked exocytosis was found to be calcium dependent and to involve the so-called SNARE proteins. Exocytosis was found to be the mechanism underlying to the release of ATP from these cells during RVD that was reported previously (321). Although exo- and endocytosis were found to require extracellular ATP and active Erk-1/2 MAP kinases, suggesting the involvement of a positive feedback loop in the release of ATP, both processes were found to be regulated independently. **Chapter 5** describes the activation of phospholipase D (PLD) by osmotic cell swelling and its role during the RVD. The results demonstrate the involvement of PLD in the regulation of compensatory anion channels (VRACs), but not in the activation of organic osmolyte release. In addition we found that PLD plays a crucial role in vesicle cycling, affecting both exo- and endocytosis as well as ATP release. In addition to PLD, the role of two other $p21^{rho}$ effectors, ROCK and mDia1, was investigated. The results of this study are presented in **Chapter 6**. The crucial role for $p21^{rho}$ in the activation of VRAC was confirmed. In addition, expression in Intestine 407 cells of constitutively active or inactive ROCK kinase or of constitutive active mDia1 was found to partially inhibit the cell swelling-induced activation of VRAC. From these results it was concluded that either active remodeling of the actin cytoskeleton is required to activate VRAC or that overexpression of these proteins indirectly affect VRAC activation by competing for other, yet unidentified, cellular factors. **Chapter 7** summarizes initial experiments of a new line of investigations aimed to identify VRAC at the molecular level. Using Hg^{2+}-treated cultures of Intestine 407 cells, we observed a strong inhibition of the cell swelling-induced anion efflux whereas the efflux of

organic osmolytes was not affected. These results suggest that initially the osmotic cell swelling was normal, in agreement with time laps video analysis of volume changes as measured under patch clamp conditions. Further experiments are needed to discriminate whether Hg^{2+}-sensitive AQPs play a role as ion channels, water channels or as both. Moreover, a possible role of Hg^{+}-sensitive AQPs (e.g. AQP 4) in cell swelling-induced release cannot be excluded yet and likewise awaits further studies.

Samenvatting

Het volume van een cel is het resultaat van het subtiele evenwicht tussen wateropname en afgifte over het plasmamembraan. De cel kan zijn volume regelen door een herverdeling van osmolieten met als gevolg een netto transport van water. Hoewel volume-regulatie op dit eenvoudige principe is gebaseerd, is de regulatie van het proces en de moleculaire identiteit van de betrokken kanalen en transporters nog niet in detail bekend. **Hoofdstuk 1** en **Hoofdstuk 2** geven een overzicht van de stand van zaken. **Hoofdstuk 3** beschrijft de rol van organisch osmolieten, zoals taurine en phospho-choline, tijdens de 'Regulated Volume Decrease' (RVD) in Intestine 407 cellen. Onze resultaten tonen aan dat in deze epitheliale cellijn de uitstroom van organische osmolieten dient als een tweede verdedigingsmechanisme. In vergelijking met de snelle activering van 'cell volume gereguleerde anion kanalen' (VRACs), begint de uitstroom van organische osmolieten pas na 1 à 2 minuten en wordt de drempel voor activering slechts bereikt na een grotere osmotische stimulatie. Onze gegevens wijzen erop dat de efflux van organische osmolieten verschilt van die van chloride ionen en ook onafhankelijk wordt gereguleerd. **Hoofdstuk 4** beschrijft de snelle activering van exo- en endocytose door osmotische cel zwelling. De door hypotoniciteit geïnduceerde exocytose bleek afhankelijk van calcium te zijn en gebruik te maken van de zogenaamde SNARE eiwitten. Uit het onderzoek kwam naar voren dat exocytose het verantwoordelijke mechanisme is dat leidt tot de extracellulaire afgifte van ATP. Hoewel voor de activatie van zowel exo- als endocytose extracellulair ATP en Erk-1/2 MAP kinase activering nodig is, hetgeen een positieve feedbackloop suggereert, is gebleken dat beide processen onafhankelijk worden gereguleerd. **Hoofdstuk 5** beschrijft de activering van phospholipase D (PLD) door osmotische cel zwelling en de rol van dit lipase gedurende de RVD responsie. De uitkomsten tonen de betrokkenheid van PLD aan in de activatie van compensatoire anion kanalen (VRACs), maar niet in de activering van de efflux van organische osmolieten. Daarnaast vonden wij dat PLD een essentiële rol in de vesicle cycling speelt, waarbij het zowel exo- als endocytose als ook de afgifte van ATP wordt gereguleerd. Behalve PLD is ook de rol van twee andere $p21^{rho}$ effectors, ROCK en mDia1, onderzocht. De resultaten van deze studie zijn weergegeven in **Hoofdstuk 6**. De essentiële rol voor $p21^{rho}$ in de activering van VRAC werd bevestigd. Bovendien bleek dat expressie in Intestine 407 cellen van een mutant constitutief actieve of inactieve ROCK kinase of van een constitutieve actieve mDia1 mutant leidde tot een gedeeltelijke remming van de, door celzwelling geactiveerde, Cl^- kanalen. Uit deze resultaten is geconcludeerd dat het remodelleren van actine cytoskelet een vereiste is om VRAC te kunnen activeren of, als alternatief, dat overexpressie van deze eiwitten VRAC activatie indirect

beïnvloeden door om andere, nog niet geïdentificeerde, cellulaire factoren te concurreren. **Hoofdstuk 7** vat de eerste experimenten samen van een nieuwe lijn van onderzoek naar de moleculaire identiteit van VRAC. Gebruikmakend van Hg^{2+}-behandelde Intestine 407 cellen, is een sterke remming van de door celzwelling veroorzaakte anion efflux waargenomen terwijl de uitstroom van organische osmolieten niet werd beïnvloed. Deze resultaten suggereren dat de cel in het begin normaal zwelt, in overeenstemming met videoanalyses waarin de volumeveranderingen van de cellen onder 'patch clamp' condities werden gemeten. Verdere experimenten zijn nodig om te onderscheiden of Hg^{2+}-gevoelige AQPs functioneren als ionenkanalen als waterkanalen of als beiden. Een potentiële rol van Hg-ongevoelige AQPs (bijv. AQP 4) in een zwelling-geïnduceerde organische osmoliet afgifte kon door onze experimenten (nog) niet uitgesloten worden en verdient nadere studie.

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Osmotic swelling provoked release of organic osmolytes in human intestinal epithelial cells: Relation to chloride channel activation and regulation by PKC.
Sebastian F.B. Tomassen, Durk Fekkes, Hugo R. de Jonge, and Ben C. Tilly. **(2004)**, *Am J Physiol Cell Physiol* 286: C1417-22.

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Cell volume regulation in intestinal epithelial cells.
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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 19 december 1970 te Oss. In 1989 behaalde hij het VWO diploma aan het Kruisheren Kollege te Uden. Van 1990 tot 1996 studeerde hij aan de Universiteit Wageningen bioprocestechnologie, oriëntatie moleculair-cellulair. Tijdens zijn studie werkte hij mee aan 3 onderzoeksprojecten. In 1994 was hij voltijds lid van de Wageningse acht van de roeivereniging Argo. De moleculaire specialisatie betrof de bestudering van het replicatiemechanisme van *Cowpea Mosaic Virus* bij de vakgroep moleculaire biologie (Prof. dr. A. van Kammen). De stage werd gelopen bij het IPO-DLO (onder begeleiding van Dr. ir. A. Schots) te Wageningen waarbij werd gewerkt aan de modificatie van de binding van bacteriële Cry-toxines. De bioprocestechnologische specialisatie betrof de optimalisatie van de groei tijdens vaste-stof-fermentatie van *Rhizopus oligosporus* bij de vakgroep levensmiddelentechnologie (Prof. dr. ir. J. Tramper). Het daarop volgende jaar heeft de auteur gewerkt in het kader van een non-profit project aan de klonering van het phenol-hydroxylase uit *Candida parapsilosis* (Dr. J. Oosterhaven) bij de vakgroep Biochemie in Wageningen. Vervolgens is een jaar gewerkt bij het ATO-DLO aan stofwisselingsstoornissen en het zoeken naar moleculaire markers, door middel van AFLP (Prof. dr. N.C.M. Laane). Extra cursussen bioinformatica (Prof. dr S.C. de Vries), ontwikkelingsbiologie (Prof. dr. T. Bisseling) en het artikel 9 certificaat (Prof. dr. L.F.M. van Zutphen) zijn behaald in deze periode. In juni 1999 werd bij de vakgroep biochemie als assistent in opleiding begonnen met het door NWO gefinancierde project 'Regulation of cell volume by p21-rho mediated signalling'. Daarbij werd uitgebreid onderzoek gedaan aan de organische osmolieten release en een mogelijke rol van aquaporines. Het daaropvolgende jaar is gewerkt aan de uitwerking van de in dit proefschrift beschreven studie.