Signal Transduction Involved in Cell Volume Regulation

Signaal-overdracht betrokken bij celvolume regulatie

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Oan Heit en Mem
Foar Klaas

Contents

Abbreviations

ABC	ATP-binding cassette
AFM	atomic force microscopy
CFTR	cystic fibrosis transmembrane-conductance regulator
EGF	epidermal growth factor
Erk	extracellular signal-regulated protein kinase
G protein	guanine nucleotide-binding regulatory protein
MAP kinase	mitogen-activated protein kinase
MBP	myelin basic protein
MEK	mitogen-activated/Erk-activating kinase
PKA	protein kinase A
PKC	protein kinase C
PtdIns-3K	phosphatidyl inositol 3 kinase
RVD	regulatory volume decrease
RVI	regulatory volume increase
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
VRAC	volume-regulated anion channel

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General introduction

CELL VOLUME REGULATION

Mammalian cells are surrounded by a selective permeable plasma membrane that allows the interior of the cell to differ in composition from the surrounding solution. The plasma membrane is formed by a bilayer of (phospho-) lipids and contains many different proteins. Hydrophobic molecules such as hydrocarbons, carbon dioxide and oxygen, rapidly dissolve into the membrane and can cross it by diffusion. Other small molecules that are polar but uncharged, for example water and ethanol, can also pass the membrane by simple diffusion. In contrast, the membrane is selectively permeable for specific ions and polar molecules that can only cross the membrane barrier with the aid of specific membrane-spanning transport proteins. Some of these proteins form hydrophilic channels through which specific molecules diffuse down their concentration gradient (passive transport). Other transport proteins use energy (direct or indirect) to pump molecules against their concentration gradient into or out of the cell (active transport).

Due to the high water-permeability of the mammalian plasma membrane, an imbalance between the osmolarity of the cytoplasm and the surrounding medium will immediately lead to a redistribution of water and to a subsequent change in cell volume. Because disturbances in cell size will finally result in a loss of function, most cell types react immediately by activating volume regulatory mechanisms. In general, these involve the activation of transport pathways in the plasma membrane, leading to either a net accumulation (regulatory volume increase or RVI) or a loss of osmotically active intracellular solutes (regulatory volume decrease or RVD). Both

processes are schematically illustrated in Figure 1. The principal inorganic ions involved in volume regulation are Na^+, K^+ and Cl^-. Cell shrinkage leads to the net uptake of NaCl, through activation of Na^+/H^+ and Cl^-/HCO_3^- antiporters or by activation of Na^+-K^+-$2Cl^-$ or Na^+-Cl^- symporters (for reviews see: [1-3]). Osmotic cell swelling, however, leads to the extrusion of K^+ and Cl^- by a parallel activation of cation and anion selective ion channels in the majority of cell types studied, or by activation of K^+/Cl^- co-transport [3-6]. In addition, the redistribution of ions is often accompanied by the release or accumulation of organic osmolytes, such as polyols, methylamines and certain amino acids, which do not interfere with cellular function (for reviews see: [2,7]).

Whereas the mode of activation of K^+-channels involved in the RVD response is well-known, involving a local rise in intracellular free Ca^{2+} [8-11], the molecular identity and the mechanism of activation of the volume-regulated anion channels (VRACs) have not yet been fully elucidated.

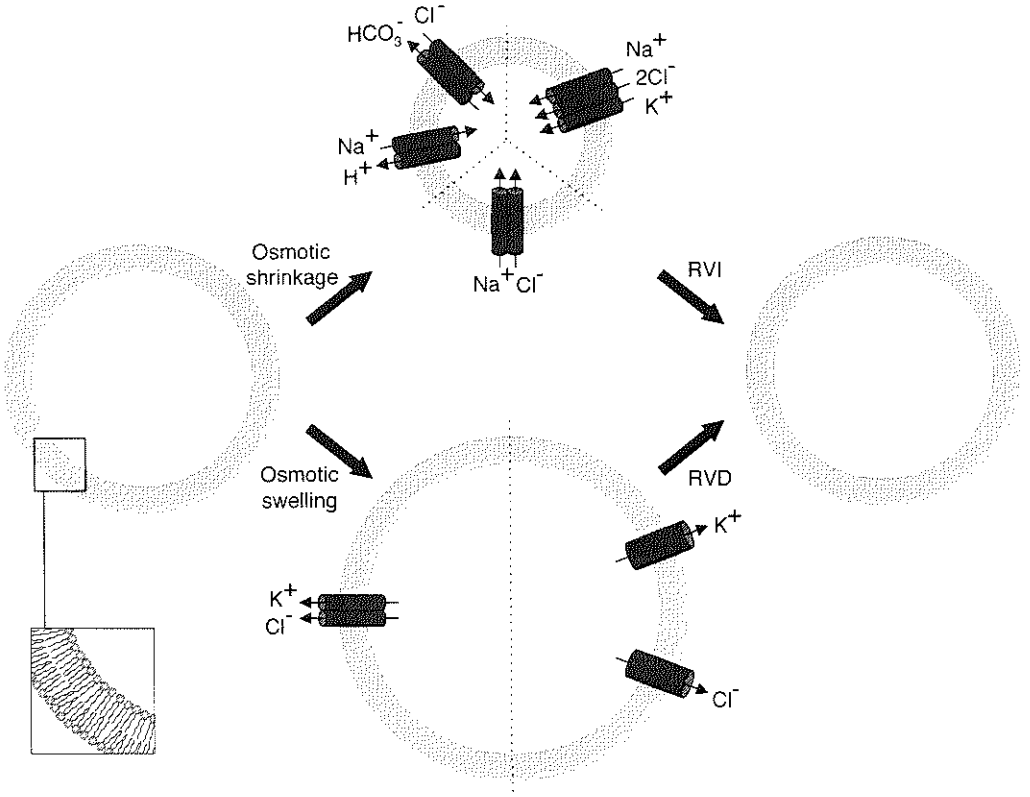

Figure 1. Schematic illustration of cell volume regulation

An increase in extracellular osmolarity or a decrease in osmolarity of the cytosol will lead to an efflux of water from the cytosol to the extracellular medium resulting in cell shrinkage. *Vice versa*, a decrease in extracellular osmolarity, or an increase in the osmolarity of the cytosol will lead to cell swelling. In reaction to cell shrinkage, ion transporters are activated resulting in the net uptake of NaCl, while cell swelling induces activation of ion channels leading to K^+ and Cl^- efflux. The inset shows the orientation of phospholipids in the lipid bilayer.

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL DISTURBANCES IN INTRA- OR EXTRACELLULAR OSMOLARITY

Physiological disturbances in osmolarity

In mammals, volume stress under physiological conditions is generally due to a change in the intracellular osmotic pressure caused by hormone-regulated metabolic processes, by bulk uptake and transport of nutrients, or by secretory processes. The osmolarity of the interstitial fluid is carefully maintained within a narrow range by interlocking endocrine systems: a change in plasma osmolarity or volume is sensed by osmoreceptors in the brain and in atrial and juxtaglomerular endocrine cells, which respond by secreting hormones that affect thirst sensation and the renal uptake or excretion of water and salts. By these mechanisms, many cell types in the body are protected against large disturbances in extracellular osmolarity. A notable exception are the intestinal and renal epithelia, which experience frequent osmotic perturbations of extracellular origin due to an isosmotic luminal fluid, or to rapid changes from hyperosmolarity to isosmolarity during transition from antidiuresis to diuresis (kidney medulla) [6].

As proposed by Häussinger and Schliess [12], a hormone-induced change in cellular volume may serve as an independent signal that regulates cellular metabolism and gene expression. For example, insulin and glucagon affect the cellular hydration by modulating ion transport systems. Insulin, by activating Na^+/H^+ exchange, Na^+/K^+-$2Cl^-$ cotransport and the Na^+/K^+-ATPase, stimulates the accumulation of K^+, Na^+ and Cl^- and subsequently induces cell swelling. Glucagon, in contrast, induces the depletion of cellular K^+, Na^+ and probably Cl^- by simultaneously activating K^+-channels and Na^+/K^+-ATPase leading to cell shrinkage. In hepatocytes, an increase in cell volume promotes a more anabolic metabolism, whereas a decrease in cell volume triggers a catabolic pattern of cellular function [2,12].

Pathophysiological disturbances in osmolarity

Several pathological conditions lead to a disturbance of the body fluid osmolarity (for review see: [13]). An increase in extracellular osmolarity of pathological origin is seen during hypernatremia, hyperglycemia and uremia. Hypernatremia is primarily caused by disturbances of the sensorium that limit free access to water, such as stroke, cranial trauma and diabetes insipidus. Other causes are excessive diarrhea, exposure to extremely hot climates or (chronic) renal failure combined with the inability to replenish water. Hyperglycemia occurs most often in the setting of diabetes mellitus. Uremia is caused by renal failure leading to an increased plasma concentration of urea. A decrease in extracellular osmolarity is seen in hyponatremia. A variety of pathological conditions lead to the impairment of free water clearance and thereby to hyponatremia. The most common are congestive heart failure, hepatic cirrhosis, chronic renal failure and inappropriate secretion of antidiuretic hormone (SIADH).

Besides alterations in extracellular environment, cell swelling occurs also during periods of an impairment of cellular metabolic activity. Due to the processes that maintain the Gibbs-

Donnan disequilibrium, a constant supply of energy is necessary to maintain cell volume. It is well known for many years that ischaemia leads to acute cell swelling in the heart (one of the most extensively studied organs), brain, kidney, gallbladder, liver and skeletal muscle [14]. Ischaemia causes a shift from aerobic metabolism to anaerobic glycolysis, resulting in the accumulation of metabolites such as inorganic phosphates and lactate, which increase tissue osmolarity. In zero-flow ischaemia, there is no metabolite washout and osmolytes accumulate in the extracellular space thereby reducing the transmembrane osmotic gradient. However, during low-flow ischaemia or reperfusion, osmolytes expelled from cells are gradually washed away, reducing extracellular osmolarity, which finally results in cell swelling (reviewed by [15]).

CHARACTERISTICS OF THE VOLUME-REGULATED ANION CONDUCTANCE

In spite of the fact that the molecular identity of the VRAC has not yet been established, the characteristics of the VRAC have been studied extensively in many different cell types using the whole cell patch clamp technique (reviewed in: [4,16,17]). The results of these studies revealed that the cell swelling-induced current displays (1) a marked outward rectification; (2) a strong voltage-dependent inactivation at depolarizing membrane potentials; (3) a permeability sequence, as determined from shifts in reversal potential, that corresponds to the Eisenman's sequence I ($SCN^->I^->Br^->Cl^->F^->$gluconate$^-$); and (4) an inhibition by common Cl^--channel blockers like 4-acetamido-4'-isothiocyanostilbene (SITS), 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and diphenylamine-2-carboxylate (DPC). In addition, extracellularly applied nucleotides, like ATP and UTP, in millimolar concentrations, as well as the purinoceptor antagonists suramin and reactive blue inhibit the cell swelling-induced anion conductance (Figure 2). This inhibition is most prominent at depolarizing membrane potentials and apparently does not involve purinoceptor activation [18].

The volume-sensitive anion conductance can also be studied in intact monolayers of intact cells using macroscopic $^{36}Cl^-$ or $^{125}I^-$ efflux assays. In such an assay, the release of isotope into the extracellular medium is quantified as a function of time. In Intestine 407 cells, the cell line used in our studies, a significant increase in anion efflux was already observed after reducing the osmolarity of the extracellular fluid by 5 - 10%, whereas saturation of the response is reached at an approx. 30% reduction in tonicity. Unlike the increase in anion conductance observed during whole-cell patch clamp experiments, which lasts as long as the bathing solution remains hypotonic, the anion efflux from intact cells is transient, even when the reduction in the tonicity of the medium is permanent, and closely resembles the actual change in cell volume as occurs during the RVD response [19].

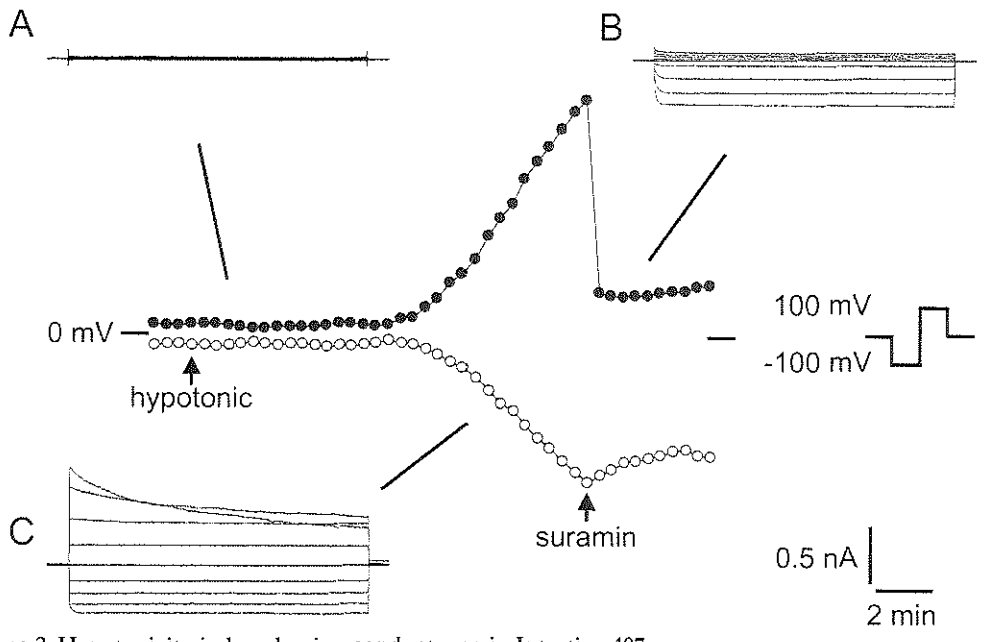

Figure 2. Hypotonicity-induced anion conductance in Intestine 407

Time course of activation of the swelling-induced whole-cell Cl^- current at -100 and +100 mV holding potentials, and its inhibition by extracellular added suramin (100 μM, final concentration). Insets (A-C) show Cl^- currents in response to step pulses (2 s duration) from -100 to +100 mV (in 25 mV increments) at a higher time resolution. The hypotonic-shock induced Cl^- current displays a marked outward rectification and a strong voltage-dependent inactivation at depolarizing membrane potentials (Inset B). The inhibition of the Cl^- current by suramin is most prominent at depolarizing membrane potentials.

CANDIDATE PROTEINS FOR VOLUME-REGULATED ANION CHANNELS

Several membrane proteins have been proposed to serve as a VRAC itself, or as an important regulator of VRAC. The most plausible candidate proteins are discussed below.

ATP-binding cassette proteins

CFTR (cystic fibrosis transmembrane-conductance regulator) and P-glycoprotein are members of the superfamily of ATP-binding cassette (ABC) proteins, and were found to display complementary patterns of expression in epithelial tissue [20]. Therefore, it was suggested that CFTR and P-glycoprotein may have overlapping physiological functions. Both proteins were found to be associated with cell volume regulation in distinct cell models. An antibody raised against a peptide corresponding with the amino acid positions 505-511 of CFTR partially attenuated the swelling-activated Cl^- current in the human colonic cell line T84 [21]. Furthermore, Valverde et al. [22] found that cell volume regulation after hypotonic challenge is defective in intestinal crypt epithelial cells isolated from *CFTR* knockout mice. However, in this cell type the

impairment of RVD in *CFTR* -/- crypts appeared to be related to the inhibition of volume-sensitive K^+-channels rather than Cl^--channels, suggesting that CFTR plays a crucial role in the opening of volume-sensitive K^+-channels [22]. Also in other cell types CFTR-mediated Cl^- currents were found to function completely independent of the volume-regulated anion currents [23-25].

P-glycoprotein (MDR-1) was suggested to be the volume-sensitive channel protein itself by Valverde et al. [26] who found that expression of the protein increased the volume-sensitive Cl^- currents significantly, whereas tamoxifen, a P-glycoprotein inhibitor, was found to inhibit both Cl^--channel activity and the RVD response. Kunzelmann et al. [27], however, reported that in HT-29 colon adenocarcinoma cells, expression of P-glycoprotein did not affect the volume-sensitive K^+ and Cl^- conductances. In addition, in NIH-3T3 cells lacking P-glycoprotein expression, osmotic cell swelling leads to the development of a normal increase in Cl^- conductance and an RVD response [28]. Nevertheless, in some selected cell types P-glycoprotein does seem to influence cell-volume recovery after swelling and may serve as a regulator of endogenous volume-sensitive Cl^--channels dependent on its (protein kinase C-modulated) phosphorylation state [26,29,30]). In other cell types, e.g. human squamous lung carcinoma epithelial cells, P-glycoprotein has been reported to increase the sensitivity of VRAC to P-glycoprotein inhibitors [31].

The complete lack of CFTR expression in Intestine 407 cells, despite the development of large Cl^- currents in response to hypotonic stimulation (this thesis) suggests that CFTR is not of crucial importance in the activation of volume-sensitive Cl^-- or K^+-channels. A similar conclusion was reached by Nilius et al. [32] for calf pulmonar artery endothelial (CPAE) cells, who likewise failed to detect forskolin- or cAMP-activited Cl- currents, the hallmark of CFTR, while large Cl^- currents were activated in these cells after hypotonic stimulation.

Both P-glycoprotein and CFTR have been reported to be involved in the regulation of cell swelling- or deformation-induced ATP release in hepatocytes and red blood cells, respectively [33,34]. As discussed in Chapter 2, ATP might subsequently act as an auto- or paracrine factor in the potentiation or even activation of VRACs. These two examples suggest that VRAC regulation by the ABC proteins is probably rather complex, and could involve very indirect mechanisms such as purinergic signalling.

ClC-2/3

In mammals, the ClC family of Cl^--channels consists of nine known members (reviewed by [35]). Two of these members have been suggested to be associated with volume-sensitive Cl^- currents: ClC-2 and ClC-3.

ClC-2 was found to contain intramolecular domains that are sensitive to both hyperpolarization and extracellular hypotonicity [36]. In T84 cells, however, hypo-osmotic cell swelling was able to increase the ClC-2 channel activity only after prior activation by hyperpolarizing voltages. Moreover, inhibition of the channel did not affect the RVD [37]. Therefore, it appears that ClC-2 channels may contribute to, but are not required for development of the volume-sensiti-

ve Cl^- current. This was confirmed by the findings of Xiong et al. [38], who report that expression of ClC-2 in Sf9 cells enhances the rate of RVD; however, the associated Cl^- current was distinct from the endogenous swelling-activated Cl^- currents on the basis of pharmacology and inhibition by an anti-ClC-2 antibody. Recently, Tewari et al. [39] demonstrated that the human recombinant ClC-2 channel expressed in HEK 293 cells are activated by cAMP dependent protein kinase A (PKA) and arachidonic acid. This again supports the notion that ClC-2 is not identical to a VRAC, because osmo-sensitive Cl^- currents are regulated independently of PKA, and are inhibited rather than activated by arachidonic acid in many cell types (Chapter 2).

Expression of ClC-3 in NIH/3T3 cells leads to a large Cl^- conductance under isosmotic conditions, which exhibits properties identical to the hypotonicity-induced anion current in native cells [40]. The current could be increased by inhibition of protein kinase C, whereas an increase in intracellular free Ca^{2+} reduced the current [41]. The latter suggests that in most cell types, ClC-3 is not responsible for the volume-sensitive Cl^- current, since an increase in intracellular Ca^{2+} was found to potentiate or even activate hypotonic shock-induced anion channels, as discussed in Chapter 2. In addition, Du and Sorota [42] reported that in canine atrial cells, PKC stimulates and does not prevent the volume-sensitive Cl^- current.

I_{Cln}

I_{Cln} is a protein originally cloned from Madin-Darby canine kidney (MDCK) cells and ubiquitously expressed in most organs and tissues (reviewed by [43]). Expression of the mRNA encoding mammalian I_{Cln} in *Xenopus laevis* oocytes induces a Cl^- current reminiscent of the volume-sensitive anion currents as found in almost all cell types studied so far [44]. Mutation of one amino acid residue (G49) in the putative nucleotide-binding domain of the protein altered both the nucleotide sensitivity and kinetics of the expressed current [44], suggesting that I_{Cln} is the channel protein itself rather than a regulating protein. Furthermore, the hypotonicity-induced Cl^- currents in NIH/3T3 cells are suppressed by antisense oligonucleotides complementary to the first 30 nucleotides of the coding region of the I_{Cln} protein [45]. Musch et al. [46], however, found that a hypotonic shock dose-dependently stimulates translocation of I_{Cln} from the cytosol to the Triton X-114-insoluble fraction. They found that I_{Cln} does not insert into but associates with the membrane, suggesting that I_{Cln} acts as a channel regulator rather than as a channel itself [46].

There are, however, observations that argue against a crucial role for I_{Cln} in the activation of VRACs. Buyse et al. [47], found that injection of CLC-6 RNA in *Xenopus* oocytes induced a Cl^- current identical to I_{Cln} and therefore suggest that the I_{Cln} current corresponds to an endogenous conductance of *Xenopus* oocytes that can be activated by expression of structurally unrelated proteins. Furthermore, some differences were found between the Cl^- current induced by I_{Cln} expression in *Xenopus* oocytes, and the endogenous hypotonicity-induced Cl^- currents of

Xenopus oocytes; for example, unlike the hypotonicity-induced Cl^- currents, the currents induced by I_{Cln} were inhibited by cyclamate, and extracellular cAMP only inhibited the outward current [48].

AIM AND SCOPE OF THIS THESIS

The aim of this thesis was to unravel the signal transduction pathways involved in regulatory cell volume decrease, with special emphasis on the regulation of VRACs. As a model, we used the human fetal jejunum-derived epithelial cell line Intestine 407, which does not express Ca^{2+}- and voltage-sensitive Cl^--channels nor cAMP/protein kinase A sensitive CFTR Cl^--channels [8, 49-51]. This low background of Cl^--channel activity and the manifestation of large volume-sensitive Cl^- currents upon hypotonic stimulation render these cells particularly suitable for studying VRACs. Chapter 2 gives an overview of the literature in relation to our own results on signalling mechanisms involved in cell volume regulation.

Erk-1/2 MAP kinase activation is a general phenomenon in hypotonic stimulation of mammalian cells. Furthermore, in homology to the yeast cell system, an important role of Erk-1/2 MAP kinase was suggested in the RVD. The role of Erk-1/2 MAP kinase in volume-sensitive ion channel activation and the signal transduction pathway leading to hypotonicity-induced Erk-1/2 activation was studied, as described in Chapter 3.

Like many kinds of mechanical stress, hypotonic stimulation leads to the release of ATP for many different mammalian cell types. Extracellular ATP is known to act as an autocrine factor by signalling through purinergic receptors on the cell membrane. Since purinergic stimulation is known to affect Cl^--channel activation in several cell systems, the role of extracellular ATP in swelling-induced anion channel activation was studied in Chapter 4. Furthermore, this chapter describes the role of extracellular released ATP in the hypo-osmotic stress-induced activation of Erk-1/2 in Intestine 407 cells.

In the experiments described in Chapter 5, we investigated the mechanism leading to the release of ATP. Because hypo-osmotic swelling coincides with an increase in the rate of vesicle recycling, a putative role for exocytosis in the release of ATP was evaluated.

The existence and regulation of hypotonicity-induced endocytosis was studied and described in Chapter 6.

Atomic force microscopy is a very sensitive tool, which is suitable for studying surfaces at nanometer resolution. Recently, attempts were made by several groups using this technique for scanning and visualizing the surface of living cells. In collaboration with the group of Prof. J. Greve and Prof. B.G. de Grooth (Department of Applied Physics, University of Twente), we started to evaluate the applicability of this technique in studying cell membrane surface events, like exo- and endocytosis or membrane unfolding, in response to hypotonicity-induced cell swelling. The first results are described in Chapter 7.

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Signalling mechanisms involved in cell volume regulation

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INTRODUCTION

Most mammalian cells have developed compensatory mechanisms to respond to the variable osmotic stress caused by changes in the concentrations of intracellular osmo-active substances (e.g. glucose, amino acids, lactate) or by variations in the osmolarity of the surrounding medium. Two distinct mechanisms can be recognized; the Regulatory Volume Increase (RVI), activated by cell shrinkage and leading to a net increase in the intracellular osmolarity, and the Regulatory Volume Decrease (RVD), which is triggered by cell swelling and directs a reduction in the tonicity of the cell. Whereas the RVI response depends on the activation of ion pumps and carriers, the RVD response principally involves the concerted opening of cation and anion selective ion channels. To date, the K^+ and Cl^- conductances activated upon hypo-osmotic stimulation have been characterized in many different cell systems. The molecular identity of the channels however, as well as the mechanism(s) involved in their activation have not yet been fully clarified and may differ between cell types. In this review, we will evaluate the different signalling pathways activated by osmotic cell swelling and discuss their putative role(s) in ion channel regulation, in maintaining cellular volume homeostasis and in paracrine signal transduction, with emphasis on intestinal epithelial cells.

INTRACELLULAR SIGNALLING MOLECULES INVOLVED IN THE RVD RESPONSE

Although present knowledge about signal transduction pathways utilised to activate VRACs is far from complete, several components of the cascade(s) have been recognised and their crucial importance in the activation or regulation of the anion conductance has been established. To study cell swelling-activated Cl^--channels in the intestine, we used Intestine 407 cells, a human fetal jejunum-derived epithelial cell line. These cells are particularly suitable for studying VRACs, because of their low background of Cl^--channel activity. Apart from a large swelling-induced anion current, these cells do not express other anion channels such as Ca^{2+}- and voltage-sensitive Cl^--channels or cAMP/protein kinase A sensitive CFTR Cl^--channels [1; 2-4]. Furthermore, in the sub-clone we used, P-glycoprotein expression was not detected using an RNAse protection assay.

The different signalling molecules that are activated upon or increased by hypo-osmotic cell swelling are listed below and their putative role in the regulation of VRACs is discussed.

Ca^{2+}

In many different cell systems, hypo-osmotic cell swelling leads to an increase in the intracellular free Ca^{2+} concentration (reviewed in: [5]). In the intestine, both in jejunal and colonic epithelial cells, the RVD response was found to be triggered by an elevation of $[Ca^{2+}]_i$ [6; 7-8]. Because inhibition of the osmosensitive K^+ and/or Cl^- conductance will result in an impaired RVD response [9], it was impossible to distinguish between the effects of Ca^{2+} on K^+- or Cl^--selective channels, in these studies. In a number of cell models, an increase in $[Ca^{2+}]_i$ was found to be critically involved in the activation of volume-sensitive Cl^- currents [10-15]. In other cell types, however, including Intestine 407 cells, a rise in $[Ca^{2+}]_i$ was not essential for the activation of swelling-induced Cl^--channels [1, 16, 17-28].

Although not always involved in a direct activation of VRACs, Ca^{2+} 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 [29, 3]. This potentiation is not due to an in increase in driving force (e.g. stimulation of Ca^{2+}-activated K^+-channels) because of the insensitivity of the anion efflux to treatment with the K^+ ionophore valinomycin [29].

Tyrosine kinases

In Intestine 407 cells, hypo-osmotic stimulation was found to trigger a rapid and transient increase in tyrosine phosphorylation of several proteins, including the focal adhesion kinase p125FAK and members of the MAP (mitogen-activated protein) kinase family [16, 30]. Treatment of the cells with tyrosine kinase inhibitors like herbimycin or genistein largely redu-

ced 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 [16]. These data clearly demonstrate that protein tyrosine phosphorylation is a prerequisite for the proper development of 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 [28, 31-33]. Sorota [31] demonstrated that the inhibition of VRACs by genistein is abolished after intracellular application of ATPγS, but that ATPγS by itself is not able to activate the channel. These data are in line with the notion that activation of a tyrosine kinase is an essential early step in the activation of volume-sensitive anion channels and argue against a direct interaction of genistein with VRACs as has been reported for CFTR Cl^--channels [34, 35]. Activation of VRACs 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 [36, 37]. In the latter cell system however, Voets et al. [33] did find evidence for tyrosine kinase involvement.

Sofar, the identity of the tyrosine kinase(s) required for the activation of VRACs has been established only for a few cell types. For Jurkat T lymphocytes, strong evidence is available that the Src-like $p56^{lck}$ tyrosine kinase is both essential and sufficient for VRAC activation [32, 38]. Using $p56^{lck}$ deficient J-Cam 1.6 cells it was demonstrated that the impaired volume regulation of these cells could be restored by transfection of $p56^{lck}$ [32]. Moreover, in these cells, a direct activation of anion channels in excised membrane patches was observed following addition of purified $p56^{lck}$ and ATP [38]. In endothelial cells, however, Src-like kinases are apparently not involved in VRAC activation, since specific Src-activating peptides did not affect volume-sensitive anion currents [33].

MAP kinases

The MAP kinases constitute a family of serine-threonine protein kinases, ubiquitously expressed in eukaryotes, that are activated by such diverse stimuli as growth factors, (neuro-)hormones, cytokines as well as by several forms of cellular stress. Signalling through MAP kinases involves the stimulation of a "three-component module" (Figure 1) consisting of a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (MKK) and, finally, the MAP kinases themselves (reviewed in: [39]). Three different subfamilies of MAP kinases have been recognized in mammalian cells: p42/p44 Erk-1/2, p38 MAP kinase and JNK, which are likely to play important roles in cell proliferation and differentiation, in (prevention of) apoptosis and in the cellular responses to stress.

In the yeast *Saccharomyces cerevisiae*, MAP kinase activation was shown to be a prerequisite for a proper compensatory response to the osmotic stress induced by changes in the extracellular tonicity [40, 41]. Cell shrinkage was found to activate the MAP kinase Hog1p, which leads to an increase in glycerol synthesis and a restoration of the osmotic gradient [40]. Hog1p

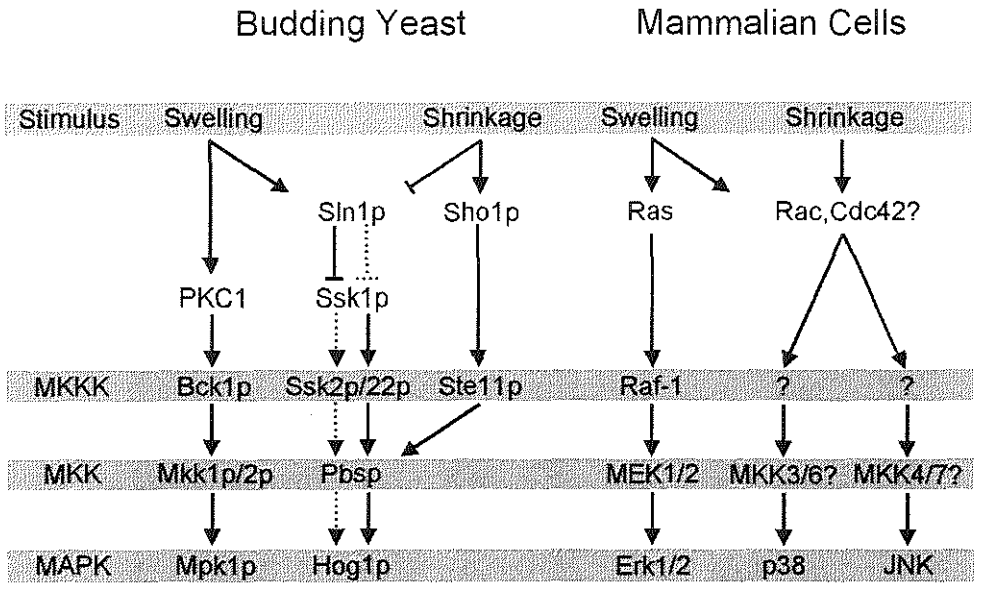

Figure 1. MAP kinase activation in yeast and mammalian cells

In budding yeast, cell swelling leads to (1) an activation of Mpk1p through PKC1 and (2) to an inactivation of the MAP kinase Hog1p, by stimulating the histidine kinase Sln1p and a subsequent inhibition of its response regulator Ssk1p. Cell shrinkage leads to activation of Hog1p through a cascade involving Sho-1, Ste11p and Pbsp, and to a release of the Ssk1p inactivation by Sln1p inhibition. In several mammalian cells, including Intestine 407, extracellular hypotonicity leads to activation of the MAP kinase Erk-1/2 (through the Ras/Raf pathway), and the stress-related MAP kinases JNK and p38. In addition to hypotonic stress, (severe) cell shrinkage is also able to activate JNK and p38 [105].

displays a high similarity with the mammalian p38/JNK MAP kinases and is activated by Pbs2p (MKK). As revealed by Maeda et al. [42] Pbs2p activity is controlled by two distinct transmembrane osmoceptors (Figure 1); an osmosensor called Sho1p and a "two-component system" consisting of the histidine kinase Sln1p and the response regulator Ssk1p. Sln1p is activated during periods of low extracellular osmolarity, leading to inactivation by phosphorylation of Ssk1p and the inhibition of Ssk2/22p (both MKKK's). Finally this resulted in a reduced activity of Pbs2p and Hog1p. During periods of high extracellular osmolarity Sho1p, an SH3 domain containing receptor, is activated, and stimulates Pbs2p directly. At the same time, Sln1p is inactivated and, as a consequence, the inhibition of Ssk2/22p is released. Unlike Hog1p, another MAP kinase, Mpk1p, is activated by osmotic cell swelling through a mechanism involving PKC1 [41] (Figure 1). Mpk1p is highly homologous to the mammalian Erk-1/2 MAP kinase and is required for growth at low osmolarity, most likely by regulating cell wall properties [41].

In plants, a transmembrane histidine kinase, structural similar to the yeast osmosensor Sln1p, was recently found: ATHK1 (*Arabidopsis thaliana* histidine kinase) protein [43]. Transcription of the ATHK1 gene was found to be upregulated in response to changes in exter-

nal osmolarity. Expression of ATHK1 gene into yeast lacking functional Sln1p and Sho1p (*sln1Δ sho1Δ* double mutant) leads to Hog1p activation and prevented high osmolarity induced cell death [43]. Taken together, these results suggest that, in plants, analogous to the yeast system, ATHK1 functions as an osmosensor signalling through a MAP kinase cascade.

Hypotonicity-induced activation of Erk-1/2 MAP kinases has been reported for all mammalian cell types studied so far, including Intestine 407 cells [16, 44], H4IIE hepatoma cells [45], primary rat hepatocytes [46], cardiac myocytes [47], astrocytes [28, 48], C6 glioma cells [49], isolated rat pancreatic acini [50] and mIMCD3 renal cells [51]. In addition, in many cell models, hypo-osmotic cell swelling also leads to the activation of p38 and JNK MAP kinases [30, 47, 51-53]. Unlike the activation of Mpk1p in yeast, the activation of Erk-1/2 by osmotic cell swelling is independent of protein kinase C [44-46, 48-50], but in several cell types including Intestine 407 cells, involves $p21^{ras}$ and Raf1 [44, 48, 49]. In primary astrocytes [48], but not in a number of other cell models [44, 49, 50], the hypotonicity-induced Erk-1/2 activation was sensitive to the PtdIns 3-kinase inhibitor wortmannin. In addition, pretreatment with pertussis toxin prevented the swelling-induced Erk activation in H4IIE rat hepatoma cells [45] and primary rat hepatocytes [46] suggesting the involvement of $G\alpha_i$ and $G\alpha_o$, but not in C6 glioma cells [48] and Intestine 407 cells [44].

Because Erk-1/2 and p38 MAP kinase activation is among the initial signalling events after hypo-osmotic cell swelling, and because their yeast homologues are critically involved in the activation of volume compensatory mechanisms, it is tempting to propose a model in which MAP kinases play an important role in the regulation of the RVD response in mammalian cells. However, in Intestine 407 cells, preventing MAP kinases activation by using specific inhibitors (Erk1/2: PD098059; p38: SB203580) or by expression of dominant negative G proteins (Erk-1/2: N17-$p21^{ras}$; p38: N17-$p21^{rac}$) did not affect the activation of the VRACs [30, 44, 54], arguing against a direct role of these kinases in cell volume control. This notion is supported by the observations that PD098059 treatment of mIMCD3 renal cells did not affect steady state cell volume after 30 min of osmotic stress [51]. In astrocytes, however, Crepel et al. [28] found that PD098059 reversibly inhibited activation of the Cl^- current induced by hypo-osmotic stimulation. The cell swelling-induced activation of JNK develops rather slowly and only after a distinct lag phase of 10-20 min, arguing against a direct role of JNK in the RVD response [47, 52, 53; Tilly et al., unpublished results].

Because the function of MAP kinases in the regulation of gene expression is well established, it is attractive to propose a role for Erk-1/2 and p38 in restoring cellular homeostasis and in promoting long-term survival. In mIMCD3 renal cells, the hypotonicity-induced Erk activation was found to be at least partially responsible for the increased expression of Elk-1 and Egr-1 [51]. Inhibition of Erk activation, however, did not affect cell viability in these cells or in H4IIE hepatoma cells [51, 52]. In addition to transcription regulation, hypotonicity-provoked Erk-1/2 and p38 activation were found to regulate hepatic taurocholate secretion and to inhibit proteolysis in hepatocytes, respectively [46, 53].

G proteins

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, which could be inhibited by GDPβS [33, 55, 56]. In addition, the RVD response was facilitated by GTPγS or NaF-AlF_4^- in cervical cancer and osteosarcoma cells [57, 58] and, in platelets, impaired by GDPβS [59]. Treatment with pertussis toxin abrogated the RVD response in platelets and cervical cancer cells, suggesting the involvement of $G\alpha_i$ [58, 59]. In calf pulmonary artery endothelial cells (CPAE), the hypotonicity-induced Cl^- current was mimicked by cytosolic GTPγS and inhibited by GDPβS [33], indicating a role for G proteins in the activation of the swelling-induced anion conductance. In other cell types, however, the intracellular introduction of non-hydrolyzable guanine nucleotides did not affect the volume-sensitive anion current or the RVD response [58, 60].

Using *Clostridium botulinum* exoenzyme C3, we previously demonstrated that the ras-related small G protein $p21^{rho}$ is involved in the activation of the osmosensitive anion efflux in Intestine 407 cells [30]. Involvement of $p21^{rho}$ is not restricted to epithelial cells; comparable results were recently obtained with bovine endothelial cells [61]. Regulation of ion channels by G proteins could be either directly, through interaction of the G protein (subunits) with the channel protein itself, or indirectly, through the activation of signalling cascades. Several downstream targets of $p21^{rho}$ have now been identified including the Rho kinase $p160^{Rock}$, a regulator of myosin light chain phosphatase (for review see: [62]). For bovine endothelial cells, but not for Intestine 407 cells and isolated human umbilical vein endothelial cells (Van der Wijk and Tilly, unpublished results), it was found that treatment of the cells with a specific $p160^{Rock}$ inhibitor (Y-27632) prevented activation of the volume-sensitive Cl^- conductance [61], suggesting the involvement of myosin phosphorylation. In Intestine 407 cells, however, the hypotonicity-provoked anion efflux was not affected by inhibitors of myosin light chain kinase [30]. In contrast, in these cells, a swelling-induced and $p21^{rho}$ dependent activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase) was found to be essential for activation of the anion efflux [30]. PtdIns 3-kinase dependency was also found for volume-induced changes in amino acid transport in skeletal muscle cells [63].

Cytoskeleton

In many different cell types, hypo-osmotic cell swelling was accompanied by a transient reorganization of the actin cytoskeleton and/or the total amount of F-actin. In several cell types, a transient depolymerization of F-actin was found (PC12 cells [64]; IMCD cells [65]; Ehrlich ascites tumor cells [66]; dogfish shark rectal gland cells [67]). In C6 rat glial cells and isolated rat hepatocytes, however, osmotic cell swelling was accompanied by an increase in total F-actin [68, 69]. No clear changes in the relative amount of F-actin were found for shark rectal gland cells [70].

Intestine 407 cells respond to hypo-osmotic stimulation with a very transient (2-5 min) increase in total F-actin that coincides with the activation of a compensatory anion efflux [30]. In addition, a clear change in the subcellular distribution of F-actin was observed, as revealed by constructing confocal laser microscope images of cells stained with rhodamine-conjugated phalloidin. An almost immediate (< 60 s) increase in F-actin stress fibers was found at the basal part of the cell, whereas the actin filaments of the apical part were largely disrupted [30].

In most cell types studied, a disruption of the F-actin microfilaments by cytochalasin B or D treatment leads to an impaired activation of VRACs and/or the RVD response (Necturus gall bladder cells [71]; rabbit proximal tubule cells [72]; PC12 cells [66, 73]; HSG cells [19]; Jurkat lymphoma cells [74]; embryonic chick cardiac myocytes [16]; prostate cancer spheroids [75]). In other cell systems, phalloidin-mediated stabilization of F-actin prevented activation of VRACs [76] and, *vice versa*, cytochalasin treatment potentiated [29, 30, 76] or even mimicked [77] the activation of VRACs by cell swelling.

Both the actin rearrangement (for review see:[78]) and VRAC are regulated by $p21^{rho}$ [30, 61] suggesting a close correlation between cytoskeletal remodeling and channel activation. To date, little is known about how cytoskeletal remodelling is coupled to the activation of osmosensitive ion channels. Several reports suggest that the cytoskeletal reorganization is involved in the hypotonicity-induced increase in intracellular Ca^{2+} levels [75, 79], which, as discussed in the section on Ca^{2+}, plays an important role in regulating the RVD response. However, Cornet et al. [66] showed 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 [86]. Alternatively, generation of actin filaments of a specific size may be crucial for activation, a notion supported by our observations that pretreatment of the cells with cytochalasin B potentiated the hypotonicity-induced anion efflux. Direct modulation of the activity of ion channels by actin filaments is not unprecedented: in toad kidney A6 cells a Na^+-channel has been described that could be regulated by exogenously added F-actin [80]. Furthermore, signalling molecules associated with the cytoskeleton may be involved. In melanoma cells lacking actin-binding protein (ABP), activation of a volume-sensitive K^+-channel was markedly decreased but could be rescued by transfecting the cells with the wild type ABP gene [81]. Finally, remodelling of the cytoskeleton may play an important role in recruiting (activated) ion channels into the plasma membrane. The different modes in which the cytoskeleton may affect ion channel activation are summarized in Figure 2.

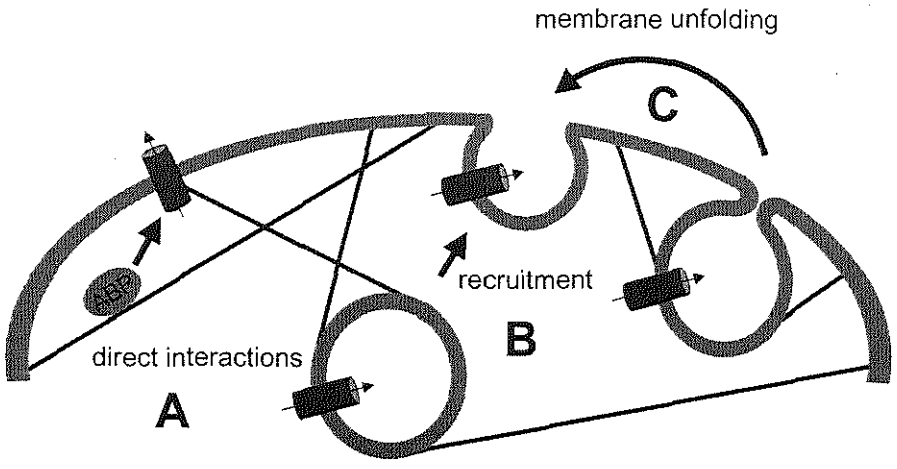

Figure 2. Mechanisms of ion channel regulation by the cytoskeleton

Three different models are presented that theoretically could be involved in the regulation of ion channels by cytoskeletal remodelling: **A)** direct activation by cytoskeletal elements or by proteins associated with the cytoskeleton, like actin-binding proteins (ABP), **B)** recruitment of ion channels by regulated vesicle fusion, and **C)** unfolding of the membrane surface.

AUTO-/PARACRINE SIGNALLING MOLECULES INVOLVED IN RVD

In addition to a direct activation of intracellular signalling cascades by cell swelling, hypo-osmotic stimulation can also lead to the extracellular release of hormones and other bioactive molecules. Through interaction with specific membrane receptors, these hormones trigger distinct signal transduction pathways which potentially could affect the RVD response of the cell or modulate the responses of surrounding cells.

ATP

Several forms of cellular stress, including hypo-osmotic stimulation, were found to increase the release of ATP. Extracellular ATP, acting through binding to $P2Y_2$-type purinoceptors, has been implicated in the activation of the osmosensitive Cl^- current in HTC hepatoma cells and in rat biliary epithelial cells [82, 83]. In Intestine 407 cells, however, degrading or trapping extracellular ATP by treating the cells with apyrase or hexokinase/glucose did not affect the volume-sensitive anion efflux, nor did it prevent the development of a swelling-induced Cl^- current [29]. Furthermore, unlike the observations in HTC hepatoma cells [82], addition of extracellular ATP was not able to activate VRACs in the absence of osmotic cell swelling in these cells [29]. Although not able to directly activate the osmosensitive conductance in Intestine 407 cells, extracellular ATP, through a $P2Y_2$-receptor-mediated increase in intracellular Ca^{2+}, may play an important role in potentiating both the K^+ and Cl^- conductances (see: section Ca^{2+} and [29]).

Involvement of $P2Y_2$-receptors is not universal; in other cell models the ATP metabolite adenosine was found to regulate VRACs through activation of the A1 type adenosine receptor ($9HTE_0$, human tracheal epithelial cells [84]; RCCT-28A rabbit cortical collecting duct cells [85]).

Besides its effects on the regulation of anion currents, extracellularly released ATP was found to be critically involved in the activation of the Erk-1/2 members of the MAP kinase family in Intestine 407 cells [29]. Not only were ATP and UTP in (sub) micromolar concentrations able to activate Erk-1/2 under isotonic conditions, but the hypotonicity-induced MAP kinase activation was completely abolished in the presence of apyrase or the purinoceptor-inhibitors suramin and reactive blue [29]. This implies that the activation of Erk-1/2 by hypotonicity is not a direct consequence of osmotic cell swelling, but instead involves an autocrine/paracrine mechanism with ATP as a messenger. The ability of ATP and purinoceptors to activate MAP kinases is not unprecedented; extracellularly added ATP was found to activate Erk-1/2 and JNK in many different model systems [86-94].

To date it is not clear how the release of ATP from Intestine 407 cells is regulated. Previously, Roman et al. [95] established an important role for the ABC transporter P-glycoprotein. Inhibition of P-glycoprotein expression (by anti-sense oligonucleotides) or function (by verapamil treatment) in Intestine 407 cells, however, did not affect the osmosensitive Cl^- current [96]. In addition, in the subclone of Intestine 407 cells we use, P-glycoprotein expression was not detected [29]. Taken together, these findings argue against a role for P-glycoprotein in the release of ATP from these epithelial cells. In contrast to the activation of VRACs, the hypotonicity-induced ATP release from Intestine 407 cells was sensitive to BAPTA-AM treatment and inhibited by cytochalasin B [29], suggesting the involvement of exocytosis. This notion is supported by the observations that an increase in intracellular Ca^{2+} could promote the release of ATP in the absence of an osmo-shock [29, 85]. In addition to Ca^{2+} and the cytoskeleton, PtdIns 3-kinase has also been implicated recently [97].

Arachidonic acid

Several studies have considered a possible role of arachidonic acid or its metabolites in the RVD response. In both human platelets and neuroblastoma cells, a hypotonicity-induced increase in the release of arachidonic acid was observed [59, 98]. In platelets, the release of arachidonic acid was found to be critically involved in activating the RVD response [59]. For other cell models, however, including HSG cells [19], Ehrlich ascites tumor cells [99], human neuroblastoma [98] and Intestine 407 cells [2] an inhibitory effect of arachidonic acid on the RVD and VRACs was reported. The mechanism of this inhibition is unknown, but, because other (un)saturated fatty acids (oleic, linoleic, linolenic and palmitoleic acid) mimic the inhibitory effect on the RVD response, a nonspecific detergent effect was suggested [99].

In Ehrlich ascites tumour cells and crypts isolated from mouse distal colon, leukotriene LTD_4, produced by lipoxygenase action on arachidonic acid, potentiated the RVD response and

volume-sensitive K^+- and Cl^--channels [8,100]. In addition, the LTD_4 receptor antagonist SK&F104353 as well as lipoxygenase inhibitors (nordihydroguaiaretic acid or ETH 615-139) inhibited volume-sensitive Cl^- currents in crypts from distal colon and jejunum [101, 102] and in human fibroblasts [103], respectively. In cultured nonpigmented ciliary epithelial cells, however, prostaglandin E2, but not leukotrienes, facilitated the RVD response [104], while an inhibition of the RVD response by prostaglandin E2 was found in mouse distal colon [8].

CONCLUDING REMARKS

A plethora of signalling cascades are activated upon hypo-osmotic stimulation of mammalian cells. Although some signalling molecules are only able to activate or modulate the RVD and/or osmosensitive anion channels in selected cell models, other signalling cascades seem to be more universally involved. These include the activation of one or more tyrosine kinases, G-proteins and a rise in intracellular free Ca^{2+} (Figure 3). Present knowledge, however, about the molecular mechanism by which these signalling molecules affect the conductance state of the channels involved in the RVD response is still rather 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.

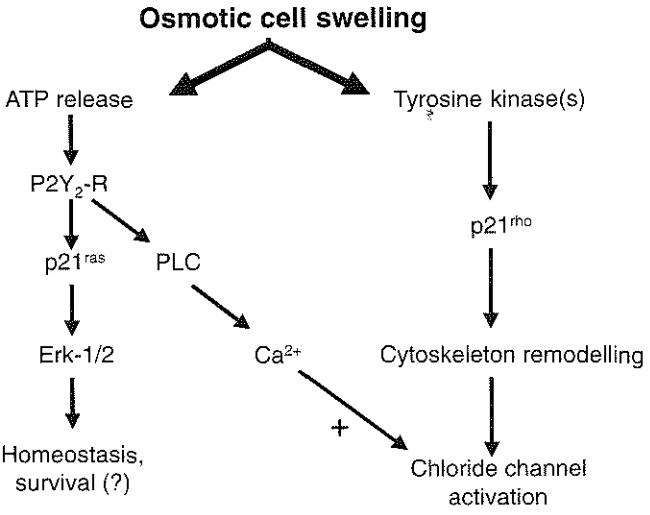

Figure 3. Hypotonicity-induced cellular signalling in Intestine 407 cells

In Intestine 407 cells, hypotonic stress leads to the parallel activation of VRACs and the MAP kinase Erk-1/2. VRAC activation was found to depend on tyrosine kinase(s), $p21^{rho}$ and the cytoskeleton, while Erk activation is caused by hyposhock-induced ATP release which, through an autocrine or paracrine mechanism, activates the purinoceptor $P2Y_2$. In addition, an ATP/$P2Y_2$ mediated increases in intracellular Ca^{2+} may serve as a modulator of VRAC activity.

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3

Osmotic swelling-induced activation of Erk-1/2 in Intestine 407 cells involves the Ras/Raf signalling pathway

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ABSTRACT

Human Intestine 407 cells respond to hypo-osmotic stress with a rapid stimulation of compensatory ionic conductances accompanied by a transient increase in the activity of the extracellular signal-regulated protein kinases Erk-1 and Erk-2. In this study, we examined the upstream regulators of hypotonicity-induced Erk-1/2 activation and their possible role in cell-volume regulation. The hypotonicity-provoked Erk-1/2 activation was greatly reduced in cells pretreated with the specific mitogen-activated/Erk-activating kinase inhibitor PD098059 and was preceded by a transient stimulation of Raf-1. Pretreatment of the cells with PMA, GF109203X, wortmannin or *Clostridium botulinum* C3 exoenzyme did not appreciably affect the hypotonicity-provoked Erk-1/2 stimulation, suggesting the osmosensitive signalling pathway to be largely independent of protein kinase C and $p21^{rho}$. In contrast, expression of dominant negative RasN17 completely abolished the hypotonicity-induced Erk-1/2 activation. Stimulation of the swelling-induced ion efflux was independent of activation of these mitogen-activated protein kinases, as revealed by hypotonicity-provoked isotope efflux from $^{125}I^-$ and $^{86}Rb^+$-loaded cells after pretreatment with PD098059 and after expression of RasN17. In addition, the epidermal-growth-factor-induced potentiation of the hypotonicity-provoked anionic response did not depend on the increase in Erk-1/2 activity but, instead, was found to depend on Ca^{2+} influx. Taken together, these results indicate that hypotonic stress induces Erk-1/2 activation through the Ras/Raf-signalling pathway and argue against a direct role for this pathway in cell-volume control.

INTRODUCTION

Most mammalian cells respond to changes in cellular volume with a net movement of water driven by a redistribution of salt and/or small organic molecules (for reviews see [1,2]). As a consequence of hyperosmotic stress, cells are triggered to accumulate osmolytes leading to a subsequent influx of water (regulatory volume increase). In reverse, hypo-osmotic stress induces a loss of cellular water due to osmolyte release [regulatory volume decrease (RVD)]. Despite the extensive research carried out on cell-volume regulation to date, the molecular mechanisms of osmosensing and osmosignalling are not completely resolved. Although differences may exist between cell types, a general feature of cell signalling in response to hypotonic stress is the rapid and transient activation of the extracellular-signal-regulated protein kinases Erk-1/2. Activation of these kinases has now been reported for a variety of mammalian cell types, including Intestine 407 cells [3], hepatoma cells [4], hepatocytes [5], cardiac myocytes [6], astrocytes [7] and C6 glioma cells [8], as well as for yeast [9].

Erk-1/2 are members of the superfamily of mitogen-activated protein kinases (MAP kinases): a group of serine/threonine kinases that are activated by dual-specificity protein kinases through phosphorylation on both threonine and tyrosine residues. Erk-1/2 are activated by diverse extracellular stimuli and affect many important cellular processes via protein phosphorylation of specific targets (for reviews see [10,11]). Several different pathways may lead to Erk-1/2 activation (reviewed in [12]). The pathway studied in most detail is the signalling cascade initiated by tyrosine kinase-containing receptors [e.g. epidermal growth factor (EGF) and platelet-derived growth factor receptors], which involves activation of the small G-protein $p21^{ras}$ (Ras). Conversion of Ras-GDP into the active GTP-bound state, via coupling of the Grb2-Sos complex to the receptor [13], recruits the Raf-1 kinase to the plasma membrane [14]. Subsequently, Raf-1 becomes activated and phosphorylates the mitogen-activated/Erk-activating kinase (MEK) [15], a dual specificity protein kinase, which in turn activates Erk-1/2 [16].

In addition to receptor tyrosine kinases, several G-proteins coupled to serpentine receptors, as well as cell-surface adhesion receptors of the integrin family, have been identified as activators of Erk-1/2 [17,18]. These receptors have been suggested to activate the Ras/Erk pathway via activation of PtdIns 3-kinase (G-protein coupled receptors) or by binding of the focal adhesion kinase $p125^{FAK}$ (integrin receptors) to the Grb2-Sos complex [19,20]. In addition, several Ras-independent pathways have been reported that lead to Erk-1/2 activation. For instance, Robbins et al. [21] and Chen et al. [22] found that expression of a dominant negative Ras mutant did not affect Erk-1/2 activation by G-protein-bound and integrin receptors. Furthermore, EGF has been reported to activate Erk-2 via Ras-independent pathways, which involve protein kinase C (PKC) activation or intracellular Ca^{2+} $[Ca^{2+}]_i$ increases [23-25].

Using the human fetal jejunum-derived Intestine 407 cell line as a model, we have recently demonstrated that hypo-osmotic cell swelling is accompanied by a rapid and transient increase in tyrosine phosphorylation of several proteins, including $p125^{FAK}$ and Erk-1/2, as well

as by activation of PtdIns 3-kinase [3,26]. Furthermore, increased tyrosine phosphorylation was found to be an essential step in eliciting an RVD response [3]. The finding that EGF not only activates the MAP kinases Erk-1/2 but also strongly potentiates the hypotonicity-induced ion efflux [3] suggested a role for Erk-1/2 in the activation and/or growth factor potentiation of the osmosensitive anion efflux. This notion was supported by the recent findings that both the yeast Hog1p and Mpk1p members of the MAP kinase family are critically involved in cell-volume regulation [9,27].

In this study we have investigated the regulatory pathway leading to hypotonicity-induced Erk-1/2 activation and evaluated the role of these MAP kinases in cell-volume regulation. The results presented indicate that the transient, osmosensitive activation of Erk-1/2 in Intestine 407 cells is downstream of the Ras/Raf pathway, and independent of PKC, Ca^{2+} influx and PtdIns 3-kinase. Treatment of the cells with the specific MEK inhibitor PD098059 or expression of dominant negative RasN17 prevented the cell swelling-induced Erk-1/2 activation, but did neither affected the osmosensitive anion efflux or its potentiation by EGF. Taken together, the results argue against a direct role for Erk-1/2 in cell-volume regulation.

MATERIALS AND METHODS

Materials

Radioisotopes ($^{125}I^-$, $^{86}Rb^+$ and [γ-^{32}P]ATP) and enhanced chemiluminescence (ECL) detection kit were purchased from Amersham Netherlands B.V. ('s Hertogenbosch, The Netherlands). Antibodies and Syntide-2 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). The MEK inhibitor PD098059, myelin basic protein (MBP) and GF 109203X were purchased from Calbiochem (La Jolla, CA, U.S.A.), Sigma (St. Louis, MO, U.S.A.) and Biomol (Plymouth Meeting, PA, U.S.A.) respectively. Protein A-Sepharose was obtained from Pharmacia Biotech (Uppsala, Sweden). Recombinant *Cl. botulinum* C3 exoenzyme was isolated and prepared as described previously [28].

Cell culture

Intestine 407 cells were routinely grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 25 mM Hepes, 10% fetal calf serum, 1% non-essential amino acids, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Before the experiments, cells were serum-starved overnight.

Assay of Erk-1/2 activity

After stimulation, cells were washed once with ice-cold PBS and 1 ml of lysis buffer was added (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 0.5%

Nonidet P40, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% aprotinin and 50 μg/ml leupeptin, pH 7.4). After 10 min (0°C), lysates were collected, cleared by centrifugation and the supernatants were incubated with polyclonal anti-Erk-2 antibodies (recognizing both Erk-2 and, to a lesser extent, Erk-1) for 1 h. Thereafter, Protein A-Sepharose was added and the mixture was incubated for another hour. The immunoprecipitates were washed three times with lysis buffer and three times with kinase buffer (10 mM Tris/HCl, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.4), and Erk-1/2 activity was determined by *in vitro* phosphorylation of MBP. Phosphorylation was carried out in 40 μl of kinase buffer supplemented with 0.5 mg/ml MBP and 25 μM ATP + 5 μCi of [γ-^{32}P]ATP (30 min; 37°C). Reactions were terminated by the addition of SDS sample buffer. The samples were boiled for 5 min and subjected to SDS/PAGE. Radioactivity of MBP was quantified by a phospho-imaging system (Molecular Imaging System GS-363; Biorad).

Assay of Raf-1 activity

Cells were lysed in 50 mM Hepes, pH 7.5, containing 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 1% aprotinin (10 min, 0°C). Lysates were collected, cleared and incubated with 1 μg/ml polyclonal rabbit anti-Raf-1. Immune complexes were bound to Protein A-Sepharose and washed three times with lysis buffer, twice with 0.5 M LiCl, 0.1 M Tris/HCl, pH 7.5 and once with kinase buffer (25 mM Tris/HCl, 10 mM $MnCl_2$, 1 mM dithiothreitol, 25 mM β-glycerolphosphate, pH 7.5). Raf-1 activity was determined using Syntide-2 as a substrate. Phosphorylation was carried out for 20 min at 30°C in 40 μl of kinase buffer containing 125 μM Syntide-2 and 25 μM ATP + 5 μCi of [γ-^{32}P]ATP. Thereafter, samples were rapidly centrifugated and 15 μl portions of the supernatants were spotted on Whatman p81 phosphocellulose paper. After extensive washing of the paper with 0.85% phosphoric acid, ^{32}P incorporation was monitored by phospho-imaging.

Kinase mobility shift assay

Monolayers of cells were stimulated as indicated in legends and incubations were terminated by replacing the medium with boiling SDS sample buffer. Proteins were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose. Proteins were stained with polyclonal anti-Erk-1 antibodies (recognizing both Erk-1 and Erk-2) or polyclonal anti-Raf-1 antibodies and an ECL Western-blotting detection system according to the instructions provided by the manufacturers.

Vaccinia virus infection

Infections with recombinant vaccinia virus encoding the RasN17 mutant and wild-type vaccinia virus were performed as described previously [23]. After infection, the medium was replaced by Dulbecco's modified Eagle's medium containing 0.25% BSA. Experiments were performed 16-18 h after infection.

Efflux assays

Confluent monolayers of Intestine 407 cells were loaded with 5 μCi/ml $^{125}I^-$ or 0.5 μCi $^{86}Rb^+$ 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 7.4) under a 95% O_2/5% CO_2 humidified atmosphere. Subsequently, the cultures were washed three 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) and radioisotope efflux was determined by replacing the medium at 1-2 min intervals. Hypotonic buffers were prepared by adjusting the concentration of mannitol. Radioactivity in the medium was determined by γ-radiation counting and expressed as fractional efflux per minute as described previously [29].

RESULTS

Activation and phosphorylation of Erk-1/2 and Raf-1 in response to hypo-osmotic cell swelling

Previously, a transient increase in Erk-1 and Erk-2 phosphorylation, as demonstrated by a shift in electrophoretic mobility on SDS/PAGE, has been observed after hypotonic stimulation of Intestine 407 cells [3]. Recently, however, it has been reported that a mobility shift could already be observed after phosphorylation of either a tyrosine or a threonine residue of Erk-1/2, whereas phosphorylation of both residues is required to induce activation of Erk-1/2 [10]. Therefore, we quantified hypo-osmotic swelling-induced Erk-1/2 kinase activity more directly, using MBP as a substrate. A time course of Erk-1/2 activity, as determined in confluent Intestine 407 cultures exposed to a 30% hypotonic shock (70% tonicity), is shown in Figure 1.

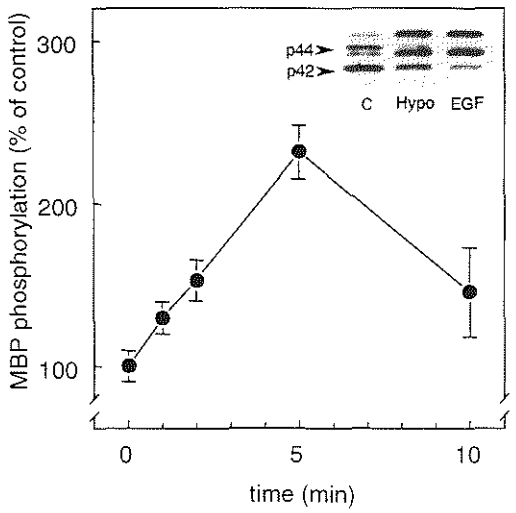

Figure 1. Time course of hypotonic shock-induced phosphorylation and activation of Erk-1/2 in Intestine 407 cells

Cells were exposed to 30% hypotonic medium for the times indicated in the presence of 200 μM sodium orthovanadate (total exposure time was 10 min at all time points). Erk-1/2 were immunoprecipitated and enzyme activity was measured using MBP as a substrate. Data are expressed as percentage of activity in cells exposed to isotonic medium (mean ± S.E.M., n=3). The insert shows the electrophoretic mobility shift of Erk-1/2 (p44/p42) induced by a 5 min exposure to 30% hypotonic medium or by EGF (50 ng/ml).

Activation started without an apparent lag phase and peaked after 5 min. Thereafter, the Erk-1/2 activity slowly declined to prestimulatory levels. No further activation of Erk-1/2 was observed within 2-4 h after osmotic stimulation (results not shown). The osmosensitive stimulation of Erk-1/2 is a direct consequence of MEK activity, since pretreatment of the cells with the specific MEK inhibitor PD098059 [30], at concentrations that effectively block EGF-induced increase in MAP kinase activity, greatly reduced Erk-mediated MBP phosphorylation (Table 1).

Table 1. Effect of the MEK inhibitor PD098059 on hypotonicity- and EGF-induced Erk-1/2 activation

Control or PD098059 (50 μM for 2 h) pretreated cultures, were exposed to isotonic medium, to 30% hypotonic medium or to 50 ng/ml EGF for 5 min. Thereafter, Erk-1/2 were immunoprecipitated and enzyme activity was determined using MBP as a substrate. Data are expressed as percentage of activity in cells exposed to isotonic medium and not pretreated with PD098059 (mean ± S.E.M., n=3).

	Erk-1/2 activity (% control)		
Pretreatment	Isotonic	Hypotonic	EGF
None	100 ± 11	279 ± 19	303 ± 32
PD098059	46 ± 5	98 ± 26	108 ± 7

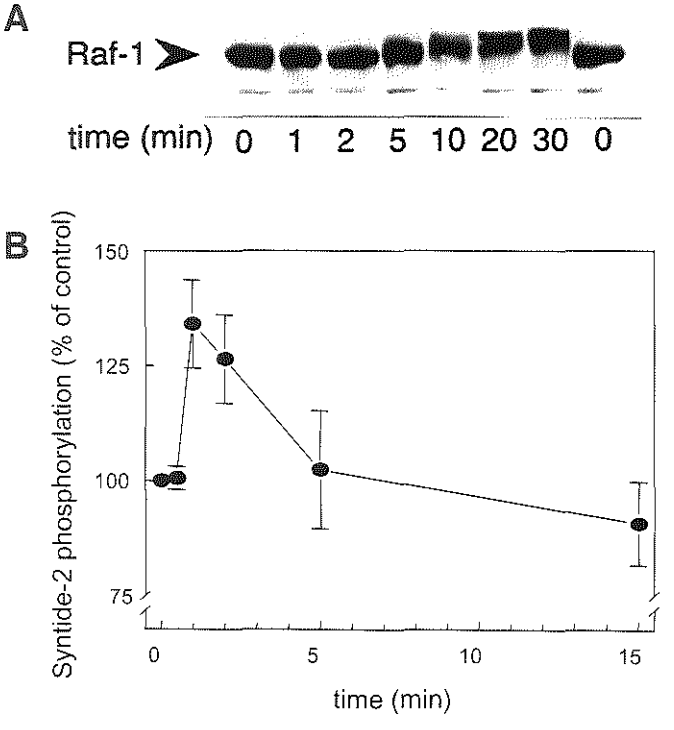

Figure 2. Time course of hypotonic shock-induced Raf-1 phosphorylation and activation

Cells were exposed to 30% hypotonic medium for the times indicated. **A**) Electrophoretic mobility shift of Raf-1. Whole cell lysates were separated by SDS-PAGE followed by western blotting. Immunoblots were incubated with anti-Raf-1 antibodies. **B**) Raf-1 kinase activity. Raf-1 was immunoprecipitated and enzyme activity was measured using Syntide-2 as substrate. Data are expressed as percentage of activity in cells exposed to isotonic medium (mean ± S.E.M., n=4).

In order to establish upstream signalling components of the hypotonicity-induced MEK/Erk activation, we studied the effects of osmotic cell swelling on both Raf-1 phosphorylation and activity. After a lag period of approx. 5 min, a marked increase in Raf-1 phosphorylation, as evidenced by a decreased electrophoretic mobility, was observed, which lasted for more than 30 min (Figure 2A). The hypotonicity-induced activation of Raf-1, however, as determined by *in vitro* phosphorylation using Syntide-2 as a substrate, started immediately and was very transient, peaking at 2 min after stimulation (Figure 2B). Subsequently, Raf-1 activity rapidly declined to levels even below the control values. Notably, the decrease in Raf-1 activity coincided with an increase in Raf-1 phosphorylation.

Osmotic activation of Erk-1/2 is largely independent of PKC

PKC has previously been identified as an activator of MEK and Erk-1/2 through a stimulation of Raf-1 kinase [31-33]. To investigate a possible regulatory role for PKC in the hypotonicity-induced activation of Erk-1/2, cells were pretreated with 100 nM PMA to either activate (5 min pretreatment) or downregulate (24 h pretreatment) PKC. Whereas activation of PKC by a brief treatment with PMA markedly stimulated Erk-1/2 under isotonic conditions, a further increase in activity of the MAP kinases was still observed after hypo-osmotic cell swelling (Table 2). Furthermore, down-regulation of PKC by prolonged PMA treatment only slightly affected hypotonicity-induced Erk-1/2 stimulation (Table 2). Pretreatment of cells with the specific PKC inhibitor GX 109203X (1 μM for 20 min) virtually abolished the PMA stimulation of Erk-1/2 activity, but caused only a minor inhibition of the hypotonicity-provoked increase in activity (Table 3). Taken together, these results suggest that hypotonic stress activates Erk-1/2 principally via a PKC-independent pathway.

Table 2. Effect of PMA on the hypotonicity-induced Erk-1/2 activation

Control and PMA pretreated (100 nM for 5 min or 24 h) cells were exposed to 30% hypotonic medium or isotonic medium for 5 min. Thereafter, the cells were lysed and Erk-1/2 enzyme activity was determined in immunoprecipitates using MBP as a substrate. Data are expressed as percentage of activity in untreated cells (mean ± S.E.M., n=4).

	Erk-1/2 activity (% control)	
Pretreatment	Isotonic	Hypotonic
None	100 ± 15	373 ± 15
PMA (5 min)	325 ± 36	460 ± 16
PMA (24 h)	93 ± 31	267 ± 33

Table 3. Effect of GF 109203X on the hypotonicity-induced Erk-1/2 activation

Cells were exposed for 5 min to isotonic medium, 30% hypotonic medium, or 100 nM PMA in control and GF 109203X pretreated (1 μM for 20 min) cultures. Thereafter, Erk-1/2 were immunoprecipitated and enzyme activity was determined using MBP as a substrate. Data are expressed as percentage of activity in untreated cells (mean ± S.E.M., n=6).

	Erk-1/2 activity (% of control)		
Pretreatment	Isotonic	Hypotonic	PMA (5 min)
None	100 ± 5	331 ± 16	368 ± 27
GF 109203X	120 ± 16	262 ± 37	135 ± 16

Hypotonic stress-induced Erk-1/2 activation depends on active Ras

To investigate the putative involvement of the small GTPase Ras in the hypotonicity-induced Erk-1/2 activation, cells were infected with recombinant vaccinia virus encoding mutant dominant negative RasN17 [23]. As compared with control cultures, infection of the cells with recombinant RasN17 virus completely abolished the hypotonic-stress-induced Erk-1/2 activation, whereas mock virus infection affected their stimulation only slightly (Figure 3). Unlike the hypotonicity-provoked Erk-1/2 stimulation, activation of these MAP kinases by EGF was only partially inhibited after expression of RasN17. This may be due to EGF-triggered activation of alternative Ras-independent pathways, as has been reported by others [23-25].

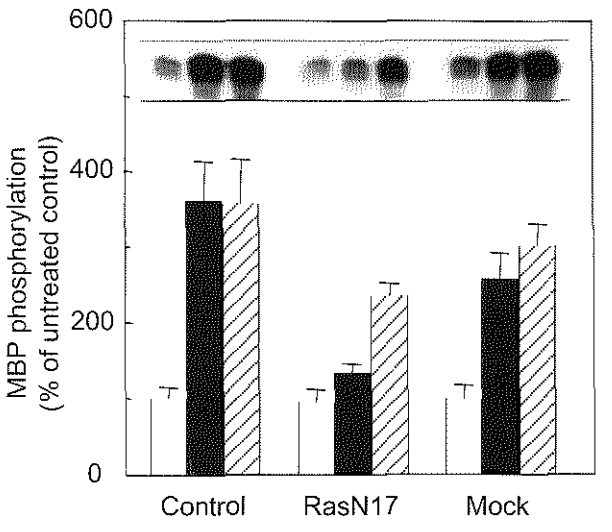

Figure 3. Effect of RasN17 expression on hypotonicity- and EGF-induced Erk-1/2 activation

Prior to exposing the cells to isotonic (open bars), 30% hypotonic (closed bars) or EGF (50 ng/ml) -containing (hatched bars) media, cells were transfected with recombinant vaccinia virus expressing RasN17 or with wild type virus (Mock). Erk-1/2 were immunoprecipitated and activity was measured using MBP as a substrate. Data are expressed as percentage of activity in uninfected (Control) cells exposed to isotonic medium (mean ± S.E.M., n=3). The insert shows a representative autoradiograph of ^{32}P-labelled MBP separated by SDS-PAGE.

In wortmannin-treated (0.1-1 μM; 30 min) cells, neither basal nor the osmosensitive Erk-1/2 activities were affected (results not shown). In addition, pretreatment of the cells with *Cl. botulinum* C3 exoenzyme under conditions that inhibited the hypotonicity-induced anion efflux, $p125^{FAK}$ phosphorylation as well as PtdIns 3-kinase activation (cf. [26]) markedly reduced basal Erk-1/2 activity but did not prevent Erk-1/2 stimulation by osmotic cell swelling (Table 4), arguing against a direct involvement of $p125^{FAK}$ and PtdIns 3-kinase in hypotonicity-induced activation of the Ras/Erk cascade.

Table 4. Effect of *Clostridium botulinum* C3 exo-enzyme on hypotonicity-induced Erk-1/2 activation
Cells were exposed for 5 min to isotonic medium or 30% hypotonic medium in the absence or presence of 50 μg/ml C3 exo-enzyme (48 h). After lysis of the cells, Erk-1/2 were immunoprecipitated and enzyme activity was measured using MBP as a substrate. Data are expressed as percentage of activity in untreated cultures exposed to isotonic medium (mean ± S.E.M., n=3).

	Erk-1/2 activity (% control)	
Pretreatment	Isotonic	Hypotonic
None	100 ± 3	188 ± 4
C3 exo-enzyme	50 ± 8	105 ± 17

Erk-1/2 activation is not involved in triggering the compensatory ion efflux

Recent studies have demonstrated that in yeast strains defective in responding to alterations in the tonicity of the medium, the Hog1p kinase was mutated and inactive, suggesting an important role for members of the MAP kinase family in cell-volume regulation [27]. To investigate the role of Erk-1/2 in the activation of the osmosensitive anion conductance, hypotonicity-provoked $^{125}I^-$ efflux was determined in PD098059-pretreated cultures. As clearly shown in Figure 4(A), PD098059 (50 μM, 2 h) inhibition of MEK did not affect the swelling-induced $^{125}I^-$ efflux, whereas Erk-1/2 activation was almost completely abolished (cf. Table 1). Likewise, the osmosensitive $^{86}Rb^+$ efflux, indicative for K^+-channel activation, remained unaffected in the presence of PD098059. In addition, no significant decrease in osmosensitive iodide efflux was observed in cells expressing RasN17 (Figure 4B).

We previously reported an EGF-induced potentiation of the osmosensitive anion efflux in Intestine 407 cells [3]. Since, in addition to the activation of the Ras/Erk signalling cascade, EGF is also able to raise $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores and by activation of plasma-membrane Ca^{2+} channels [34,35], we studied the effects of EGF in PD098059-

and EGTA-treated cells. As shown in Figure 5, EGF potentiation of the hypotonicity-induced $^{125}I^-$ efflux was still present in PD098059-treated cells, but was completely absent from cultures pretreated with EGTA, indicating that the EGF-mediated increase in anion conductance is not due to activation of Erk-1/2 but instead involves a rise in $[Ca^{2+}]_i$. Importantly, Erk-1/2 activation by EGF or osmotic cell swelling was not reduced in EGTA-containing media (data not shown). To summarize, these results indicate that Erk-1/2 activity is involved in neither eliciting nor potentiating the compensatory osmosensitive anion efflux.

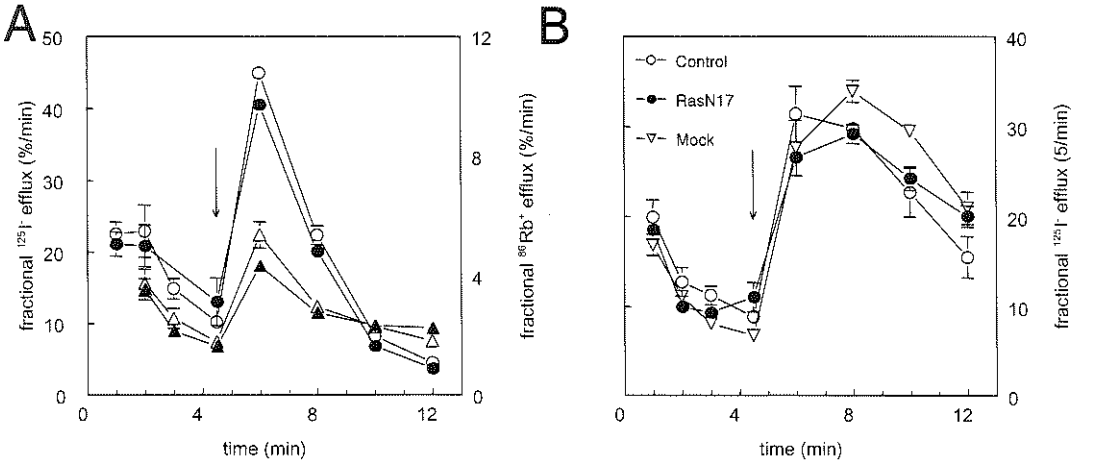

Figure 4. Effect of PD098059 and dominant negative RasN17 on osmosensitive $^{125}I^-$ and $^{86}Rb^+$ efflux

A) Radioisotope efflux was determined from control (open symbols) and PD098059 treated (50 μM, 2 h; closed symbols) cultures. Circles and triangles represent $^{125}I^-$ and $^{86}Rb^+$ efflux respectively. Data are expressed as fractional isotope efflux (mean ± S.E.M., n=3). Arrow indicates a shift from isotonic to 30% hypotonic medium.
B) Control cultures and cells infected with either recombinant vaccinia virus expressing RasN17 or with wild type vaccinia virus (Mock) were osmotically stimulated (30% hypotonicity) and fractional $^{125}I^-$ efflux was determined. Data are expressed as mean ± S.E.M. (n=6).

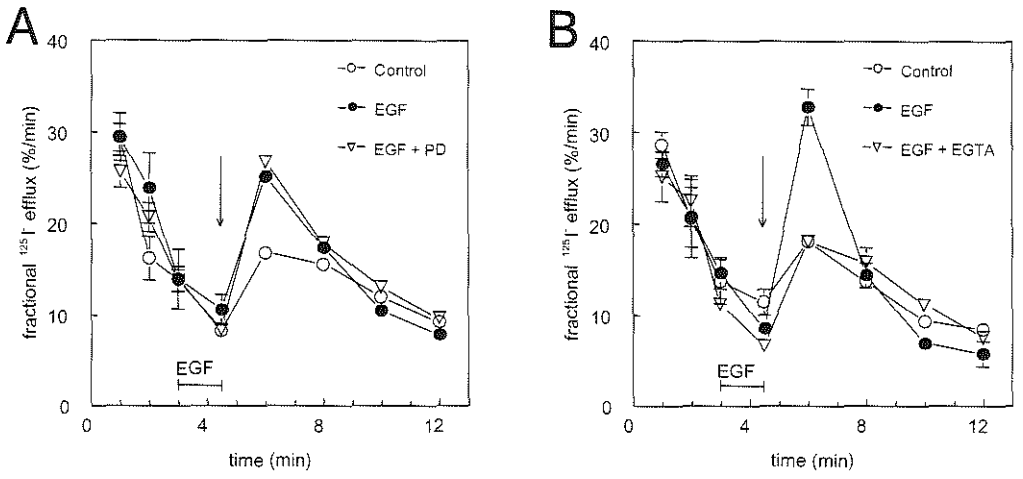

Figure 5. Effect of PD098059 and EGTA on EGF-induced potentiation of osmosensitive $^{125}I^-$ efflux

Control (open circles) and EGF-treated (50 ng/ml for 90 s prior to osmotic stimulation; closed circles and open triangles) cells were exposed to a 20% hypotonic medium and fractional $^{125}I^-$ efflux was determined. **A**) Effects of pretreatment with 50 μM PD098059. **B**) Effects of chelating extracellular Ca^{2+} by the addition of excess EGTA (1.3 mM) during the assay. Data are expressed as mean ± S.E.M. (n=3). Arrow indicates a shift from isotonic to hypotonic medium.

DISCUSSION

Osmotic cell swelling rapidly leads to the phosphorylation and/or activation of a number of signalling enzymes concomitant with an increase in the conductance of compensatory ion channels. Among these enzymes, the Erk-1/2 members of the MAP kinase family are of particular interest since (1) they are rapidly and transiently activated, without an apparent lag phase; (2) treatment of the cells with the Erk-1/2 activator EGF potentiates the hypotonicity-induced anion efflux [3]; (3) in yeast, the MAP kinases Hog1p and Mpk1p were found to be crucial for osmoresponse [9,27]. In this paper we analysed the signalling pathway(s) involved in hypotonicity-induced Erk-1/2 activation as well as their role in ionchannel activation.

Hypotonic activation of Erk-1/2 in Intestine 407 cells was accompanied by a rapid and transient increase in Raf-1 activity and was greatly reduced in cells pretreated with the MEK inhibitor PD098059. In addition, expression of dominant-negative RasN17 in the cells abolished hypotonicity-induced Erk-1/2 activation. These results clearly indicate that the osmotic cell swelling-induced activation of Erk-1/2 is a direct consequence of Ras GDP/GTP exchange, analogous to the activation of Erk-1/2 by tyrosine kinase-containing receptors. This notion is supported by our observations that the osmosensitive Erk-1/2 stimulation was largely independent of PKC acting directly on Raf-1. A hypotonicity-induced phosphorylation of Raf-1, as reported in this study, was also found in H4IIE hepatoma and C6 glioma cells [4,8]. However, a clear discrepancy exists between Raf-1 activation and its phosphorylation. As is evident from our results,

cell swelling-induced Raf-1 activation was rapid and very transient, returning to a level even below prestimulatory activity within 2-5 min, whereas Raf-1 phosphorylation was only observed after 5 min of exposure to hypotonic medium. These observations are in agreement with the findings of Wartmann et al. [36], who recently reported that subsequent to its activation by Ras, Raf-1 is immediately inactivated by hyperphosphorylation.

It still remains to be established how osmotic cell swelling activates Ras. A possible mechanism involves the Ras-related GTPase $p21^{rho}$, which has been identified as an intermediate in the lysophosphatidic acid- and integrin-receptor-induced Erk-1/2 activation [28,37,38]. Using *Cl. botulinum* C3 exoenzyme-treated cells, we previously demonstrated the involvement of $p21^{rho}$ in the osmosensitive increase in anion conductance as well as in $p125^{FAK}$ phosphorylation and PtdIns 3-kinase activation [26]. Phosphorylated $p125^{FAK}$ as well as PtdIns 3-kinase are able to activate Ras by respectively direct binding to the Grb2-SH2/SH3 adaptor protein or by stimulating a Src-like kinase, which subsequently activates the Shc-Grb2-SOS complex [19,20]. Our results, however, argue against a role for $p21^{rho}$, $p125^{FAK}$ or PtdIns 3-kinase in the signalling cascade leading to Ras activation, since pretreatment of the cells with C3 exo-enzyme did not prevent hypotonicity-induced Erk-1/2 activation. Furthermore, pretreatment of the cells with the PtdIns 3-kinase inhibitor wortmannin did not affect Erk-1/2 activation in Intestine 407 cells. PtdIns 3-kinase independence was also observed in C6 glioma cells [8]; however, Erk-1/2 activation in astrocytes was found to be completely abolished after PtdIns 3-kinase inhibition [7]. Alternatively, several heterotrimeric G-proteins have been identified as activators of Ras-type GTPase (reviewed in [39]). Both pertussis toxin-sensitive $G_i\beta\gamma$ subunits as well as pertussis toxin-insensitive $G\alpha_{12}$ and $G\alpha_{13}$ subunits have been implicated in the activation of MAP kinases. Whereas a G_i involvement in the hypotonicity-provoked Erk-1/2 phosphorylation was observed in hepatoma cells [4], G_i is not likely to be a candidate for Ras activation in Intestine 407 cells since pretreatment of the cells with pertussis toxin did not affect Erk-1/2 activation (T.van der Wijk and B.C.Tilly, unpublished works). These discrepancies in PtdIns 3-kinase and G_i involvement in the hypotonicity-induced Erk-1/2 activation clearly suggests that upstream regulators may differ between model systems.

In the yeast *Saccharomyces cerevisiae*, both the HOG1 and the PBS2 genes, which encode respectively a MAP kinase (Hog1p) and a MAP kinase kinase, are essential for adequately responding to an increase in extracellular tonicity [27]. Recently, the yeast osmosensor involved in activating the HOG1 cascade has been found [40,41]. The osmosensing mechanism belongs to the so called "two-component signal-transduction systems" and consists of the transmembrane histidine kinase Sln1p (the "sensor") and the cytosolic Ssk1p response regulator. As, in most mammalian cell types studied, a rapid activation of Erk-1/2 is among the initial signalling events after osmotic cell swelling, it is tempting to propose a model in which the Ras/Erk cascade plays an important role in the RVD response. This notion is supported by several observations that cellular and oncogenic Ras can affect ion-transport systems [42-44]. Our results,

however, using the specific MEK inhibitor PD098059 as well as the expression of dominant-negative RasN17, clearly demonstrate that the Ras/Raf/MEK/Erk cascade is not involved in the activation of compensatory ionic conductances in hypotonically stimulated Intestine 407 cells. Moreover, EGF potentiation of the hypotonicity-induced anion efflux occurs through elevation of $[Ca^{2+}]_i$ rather than Erk-1/2 activation. A potential role for Ca^{2+} signalling in the potentiation of the hypotonicity-induced ionic conductances in Intestine 407 cells was previously established by the observations that Ca^{2+}-mobilizing hormones as well as thapsigargin and the Ca^{2+}-ionophore A23187 were able to enhance, but not to trigger, the osmosensitive ionic fluxes [45].

In addition to Erk-1/2, several other members of the MAP kinase family have recently been reported. Among these, the p38 MAP kinase is of a particular interest because of its high homology to Hog1p and, in Intestine 407 cells, its rapid activation on osmotic cell swelling [46]. It is very unlikely, however, that p38 is involved in the activation of osmosensitive ionic channels, because inhibition of p38 MAP kinase, using the specific p38 inhibitor SB203580, did not block the osmosensitive anion efflux and, unlike Erk-1/2, activation of p38 MAP kinase using cytokines or anisomycin did not potentiate the ionic response [46]. The role of c-Jun N-terminal kinase (JNK), a third member of the MAP kinase family which becomes activated upon cell swelling in cardiac myocytes [6] as well as Intestine 407 cells (B.C.Tilly, unpublished work), in the regulation of osmosensitive ionic fluxes remains to be established.

To date, little is known about the physiological role of Erk-1/2 activation during the RVD response. An increased expression of the intermediate early genes c-*fos* and c-*jun* has been observed in osmotically stimulated hepatoma cells and cardiomyocytes [4,6], suggesting a role for Erk-1/2 in transcription regulation, maintaining cellular homeostasis and/or long-term survival. Activation of the Ras/Erk-signalling cascade has been widely recognized as the key event leading to increased cell proliferation. In addition to a rapid and transient first phase, a second more sustained phase of Erk-1/2 activation as well as a subsequent translocation to the nucleus was found to be a prerequisite for G_0-S transition and cell division to occur [47,48]. Yet, in Intestine 407 cells, Erk-1/2 activation is transient and a second phase is lacking, rendering a role for cell swelling-induced Erk-1/2 activation in cell proliferation unlikely. A more acute role for hypotonicity-activated Erk-1/2 has been proposed for hepatocytes [1,5]. In these cells, a relation has been found between Erk-1/2 activation and the rapid cell swelling-induced excretion of taurocholate into bile. In addition, Erk-2 has been reported to activate phospholipase A_2, the enzyme regulating arachidonic acid release, suggesting a possible involvement of Erk-1/2 in intercellular signalling [49]. However, for epithelial cell lines such as Intestine 407, a functional role for hypotonicity-provoked Erk-1/2 activation remains to be established.

The results of this study also have implications for the interpretation of our original observation that protein tyrosine phosphorylation is an essential event in the activation mechanism of cell swelling-induced compensatory ionic fluxes [3]. Since the Ras/Erk cascade is apparently not involved in the regulation of osmosensitive ionic channels, it follows that the tyrosine kinase(s) or phosphatase(s) involved in cell volume regulation are distinct from MEK.

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Osmotic cell swelling-induced ATP release mediates the activation of Erk-1/2 but not the activation of osmo-sensitive anion channels

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ABSTRACT

Human Intestine 407 cells respond to hypo-osmotic stress by the rapid release of ATP into the extracellular medium. A difference in time course of activation as well as in the sensitivity to cytochalasin B treatment and BAPTA-AM [1,2-bis-(2-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester] loading suggests that ATP leaves the cell through a pathway distinct from volume-regulated anion channels. To evaluate a putative role for nucleotides as autocrine/paracrine factors in osmotic signalling, the effects of extracellular ATP on the regulation of volume-sensitive anion channels as well as on the hypotonicity-induced activation of extracellular signal-regulated protein kinases (Erk-1/2) were investigated. Micromolar concentrations of ATP were unable to elicit an isotope efflux from $^{125}I^-$-loaded cells by itself, but strongly potentiated the hypotonicity-provoked anion efflux through a Ca^{2+}-dependent mechanism. The order of potency of nucleotides (ATP = UTP = ATPγS > ADP = AMP >> adenosine = cAMP) indicated the involvement of $P2Y_2$ receptors. In contrast, millimolar concentrations of ATP markedly inhibited both the osmotically induced isotope efflux and whole-cell Cl^- currents. Inhibition of whole-cell Cl^- currents, not only by millimolar concentrations of ATP but also by the purinoceptor antagonists suramin and reactive blue, was observed most prominently at depolarizing holding potentials, suggesting a direct interaction with volume-sensitive Cl^- channels rather than interaction with purinoceptors. Both ATP and UTP, at submicromolar levels, were found to act as potent activators of Erk-1/2 in Intestine 407 cells. Addition of the ATP

hydrolase apyrase to the bath greatly reduced the hypotonicity-induced Erk-1/2 activation, but did not affect the swelling-induced isotope efflux or whole-cell Cl^- currents. Furthermore, pre-treatment with suramin or reactive blue almost completely prevented the hypo-osmotic activation of Erk-1/2. The results indicate that extracellularly released ATP functions as an autocrine/paracrine factor that mediates hypotonicity-induced Erk-1/2 activation but does not serve as an activator of volume-sensitive compensatory Cl^- currents.

INTRODUCTION

Most cell types studied to date respond to alterations in cell volume by evoking specific compensatory mechanisms. In general, osmotic cell swelling leads to the activation of K^+ and Cl^- selective ionic channels and, depending on cell type and experimental conditions, to an accompanying efflux of organic osmolytes (amino acids, polyols and methylamines; reviewed in [1]). Although the mechanism of activation of the volume-sensitive Cl^- channels is as yet elusive, protein tyrosine phosphorylation was found to be critically involved in multiple cell types [2-6]. Furthermore, activation of the ras-related G-protein $p21^{Rho}$ appeared to be a prerequisite for activation of the volume-regulated Cl^- channels in bovine endothelial cells and human Intestine 407 cells [6, 7]. Hypo-osmotic cell swelling is accompanied by a rapid phosphorylation and/or activation of a number of other signalling enzymes including members of the MAP kinase (mitogen-activated protein kinase) family [2, 8-14]. Activation of MAP kinases, however, was not mandatory for the regulation of osmosensitive Cl^- channels [13, 14]. Although the molecular identity of the Cl^- channels involved in cellular volume regulation is not yet clear, the bioelectrical properties of osmosensitive Cl^- currents have been studied in many cell types. These currents are characterized by a marked outward rectification, a strong inactivation at positive holding potentials and an inhibition by extracellularly applied nucleotides (reviewed in [15,16]).

Recent evidence indicates that ATP is released rapidly from cells in response to increases in cellular cAMP, mechanical stress or osmotic cell swelling [17-19]. Furthermore, extracellular ATP has been recognized as an autocrine factor involved in increasing transmembrane Cl^- permeabilities and in the activation of volume-sensitive Cl^- channels [17, 18, 20]. Binding of ATP to a specific subclass of G-protein-coupled purinoceptors (the P2Y subtypes) not only leads to phospholipase C-mediated activation of protein kinase C and mobilization of Ca^{2+}, but additionally results in the activation of MAP kinases, including extracellular signal-regulated protein kinases (Erk-1/2) [21-27] and the stress-activated protein kinase SAPK/JNK [28, 29].

In this study, we used the human fetal jejunum-derived Intestine 407 cell line as a model to investigate the role of extracellularly released ATP in the regulation of volume-sensitive Cl^- channels as well as its putative involvement in the activation of Erk-1/2 by hypotonicity. Hypo-osmotic cell swelling was found to result in a dose- and time-dependent release of ATP into the

medium, through a mechanism independent of the activation of volume-sensitive Cl^- channels. Furthermore, in contrast with previous observations in other cell models [18, 30, 31], autocrine/paracrine ATP signalling did not serve as a trigger to activate volume-sensitive Cl^- channels. ATP signalling through P2Y receptors, however, was found to be fully responsible for the hypo-osmotic activation of Erk-1/2.

MATERIALS AND METHODS

Materials

Radioisotopes ($^{125}I^-$, $^{45}Ca^{2+}$, and [γ-^{32}P]ATP) and enhanced chemiluminiscence (ECL) kit were purchased from Amersham Netherlands B.V. ('s Hertogenbosch, The Netherlands). Polyclonal anti-Erk antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). The Ca^{2+}-ionophore A23187 and cytotoxicity detection kit (lactate dehydrogenase) were from Boehringer (Mannheim, Germany), and MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide), Protein A-Sepharose and luciferin/luciferase reagent were obtained from Janssen Chimica (Geel, Belgium), Pharmacia Biotech (Uppsala, Sweden) and Promega (Medison, WI, U.S.A.) respectively. Other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell culture

Intestine 407 cells were grown routinely as monolayers in Dulbecco's modified Eagle's medium supplemented with 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_2/5% CO_2 at 37°C. Prior to the experiments, cells were serum-starved overnight. Cell viability was assessed by determining lactate dehydrogenase leakage according to the instructions provided by the manufacturer (Boehringer Mannheim) and by quantifying MTT conversion as described by Hansen et al. [32].

Efflux assays

Confluent monolayers of Intestine 407 cells were loaded with 5 μCi/ml $^{125}I^-$ or 0.5 μCi $^{45}Ca^{2+}$ 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 7.4) under a 95% O_2/5% CO_2 humidified atmosphere. Subsequently, the cultures were washed three 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). Hypotonic buffers were prepared by adjusting the concentration of mannitol. Radio-isotope efflux was determined by replacing the medium at 1-2 min intervals, quantified by β- or γ-radiation counting and expressed as fractional efflux per minute as described previously [33].

Luciferin/luciferase assay

Cells were seeded at a concentration of $10^4/cm^2$ and incubated for 4 h under a humidified atmosphere of 95% O_2/5% 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). ATP release was measured in real time immediately after adding isotonic or hypotonic buffer containing the luciferin-luciferase reagent using a Topcount.NXT luminometer (Packard, Meriden, CT, U.S.A.). Values are expressed as accumulation of extracellular ATP in time and corrected for the consumption of ATP by the luciferase reaction. Hypotonic buffers were prepared by adjusting the concentration of mannitol and osmolarity was assessed using a cryoscopic osmometer (Osmomat 030; Salm & Kipp B.V., Breukelen, The Netherlands).

Measurement of whole-cell Cl^- currents

Cells were bathed in a solution containing 110 mM CsCl, 5 mM $MgSO_4$, 3.5 mM Na-gluconaat, 12 mM Hepes, 8 mM Tris, 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, 50 mM mannitol pH 7.4. Patch pipettes were pulled from borosilicate glass (Clark Electromedical Instruments, Pangbourne, Berks., U.K.) and had a resistance of 2-3 MΩ. To monitor the time-course of current activation, 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 intervals) from -100 mV to +100 mV with 25 mV increments. For command pulse control, data acquisition and analysis pCLAMP 6 software (Axon Instruments, Foster City, CA, U.S.A.) was used. All data were sampled at 5 kHz after being low-pass filtered at 500 Hz with a Bessel filter (Axon Instruments).

Erk-1/Erk-2 activity assay

After stimulation, cells were washed once with ice-cold PBS and 1 ml of lysis buffer was added (1% Triton X-100, 150 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% aprotinin and 50 μg/ml leupeptin, pH 7.4). After 10 min (0°C), lysates were collected, cleared by centrifugation and the supernatants were incubated with polyclonal anti-Erk antibodies (recognizing both Erk-2 and, to a lesser extent, Erk-1) for 1 h. Thereafter, protein A-Sepharose was added and the mixture was incubated for a further hour. The immunoprecipitates were washed three times with lysis buffer and three times with kinase buffer (10 mM Tris/HCl, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.4) and Erk-1/2 activity was determined by *in vitro* phosphorylation of myelin basic protein (MBP). Phosphorylation was carried out in 40 μl of kinase buffer supplemented with 0.5 mg/ml MBP, 25 μM ATP + 5 μCi of [γ-^{32}P]ATP (30 min, 37°C). After termination of the reactions by rapid centrifugation, boiling SDS sample buffer was added to the supernatants. The samples

were boiled for 5 min and subjected to SDS/PAGE. Radioactivity of MBP was quantified by a phospho-imaging system (Molecular Imaging System GS-363; Biorad, CA, U.S.A.).

RESULTS

Osmotic cell swelling stimulates ATP release

The release of ATP from hypotonicity-provoked Intestine 407 cells was monitored continuously using a luciferin/luciferase bioluminescence assay. As shown in Figure 1, a shift from isotonic to hypotonic medium caused a rapid increase in extracellular ATP. The ATP release was stimulus-dependent and continued for at least 15 min; only after mild hypo-osmotic stimulation (20-30% reduction in tonicity) was a tendency to level off observed. Notably, the regulatory volume decrease response under these conditions is completed within approx. 2-3 min [2]. Under all conditions tested, no detectable increase in luminescence was observed in the presence of the nucleotide hydrolase apyrase (results not shown).

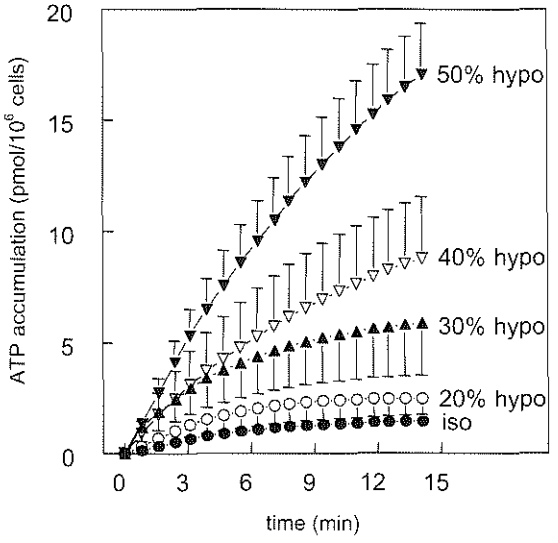

Figure 1. ATP release in response to hypo-osmotic cell swelling

ATP release from Intestine 407 cells was measured in real time using the luciferin/luciferase assay immediately after addition of isotonic or hypotonic buffer (t=0). Data are expressed as mean ± S.E.M. (n=5). The amount of ATP present at t=0 (immediately after adding isotonic (iso) or hypotonic (20%-50% hypo) buffer containing luciferin/luciferase reagent) was taken as a baseline and was not significantly different between the experiments and the buffers used (9.5 ± 0.1 pmol/10^6 cells).

Pretreatment of the cells with 1,2-bis-(2-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) or cytochalasin B significantly inhibited the hypotonicity-induced ATP release (Table 1). In contrast, the hypotonicity provoked conductive $^{125}I^-$-efflux was unaffected by BAPTA-AM loading and was strongly potentiated by pretreatment with cytochalasin B after submaximal stimulation (20% hypotonicity; Table 1). Cytochalasin B, however, did not affect the response to a saturating stimulus (30-50%; Table 1). These results, together with a clear difference in the time-course of activation/inactivation of osmosensitive ATP release and anion efflux (peaks within 1-2 minutes), suggest that ATP leaves the cell through a

pathway separate from the volume-sensitive anion channels. Notably, no significant differences were observed in either lactate dehydrogenase leakage or MTT conversion within 15 min of osmotic stimulation (50% tonicity, results not shown), indicating, together with our findings that the release of ATP is regulated by intracellular calcium and requires an intact cytoskeleton, that the increase in extracellular ATP is due to enhanced release from viable cells and not due to cell lysis.

Table 1. Effect of BAPTA-AM and cytochalasin B treatment on ATP release and hypotonicity-induced anion efflux

ATP release and fractional $^{125}I^-$ efflux was quantitated from control, BAPTA-AM loaded (25 μM, 1h) and cytochalasin B pretreated (50 μM, 30 min) Intestine 407 cells. ATP accumulation was determined after 15 min of incubation with isotonic or hypotonic medium. Activation of the anion conductance was determined by measuring the initial increase in fractional $^{125}I^-$ efflux at 90 s after changing the medium for an isotonic or hypotonic medium. Data are expressed as mean ± S.E.M. (n=3). Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

	ATP accumulation (pmol/10^6 cells)		Increase in fractional iodide efflux (%/min)		
Experimental condition	Isotonic	50% Hypotonic	Isotonic	20% Hypotonic	50% Hypotonic
Control	4.3 ± 1.0	23.1 ± 4.1	-0.7 ± 0.4	6.7 ± 1.7	32.4 ± 0.3
BAPTA-AM	1.9 ± 0.8	7.6 ± 2.3*	-0.2 ± 0.1	6.4 ± 0.5	31.1 ± 1.4
Cytochalasin B	0.3 ± 0.2*	8.0 ± 2.0*	1.4 ± 1.5	15.3 ± 1.1*	29.5 ± 1.9

Effects of extracellular nucleotides on osmosensitive anion efflux

The effects of extracellular ATP on anion transport were investigated by quantifying radioisotope efflux from $^{125}I^-$-loaded monolayers of Intestine 407 cells. Whereas extracellular ATP (0.1 μM-10 mM) was unable to evoke a $^{125}I^-$ efflux by itself (Figure 2A), micromolar concentrations (10-100 μM) greatly potentiated the anion efflux in response to a submaximal (20% reduction in tonicity) osmotic stimulus (Figure 2A). Potentiation of the swelling-induced anion efflux by ATP could have been due to an increase in iodide permeability (genuine potentiation) or to an increase in driving force (stimulation of Ca^{2+}-activated K^+ channels). Two different experiments were performed to investigate whether the isotope efflux is mainly a function of the number of activated anion channels or is affected also by the membrane potential. (i) Potentiation of the efflux by ATP was not observed after maximal (30% reduction in tonicity) hypotonic stimulation: peak $^{125}I^-$ efflux: 38.7 ± 0.8%/min in the absence and 39.8 ± 1.5%/min in the presence of 100 μM ATP (mean ± S.E.M., n=3). Under these conditions the

$^{86}Rb^+$ efflux (indicative for the K^+ permeability) was increased approx. 2-fold: peak $^{86}Rb^+$ efflux: 3.5 ± 0.2%/min in the absence and 5.9 ± 0.4%/min in the presence of 100 μM ATP (n=3). (ii) Treating the cells with the K^+ ionophore valinomycin (10 μM) did not further increase the $^{125}I^-$ efflux in response to a 30% hypotonic medium: peak $^{125}I^-$ efflux was 32.2 ± 2.8%/min in the control versus 31.7 ± 1.2%/min in the presence of valinomycin (n=3). Furthermore, as published previously [34], brief treatment of the cells with PMA completely inhibited the hypotonicity-induced $^{86}Rb^+$ efflux, but did not affect the osmosensitive $^{125}I^-$ efflux. These results show that the isotope efflux is virtually independent of the membrane potential. Taken together, micromolar concentrations of extracellular ATP potentiate the $^{125}I^-$ efflux through an increase in membrane permeability rather than by increasing the driving force for anion efflux by enhancing the K^+ conductance.

Increasing concentrations of ATP (≥ 1 mM), not only reduced the potentiation of the hypotonicity-provoked $^{125}I^-$ efflux, but eventually abolished the isotope efflux almost completely (Figure 2A). Similar results (not shown) were obtained when Mg-ATP was used, indicating that the inhibition observed is not due to a reduction in extracellular $[Mg^{2+}]$.

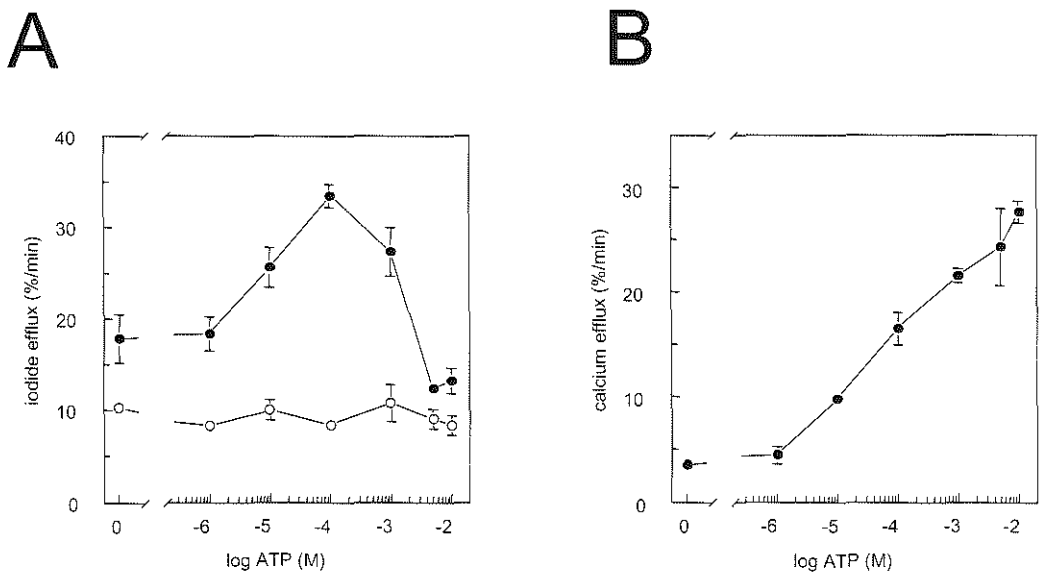

Figure 2. Effects of ATP on radioisotope release from $^{125}I^-$ or $^{45}Ca^{2+}$ loaded cells.

A) Dose-dependency of the effects of ATP on the fractional $^{125}I^-$ efflux from control (open symbols) and osmotically stimulated (20% hypotonicity, closed symbols) cultures. **B**) Dose-dependency of the ATP-induced fractional $^{45}Ca^{2+}$ efflux from radioisotope loaded cells under isotonic conditions. Data are expressed as mean ± S.E.M.(n=3).

Pretreatment of the cells with BAPTA-AM (25 μM, 1h) did not affect the anion-efflux response to hypo-osmotic stimulation but completely abolished the potentiation of the efflux by micromolar concentrations of ATP (Figure 3). These results suggest that the potentiation of the hypotonicity-induced anion efflux by extracellular ATP is due to an increase in intracellular Ca^{2+}. Indeed, addition of ATP to the medium was found to result in an increased cellular mobilization of Ca^{2+}, as quantified by $^{45}Ca^{2+}$ efflux from isotope-loaded cells (Figure 2B). The ATP-mediated Ca^{2+} release starts, like the potentiation of the hypotonicity-provoked anion efflux, at micromolar conditions and saturates at approx. 0.1-1 mM, but, in contrast with ATP effects on Cl^- channel activation, no inhibition of the Ca^{2+} efflux was observed at millimolar concentrations (Figure 2A/B). Taken together, these results suggest that different mechanisms underlie the potentiation and inactivation of the anion conductance.

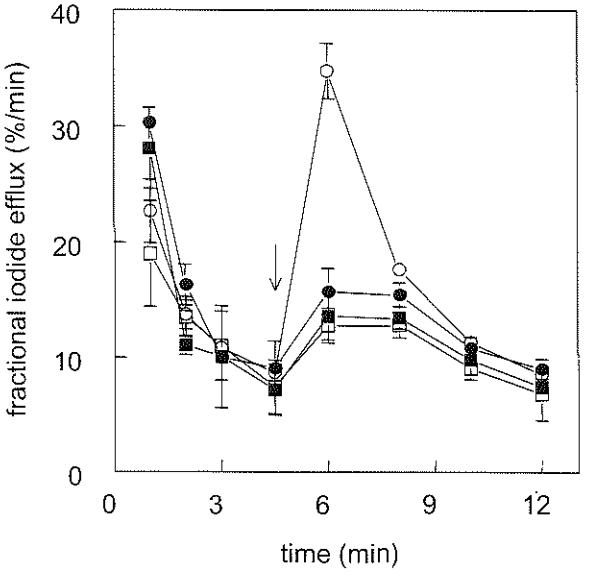

Figure 3. Calcium dependence of ATP-mediated potentiation of the osmosensitive anion efflux

Time course of the osmo-shock induced (20% hypotonicity) fractional $^{125}I^-$ efflux from control (open symbols) or BAPTA-AM-loaded (25 μM, 1h; closed symbols) cultures in the presence (circles) or absence (boxes) of ATP (50 μM). ATP or vehicle was added from time point 3 min on; arrow indicates shift to hypo-osmotic medium. Data are expressed as mean ± S.E.M. (n=3).

Extracellular ATP is known to induce Ca^{2+} mobilization by activation of G-protein-coupled purinoceptors, which are divided into subtypes on the basis of differing agonist-affinity series (reviewed in [35]). To investigate the receptor involved in the ATP effect on the osmosensitive anion efflux, the relative potencies of adenine- and uridine-containing nucleotides were determined. As shown in Table 2, this affinity profile (UTP=ATP=ATPγS>ADP=AMP; cAMP and adenosine ineffective) closely resembles the agonist specificity of the $P2Y_2$ (=P2U) type of receptor as characterized by Lustig et al. [36].

Table 2. Order of potency of nucleotides in potentiating the hypotonicity induced $^{125}I^-$ efflux in Intestine 407 cells

Increase in fractional $^{125}I^-$ efflux in response to a 20% hypotonic shock was determined in the absence or presence of equimolar concentrations (10 μM) of extracellular nucleotides. Data are expressed as percentage of the control. Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

Experimental condition	Mean ± S.E.M. (n)
Control	100 ± 6 (8)
ATP	194 ± 8* (8)
ATPγS	186 ± 7* (5)
UTP	185 ± 17* (3)
ADP	153 ± 8* (3)
GDP	140 ± 12 (3)
GTPγS	138 ± 9* (3)
AMP	134 ± 14 (3)
GTP	122 ± 2* (3)
Adenosine	107 ± 3 (3)
cAMP	91 ± 15 (3)

Effect of purinoceptor antagonists and the ATP hydrolase apyrase on osmosensitive Cl^- currents

Although extracellular ATP alone was not sufficient to activate an anion current in Intestine 407 cells, hypo-osmotically released ATP may play an important role in the feed-forward regulation of compensatory Cl^- channels. To verify this possibility, both the hypotonicity-provoked whole-cell Cl^- currents as well as the $^{125}I^-$ efflux in intact cells were measured in the absence and presence of apyrase (3 units/ml).

When exposed to a hypo-osmotic medium, Intestine 407 cells respond by developing a large, Cl^--selective, outwardly rectifying current (Figure 4A and B). The anion current displayed a slow activation after strong hyperpolarizing pulses and a marked voltage-dependent inactivation at depolarizing holding potentials. Addition of millimolar concentrations of extracellular ATP blocks the hypotonicity-induced anion efflux in a voltage-dependent manner (Figure 4C). Similar currents have been reported by others for this cell line and for many other cell systems (for review, see [16, 37]). Addition of apyrase to the bathing solution did not prevent the activation of the osmosensitive current (Figure 5A) nor did it affect the magnitude of the cell swelling-induced $^{125}I^-$ efflux from intact cells (Table 3). Similar results were obtained when a hexo-

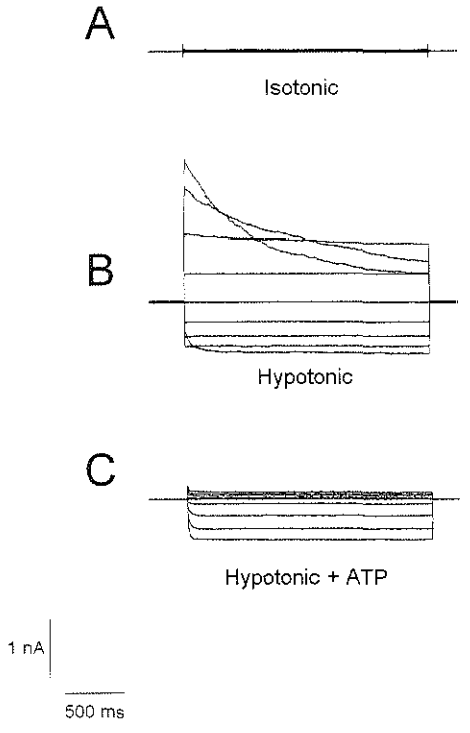

Figure 4. Volume-sensitive chloride currents in Intestine 407 cells
Whole cell Cl^- currents in control (isotonic; **A**) and hypo-osmotic stimulated (**B**) cells in response to step pulses from -100 mV to +100 mV (25 mV increments). **C**) Inhibition of the cell swelling induced Cl^- current by extracellular ATP (5 mM). Traces are representative for 20 (A/B) and 6 (C) experiments respectively.

kinase/glucose system was used to trap released ATP (results not shown). Furthermore, addition of micromolar concentrations of ATP to the bathing solution did not induce an increase in Cl^- current under isotonic conditions (Figure 5B). Taken together, these findings again indicate that, under our conditions, extracellularly released ATP triggered by osmotic cell swelling is not involved in the activation or potentiation of volume-sensitive Cl^- currents.

Paradoxically, after treatment of the cells with the purinoceptor antagonists suramin and reactive blue, a strong inhibition of the hypotonicity-induced anion efflux was observed (Table 3), suggesting a role for purinoceptors in the activation of the chloride efflux. Furthermore, addition of suramin (Figure 5A) or reactive blue (results not shown) to the bathing solution effectively decreased swelling-induced whole-cell Cl^- currents. Inhibition by both antagonists, like the inhibition by millimolar concentrations of extracellular ATP (Figure 5B), was voltage-dependent (outward currents were inhibited much more prominently than inward currents). Combined with the observation that addition of apyrase to the bathing solution had no effect on the development of hypotonicity-induced Cl^- currents (Figure 5A and Table 3), these results indicate that the inhibition of hypotonicity-induced Cl^- channels by suramin and reactive blue is unrelated to their action as purinoceptor blockers.

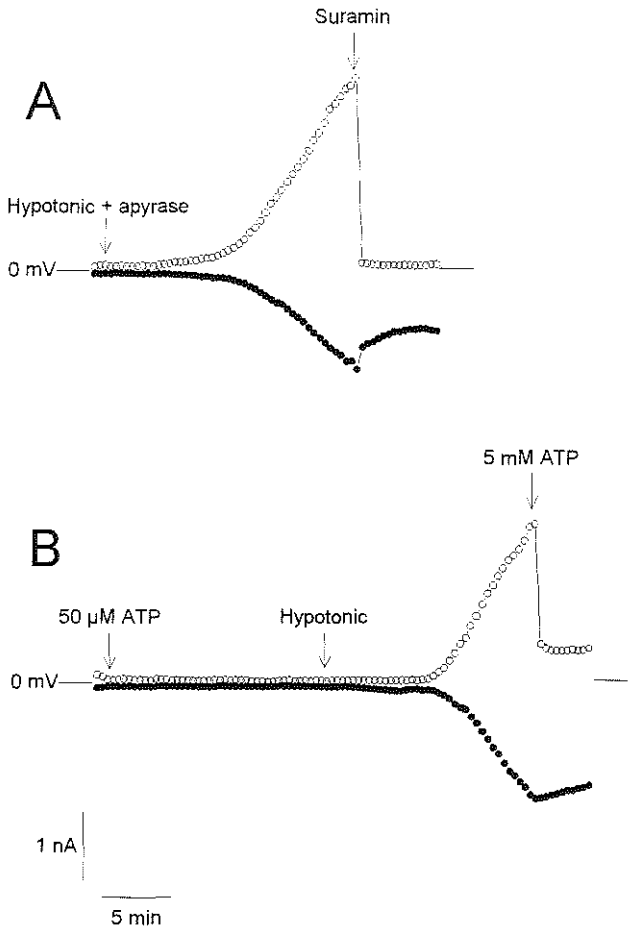

Figure 5. Time course of activation and inhibition of osmosensitive chloride currents

A) Hypotonicity-provoked whole cell Cl^- currents at –100 mV and +100 mV in the presence of apyrase (3 I.U./ml) and after subsequent addition of suramin (100 µM). **B**) Magnitude of the whole cell Cl^- currents after addition of ATP (50 µM), a shift to a hypotonic medium (30% hypotonicity) and subsequent addition of 5 mM ATP. Traces are representative for 6 experiments.

Table 3. Effect of ATP hydrolase apyrase and purinoceptor antagonists on hypotonicity-induced $^{125}I^-$ efflux

Fractional $^{125}I^-$ efflux was determined in response to a 20% and 30% hypotonic shock in the presence or absence (control) of 3U/ml apyrase, 100 µM suramin or 100 µM reactive blue. Data are expressed as percentage of control (mean ± S.E.M., n=3). Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

	Hypotonicity	
Experimental condition	20%	30%
Control	100 ± 5	100 ± 5
Apyrase	93 ± 8	108 ± 5
Suramin	38 ± 4*	55 ± 6*
Reactive blue	34 ± 4*	75 ± 2*

Autocrine release of ATP mediates swelling-induced Erk-1/2 activation

Recently, we demonstrated that hypo-osmotic stimulation of Intestine 407 cells transiently activates Erk-1/2 through the Ras/Raf signalling pathway [14]. Because ATP has been reported to activate MAP kinases in some other cell types [21-27], we investigated the possible autocrine/paracrine involvement of osmotically released ATP. It was found that extracellular ATP under isotonic conditions acts as a potent activator of Erk-1/2 in Intestine 407 cells (Figure 6). For comparison, the effect of a 30% hypotonic shock on Erk-1/2 activation is also shown in Figure 6. Stimulation of Erk-1/2 started at concentrations of 0.1-0.5 μM, indicating that the threshold for MAP kinase activation by ATP is two orders of magnitude lower than its threshold for the activation of Ca^{2+} efflux and for the potentiation of osmosensitive anion efflux (see Figure 2). As shown in Figure 7(A), treatment of the cells with apyrase (3 units/ml) not only abolished the ATP/UTP-induced Erk-1/2 activation, but also almost completely inhibited its activation by hypo-osmotic stress. Moreover, pretreatment of the cells with suramin (100 μM, 15 min) or reactive blue (100 μM, 15 min) significantly inhibited the hypotonicity-induced Erk-1/2 activation (Figure 7B). These results indicate that extracellular ATP, acting as an autocrine factor, is largely responsible for the hypotonicity-induced Erk-1/2 activation and, because UTP was equipotent, mediates its effects through $P2Y_2$ receptor signalling.

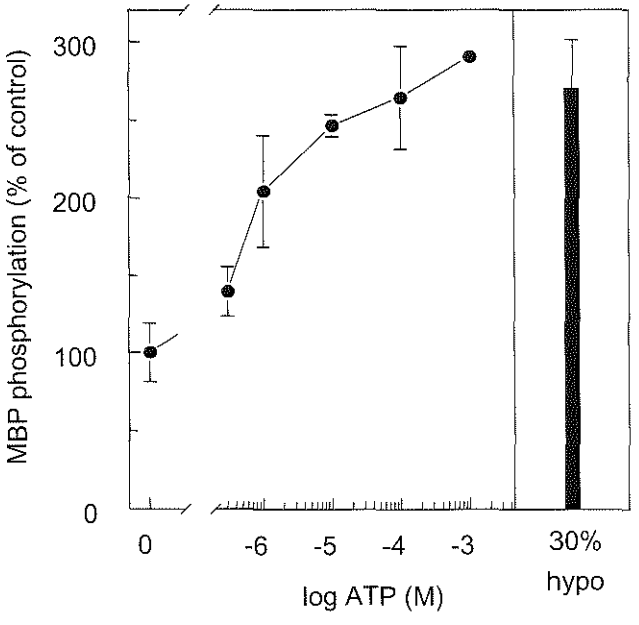

Figure 6. Dose-dependency of ATP-induced Erk-1/2 activation

Cells were exposed for 5 min to isotonic medium containing different concentrations of ATP or to a 30% hypotonic medium in the presence of 200 μM Na_3VO_4. After lysis of the cells, Erk-1/2 was immuno-precipitated and enzyme activity was determined using MBP as a substrate (as described in Experimental Procedures). Data are expressed as percentage of the control (mean ± S.E.M. for n=3).

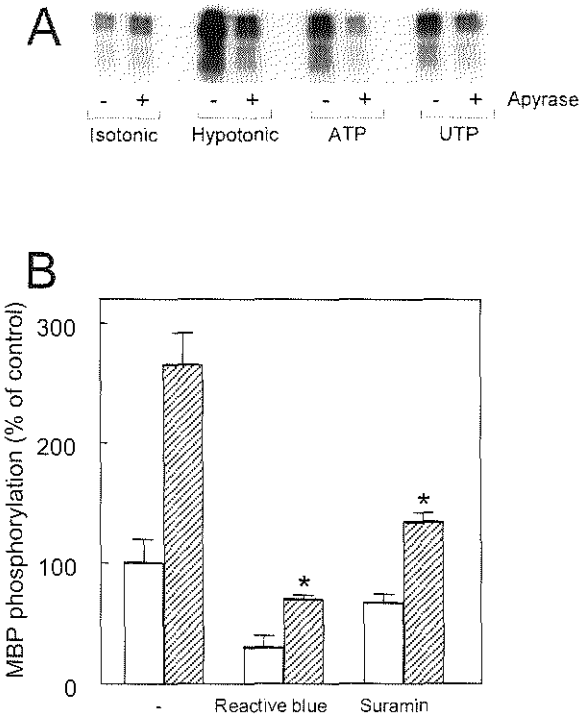

Figure 7. Extracellular released ATP activates Erk-1/2

Erk-1/2 was immuno-precipitated and enzyme activity was determined using MBP as a substrate. **A**) Following a 5 min incubation with vehicle (-) or with 3 U/ml apyrase (+), cells were exposed for another 5 min to either isotonic medium, 30% hypotonic medium, 100 μM ATP or 100 μM UTP in the presence of Na_3VO_4 (200 μM). Shown is an autoradiograph of ^{32}P-labelled MBP separated by SDS/PAGE; data are representative for 3 independent experiments. **B**) Following 15 min incubation with vehicle (-), with reactive blue (100 μM) or suramin (100 μM), cells were exposed for 5 min to isotonic (open bars) or 30% hypotonic medium (hatched bars). Data are expressed as percentage of the untreated control (mean ± S.E.M.; n=3). Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

DISCUSSION

Hypo-osmotic cell swelling induces a rapid increase in the K^+- and Cl^--selective conductances and the concomitant activation of multiple signalling pathways. In Intestine 407 cells, some components of these pathways, like $p21^{rho}$ and PtdIns 3-kinase, were found to be indispensable for a proper activation of the volume-sensitive Cl^- channels, whereas others, like the members of the MAP kinase superfamily, are apparently not involved [2, 7, 13, 14]. Recent studies in HTC hepatoma cells suggest that both the hypotonicity-induced Cl^- current activation and the subsequent cell-volume recovery completely depend on a cell swelling-induced conductive ATP efflux [18, 30]. Similar to HTC hepatoma cells, Intestine 407 cells respond to a hypo-osmotic shock with a release of ATP into the medium. The ATP release was stimulus-dependent and relatively sustained, lasting for at least 10-15 minutes. Notably, the regulatory volume decrease response under these conditions is completed in approx. 2-3 min [2]. Both the ATP-binding-cassette proteins CFTR (cystic fibrosis transmembrane-conductance regulator) and P-glycoprotein have been proposed to function as ATP-release channels or to regulate an, as-yet-

unidentified, associated ionic channel [17, 30, 38-40]. Other investigators, however, failed to establish a link between CFTR/P-glycoprotein and ATP release [19, 41-43]. In Intestine 407 cells, CFTR Cl^- channels are not expressed [2] and, in the subclone we use, P-glycoprotein expression is not detectable by RNAse protection assay (results not shown), arguing against a role for these ATP-binding-cassette transporters in ATP release. This notion is supported by the findings of Tominaga et al. [44], using a different, P-glycoprotein-expressing, subclone of Intestine 407 cells. They found that inhibition of P-glycoprotein expression or function by treatment with antisense oligonucleotides or verapamil did not affect the osmosensitive Cl^- current [44]. Hypotonicity-induced ATP release was inhibited by BAPTA-AM and cytochalasin B whereas cell swelling-induced anion efflux was strongly potentiated by cytochalasin B treatment and unaffected by Ca^{2+} chelation (see Table 1). These findings, together with the difference in kinetics between hypotonicity-induced ATP release and anion efflux, argue against conductive ATP efflux through volume-regulated Cl^- channels. In contrast, although rather speculative, the dependency of the ATP release on intracellular Ca^{2+} and an intact cytoskeleton suggests the involvement of an exocytotic process.

Based upon the inhibitory action of apyrase and of the purinoceptor antagonists suramin and reactive blue, Wang et al. [18] concluded that, in HTC rat hepatoma cells, extracellularly released ATP, through a P2-type receptor other that $P2Y_2$, was obligatory for the activation of volume-sensitive anion channels. The results of our study, however, demonstrate clearly that extracellularly released ATP is not involved in the activation of osmo-regulated Cl^- channels in Intestine 407 cells, as evidenced by the inability of applied ATP to evoke an anion conductance under isotonic conditions and by the insensitivity of the hypotonicity-provoked anion efflux to apyrase. Instead of a direct activation of Cl^- conductances, micromolar concentrations of ATP were found to greatly potentiate the ionic response to a submaximal hypotonic stimulus, most likely through $P2Y_2$-receptor activation (as evidenced by the order of potency of different nucleotides) and subsequent Ca^{2+} mobilization (as evidenced by $^{45}Ca^{2+}$-efflux studies and potentiation studies in BAPTA-AM loaded cultures).

Potentiation of the ionic responses to osmotic cell swelling is not unprecedented. Indeed, in Intestine 407 cells, Ca^{2+}-mobilizing hormones as well as the phosphotyrosine phosphatase inhibitor (peroxo)vanadate and cytochalasin B were found to increase the anion efflux [2, 7, 34]. These data confirm the previous notion that Intestine 407 cells do not express Ca^{2+}-activated anion channels [2, 34] implying that ATP/Ca^{2+} potentiation of anion efflux is a regulatory property of the volume-sensitive Cl^- channels.

Importantly, the inhibition of volume-sensitive Cl^- channels by millimolar concentrations of ATP appeared to be independent of $P2Y_2$-receptor signalling because at those concentrations the ATP activation of $^{45}Ca^{2+}$ efflux was unaffected. Not only high concentrations of extracellular ATP (>5 mM), but also the purinoceptor antagonists suramin and reactive blue, were found to inhibit both the volume-sensitive anion efflux and the hypotonicity-provoked

whole-cell Cl^- current significantly. These results are in line with the recent findings of Gallietta et al. [45], who reported an inhibition of swelling-induced taurine efflux and Cl^- currents by purinoceptor antagonists in $9HTE_0^-$ cells. The inhibition by ATP, suramin and reactive blue (i.e. by both purinoceptor agonist and antagonists) was voltage dependent and therefore suggests a direct interaction with the extracellular domains of the channel protein. This property renders these antagonists less suitable for investigating the involvement of purinoceptors in Cl^- channel activation, but makes it tempting to speculate that volume-regulated anion channels share homologous nucleotide-binding domains with the $P2Y_2$ receptors. Notably, the ATP concentrations reached in the close proximity of the cells *in vivo* are difficult to assess. As recently shown by Beigi et al. [46], who used cell surface-attached luciferase to measure ATP release from platelets, the ATP concentration measured by soluble luciferase in bulk extracellular fluids, as done in our studies, may greatly underestimate the concentration of ATP at the cell surface. Considering the micromolar rather than millimolar concentrations of released ATP found at the cell surface by Beigi et al. [46], the physiological role for cell swelling-induced ATP release is most plausibly potentiation of channel activity (requiring micromolar levels of extracellular ATP) rather than channel inhibition.

A general feature of cell signalling in response to hypotonic stress is the activation of the extracellular signal-regulated protein kinases Erk-1 and Erk-2 [2, 8-14]. In this study, we demonstrate that the activation of these MAP kinases is not a direct consequence of osmotic cell swelling, but instead involves an autocrine/paracrine loop with ATP as the first messenger. The ability of exogenously added ATP to potentiate the osmosensitive anion efflux is somewhat contradictory to the observations that released ATP is responsible for Erk-1/2 activation but is apparently not involved in the regulation of $^{125}I^-$ efflux or Cl^- current (e.g. no reduction in magnitude in the presence of apyrase; Table 3 and Figure 5A). We suggest that there are at least two plausible explanations for this apparent discrepancy. First, comparison of Figures 2A and 6 shows that the threshold for Erk-1/2 activation by ATP is about two orders of magnitude lower compared with ATP-potentiation of the anion efflux or activation of Ca^{2+} channels. Second, in contrast with the conditions of the Erk-1/2 experiments, in the efflux assay, the extracellular medium is replaced at 1 min intervals to assess the release of radioisotope, thereby reducing unstirred layer dimensions and preventing ATP to accumulate.

The finding that UTP is as effective as ATP suggests a second role for the $P2Y_2$ receptor, a G-protein coupled receptor, which was also found to be responsible for the potentiation of the swelling-induced anion efflux. In several cell systems, receptors coupled to the G-protein subfamilies $G_{q/11}$, $G_{i/0}$, G_s or $G_{12/13}$ were found to activate the Ras/Raf/Erk cascade (reviewed in [47, 48]). However, since the hypotonicity-provoked activation of Erk-1/2 was insensitive to pertussis toxin in Intestine 407 cells [14], involvement of $G_{i/0}$ is not likely. Notably, Erk-1/2 activation was found to be more sensitive to extracellular ATP than the Ca^{2+}-mediated potentiation of hypotonicity-induced anion efflux (submicromolar versus micromolar levels). The reason for

this difference is unknown, but may relate to the involvement of multiple $P2Y_2$-receptor subtypes, or to a different sensitivity of the signalling pathways to submaximal receptor activation.

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Increased exocytosis in response to osmotic cell swelling; a putative mechanism for the hypotonicity-provoked release of ATP

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In preparation

ABSTRACT

Osmotic swelling of Intestine 407 cells leads to a rapid release of ATP, through a mechanism involving a rise in intracellular free Ca^{2+} and depending on an intact cytoskeleton (Van der Wijk et al., Biochem. J. 343, 579-586, 1999). In this study, we further investigated a possible role of regulated exocytosis in this process. Using the fluorescent membrane probe FM 1-43, an immediate increase in cell surface membrane area was observed after hypo-osmotic stimulation. Treatment of the cells with N-ethylmaleimide, an inhibitor of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins, completely abolished this increase. In addition, after labelling the intracellular vesicle compartments with FM 1-43, hypo-osmotic stimulation led to a prominent reduction of intracellular fluorescence. Taken together, these results strongly suggest that osmotic cell swelling is accompanied by a stimulation of exocytosis. Like the cell swelling-induced ATP release, but in contrast to the activation of volume-regulated anion channels (Van der Wijk et al., Biochem. J. 343, 579-586, 1999), the hypotonicity-induced increase in membrane area was diminished after BAPTA-AM loading or cytochalasin B treatment. In addition, both the increase in plasma membrane area triggered by osmotic cell swelling as well as the release of ATP were markedly reduced after inhibition of phospholipase D-catalysed formation of phosphatidic acid by n-butanol. Our results suggest the involvement of exocytosis in the release of ATP triggered by osmotic cell swelling but argue against a role for exocytosis in the recruitment or activation of volume-sensitive anion channels. Unexpectedly, we observed that pretreatment of the cells with the ATP hydrolase apyrase abolished the osmotic

swelling-induced increase in membrane surface area, indicating that extracellular ATP is required for the cell swelling-induced exocytosis. These data suggest the existence of a positive feedback loop in the release of ATP from hypo-osmotic stimulated cells.

INTRODUCTION

In mammalian cells, hypotonic cell swelling leads to the activation of cell volume regulatory processes, which in general comprises a transient increase in K^+ and Cl^- conductances of the membrane (for reviews see: [1-4]. As a result, KCl and water exit the cell and cell 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 paracrine factor acting through plasma membrane purinoceptors [5-8]. Extracellular ATP has been shown to regulate the RVD response in a number of different cell types [5,6,9,10], either through the stimulation of a Ca^{2+}-dependent K^+ efflux [10], or by the activation of volume-sensitive Cl^--channels [5,9].

In Intestine 407 cells, extracellular ATP was not required for the direct activation of volume-sensitive Cl^--channels [8]. However, (sub-)micromolar concentrations of extracellular ATP were found to potentiate the hypotonicity-provoked anion efflux in a Ca^{2+}-dependent manner [8]. Because the volume-sensitive K^+-channels in Intestine 407, in contrast to osmo-regulated anion channels, are Ca^{2+} dependent [11], a putative role for ATP in the activation of K^+-channels is appealing. Unlike the activation of volume-sensitive anion channels, osmotically induced ATP release was found to be critically involved in the activation of extracellular-signal-regulated protein kinase (Erk)-1/2 activation in Intestine 407 cells [8]. Although the role of hypotonicity-stimulated 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 [12-22].

To date, a number of possible pathways have been proposed to explain the release of ATP from cells. These include; (1) leakage due to (local) membrane damage; (2) specific channel(s) or transporter(s) and (3) exocytotic events. Previously, members of the ABC-type of transporters were suggested to permeate ATP (for reviews see: [23,24]). Intestine 407 cells, however, lack CFTR expression and, in the subclone we use, P-glycoprotein expression was not detected [8,12], arguing against a role for these ABC transporters in the release of ATP from Intestine 407 cells. Because the cell swelling-induced ATP release differs from the activation of osmo-sensitive Cl^--channels in both the time-scale of activation/inactivation as well as in its sensitivity to inhibitors [7,8], 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 [8], suggesting the involvement of exocytosis.

In this study, changes in membrane surface area in response to osmotic cell swelling were monitored in Intestine 407 cells using the fluorescent membrane dye FM 1-43. The results strongly suggest that exocytosis is involved in the hypotonicity-induced ATP release. In addition, the results argue against an exo-/ endocytotic cycle involved in the activation of volume-sensitive anion channels.

MATERIALS AND METHODS

Materials

Luciferin/luciferase reagent was obtained from Promega Corporation (Medison, WI); FM 1-43, jasplakinolide and paclitaxel were from Molecular Probes (Eugene, OR). The Ca^{2+}-ionophore A23187 was obtained from Boehringer (Mannheim, Germany). 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.

Luciferin/luciferase assay

Cells were seeded at a concentration of 10,000/cm^2 and incubated for 4 h 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). ATP release was measured in real time immediately after adding isotonic or hypotonic buffer containing the luciferin/luciferase reagent using a Topcount.NXT luminometer (Packard, Meriden, CT). Values are expressed as accumulation of extracellular ATP in time and corrected for the ATP consumption by the luciferase reaction. Hypotonic buffers were prepared by adjusting the concentration of mannitol and osmolarity was assessed using a cryoscopic osmometer (Osmomat 030; Salm & Kipp B.V., Breukelen, The Netherlands).

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 (excitation wavelength = 479 nm; emission wavelength = 598 nm) in a fluorescence spectrophotometer (Hitachi F4500, Tiel, The Netherlands) 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. Washout experiments were performed with cells grown on petri dishes, preloaded for 2 hours with FM 1-43, and visualized during the experiment using a fluorescence microscope. Washout was studied by a continuous perfusing (2 ml/min) using medium without FM 1-43.

RESULTS

Osmotic cell swelling leads to an increase in membrane labelling

The effects of hypo-osmotic stimulation on the cell surface membrane area was studied using the styryl dye FM 1-43, a fluorescent probe that reversibly stains the lipid-liquid interface and does not penetrate the membrane [25]. Upon fusion of vesicles with the plasma membrane or by inducing the unfolding of FM 1-43 inaccessible plasma membrane, additional membrane area is exposed to the dye-containing bathing medium and fluorescence increases. As shown in Figure 1, a shift from isotonic to a 40% hypotonic medium induced an increase in fluorescence above basal values. Like the release of ATP, the hypotonicity-provoked increase in FM 1-43 fluorescence was inhibited by loading the cells with BAPTA-AM or by treatment with cytochalasin B (Figure 1), suggesting that, the membrane expansion 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, abolished the cell swelling-induced increase in

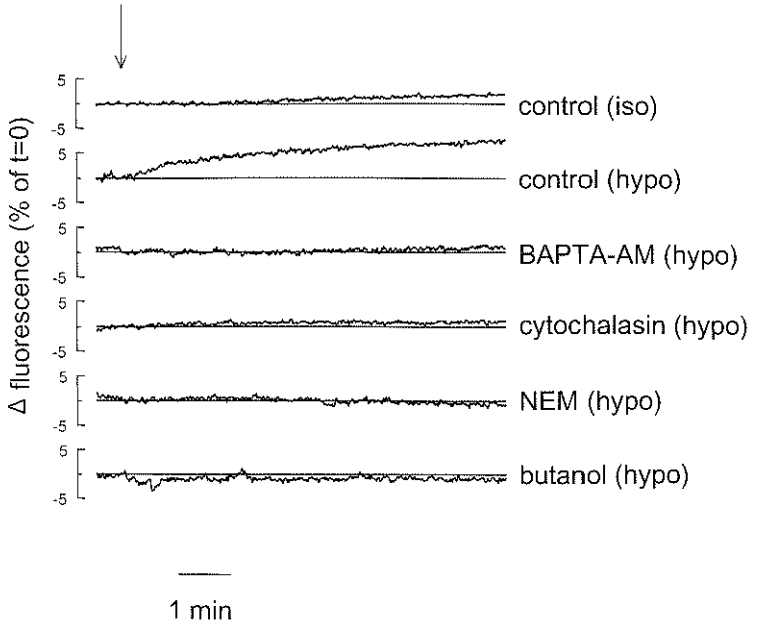

Figure 1. Time course of hypotonic shock-induced increase in FM 1-43 fluorescence and its inhibition by cytochalasin B, BATPA-AM, NEM and n-butanol

After preincubation of the cells in isotonic medium with or without cytochalasin B (50 μM, 30 min), BAPTA-AM (25 μM, 1h), NEM (1 mM, 15 min) or n-butanol (1%, 1h), as indicated in the figure, Intestine 407 cells grown on coverslips were placed in a cuvette with 1 μM FM 1-43 present in the bath solution throughout. After at least 5 min of preincubation, cells were exposed to isotonic or 40% hypotonic media with or without cytochalasin B, BAPTA-AM, NEM or n-butanol; the arrow indicates a shift in medium. Traces are representative of at least 3 experiments.

fluorescence, supporting our notion that exocytosis occurs upon osmotic cell swelling. Addition of n-butanol (1% for 1h) to the medium, leading to an inhibition of phospholipase D-catalysed formation of phosphatidic acid [26], also prevented the hypotonicity-induced increase in fluorescence (Figure 1).

Osmotic cell swelling is accompanied by exocytosis

To further investigate the notion that osmotic cell swelling triggers exocytosis, intracellular vesicular compartments were labelled by loading the cells with FM 1-43 for 15 minutes followed by a continuous perfusion with medium to remove plasma membrane-associated dye. As shown in Figure 2, changing the perfusion medium from isotonic to a 40% hypotonic one, leads to a rapid decline of approx. 10% of intracellular FM 1-43 fluorescence, indicative for fusion of intracellular vesicles with the plasma membrane. After 2-3 minutes a plateau value was reached. Notably, continuous perfusion of the cells with isotonic buffer did not diminish intracellular fluorescence.

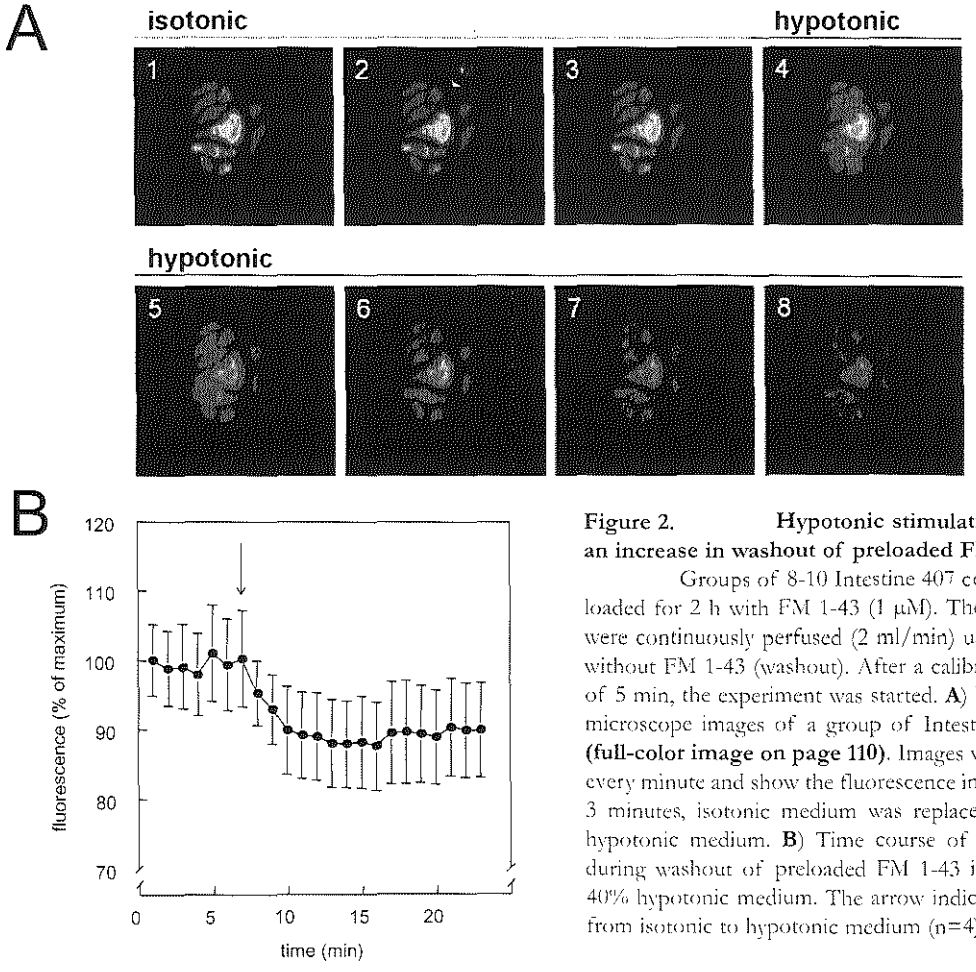

Figure 2. Hypotonic stimulation leads to an increase in washout of preloaded FM 1-43

Groups of 8-10 Intestine 407 cells were preloaded for 2 h with FM 1-43 (1 μM). Thereafter, cells were continuously perfused (2 ml/min) using medium without FM 1-43 (washout). After a calibration period of 5 min, the experiment was started. **A**) Fluorescence microscope images of a group of Intestine 407 cells **(full-color image on page 110)**. Images were sampled every minute and show the fluorescence intensity. After 3 minutes, isotonic medium was replaced by a 40% hypotonic medium. **B**) Time course of fluorescence during washout of preloaded FM 1-43 is isotonic or 40% hypotonic medium. The arrow indicates the shift from isotonic to hypotonic medium (n=4).

Osmotic cell swelling induced ATP release correlates with the activation of exocytosis

As described above, it is tempting to propose a model in which the release of ATP by osmotic cell swelling is mediated by exocytosis. There is not only a close correlation between the time course of the two processes, starting almost immediately after osmotic stimulation and lasting for 15 - 20 min, but in addition both processes were largely abolished after BAPTA-AM loading or after treatment of the cells with cytochalasin B (Figures 1 - 3 and [8]). To further investigate this interrelationship, cells were treated with NEM or n-butanol, inhibitors of cell swelling-induced exocytosis in these cells (cf. Figure 1). n-Butanol was also found to reduce the hypotonicity-provoked ATP release (76 ± 1 %; n=3), supporting the notion that exocytosis is likely to be involved. Pretreatment of the cells with NEM abolished the hypotonicity-induced ATP release as well, however, NEM pretreatment also strongly reduced the intracellular ATP concentration by approx. 90% as measured after cell lysis. Therefore no conclusion can be deduced from the inhibitory effect of NEM on hypotonicity-induced ATP release. As shown in Table 1, increasing the extracellular K^+ concentration dose-dependently inhibited the ATP release. Inhibition of K^+ release has already been reported to suppress Ca^{2+}-dependent exocytotic events [27]. Notably, an increase in $[Ca^{2+}]_i$ by treatment with the Ca^+-ionophore A23187 (5 µM) could promote the release of ATP in the absence of an osmotic shock by 227 ± 2 % (n=3).

Table 1. Dose-dependent inhibition of ATP release by high extracellular K^+

Intestine 407 monolayers were preincubated for 5 min with medium with different K^+ concentrations (K^+ concentration was enhanced by replacing Na^+). Thereafter, ATP release was determined after 15 min of incubation with isotonic or 40% hypotonic medium with K^+ concentrations as indicated. Data are expressed as mean ± S.E.M. (n=3). Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

	ATP accumulation (% of hypotonic control)	
$[K^+]_e$ (mM)	Isotonic	Hypotonic
5 (control)	15 ± 2	100 ± 8
50	15 ± 1	84 ± 5
75	11 ± 0	47 ± 7*
100	7 ± 2*	29 ± 8*

Table 2. Effect of actin- and microtubulin-cytoskeleton disturbing agents

ATP release from Intestine 407 monolayers, pretreated as indicated, was determined after 15 min of incubation with isotonic or 40% hypotonic medium. Data are expressed as mean ± S.E.M. (n=3).

	ATP accumulation (% of hypotonic control)	
Experimental condition (concentration, preincubation period)	Isotonic	Hypotonic
Control	17 ± 3	100 ± 6
Jasplakinolide(100 nM, 1h)	27 ± 2	116 ± 7
Nocodazol (10µg/ml, 2h)	21 ± 2	116 ± 2
Paclitaxel (50µM, 2h)	19 ± 5	98 ± 2

An intact actin-, but not tubulin-cytoskeleton, is a prerequisite for hypo-osmotic ATP release

As reported previously, disruption of the actin cytoskeleton by treating the cells with cytochalasin B prevented the cell swelling induced accumulation of extracellular ATP [8]. To further investigate the involvement of the cytoskeleton, cells were treated with jasplakinolide, a cell permeable agent that promotes actin polymerization and stabilization [28]. In addition, nocodazol and paclitaxel were used, leading to a disruption (nocodazol, [29]) or an increased stability (paclitaxel, [30]) of the microtubular system. As shown in Table 2, neither jasplakinolide nor nocodazol or paclitaxel treatment did affect the cell swelling induced ATP release dramatically, indicating that an intact actin cytoskeleton, but not actin remodelling or intact microtubules, is required.

Extracellular ATP is required for the hypotonicity-induced exocytosis

Pretreatment of the cells with the ATP hydrolase apyrase strongly inhibited the osmoshock-induced increase plasma membrane surface area as measured by the increase in FM 1-43 fluorescence (Figure 3). Importantly, pretreatment of the cells with apyrase did not reduce the intracellular ATP concentration as measured after cell lysis (results not shown). The effect of suramin or reactive blue (another purinoceptor inhibitor) on FM 1-43 fluorescence could not be tested because of their interference with fluorescent detection. These data indicate that extracellular ATP is required for the cell swelling-induced exocytosis and suggests the existence of a positive feedback loop.

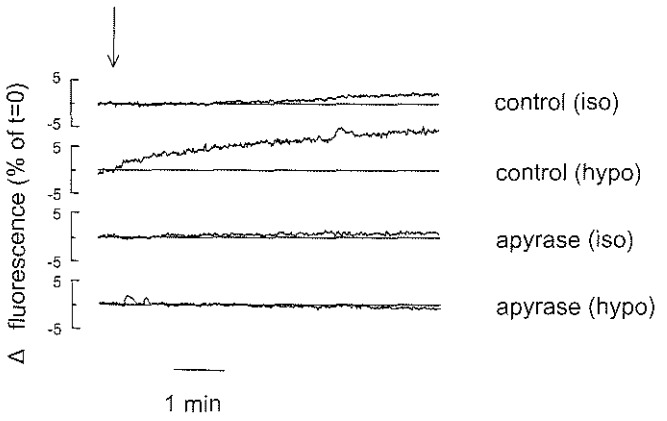

Figure 3. Apyrase inhibits the hypotonic shock-induced increase in FM 1-43 fluorescence

Intestine 407 cells grown on coverslips were placed in a cuvette with 1 μM FM 1-43 present in the bath solution throughout. After at least 5 min of preincubation, cells were exposed to isotonic or 40% hypotonic media with or without apyrase (3 units/ml); the arrow indicates a shift in medium. Traces are representative of at least 3 experiments.

DISCUSSION

In many different cell models, mechanical stress, such as osmotic cell swelling, leads to a regulated release of ATP into the surrounding fluid [5-8]. Several potential mechanisms have been proposed to be involved in ATP release, including conductive efflux through anion channels (such as CFTR), utilization of specific (ABC-type of) transporters as well as exocytosis [31,32]. In Intestine 407 cells, results from previous studies indicated that the involvement of anion channels is unlikely because 1) these cells do not express CFTR and 2) the regulation of osmo-sensitive anion channels and release of ATP is clearly distinct [7,8,12,33]. 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 [8]. Importantly, however, actin remodelling as well as the microtubular system is apparently not involved (Table 2). In Intestine 407 cells, treatment with n-butanol largely inhibited the cell swelling-induced ATP release. In the presence of n-butanol, hypotonicity-induced activation of phospholipase D (S.F.B. Tomassen et al., manuscript in preparation) leads to the formation of phosphatidyl-butanol instead of phosphatidic acid, preventing the synthesis of polyphosphorylated inositol lipids that are essential for membrane trafficking (reviewed by [26]). Taken together, the results suggest that exocytosis is involved in the osmotic cell swelling-induced release of ATP. This notion is supported by the observations that an increase in $[Ca^{2+}]_i$ alone is able to trigger release of ATP [34; Results section of this chapter].

Increase of extracellular K^+, which is known to block the release of K^+ and thereby the RVD response, dose-dependently blocked the hypotonic shock-induced ATP release in Intestine 407 cells. These findings are in line with the findings of Light et al. [10], who showed that K^+-channel blockers inhibit hypotonic shock-induced ATP release. Fujiwara et al., found that inhibition of KCl release, by increasing extracellular KCl under isosmotic conditions or by selectively inhibiting K^+- or Cl^--channels, suppressed the Ca^{2+}-dependent exocytotic events evoked by acetylcholine in guinea-pig antral mucous cells [27]. These results are in line with our suggestion that ATP is released by an exocytotic mechanism and may also explain the reported modulatory role of CFTR Cl^--channel activity in hypotonic shock-induced ATP release [33]. The mechanism by which KCl release is related to exocytosis is unknown, however, Fujiwara et al. [27] suggest that cell shrinkage induced by KCl release is the trigger for exocytosis. A more remote possibility is that depolarization of the cell membrane by increasing extracellular K^+ is responsible for the reduction in exocytosis. This explanation is not likely since depolarization is known to be in favour of exocytosis through the activation of voltage sensitive Ca^{2+}-channels [35]. The role of membrane polarization in hypotonicity-induced ATP release could be clarified by testing the effect of Cl^-- and K^+-channel inhibitors. However, most of these inhibitors (suramin, SITS, Gd^{3+} and quinidin) interfere with the luciferin/luciferase assay and can therefore not be used.

The observation of an activation of exocytosis by osmotic cell swelling in Intestine 407 cells is not unprecedented. Morphometric analysis of electron micrograph images of rat hepa-

tocytes revealed a marked enlargement of the membrane surface area within 5 min of hypotonic exposure [36]. In addition, Bruck et al. [37] observed an increase in the release of horseradish peroxidase (HRP) after hypotonic stimulation from an HRP loaded perfused liver. Similarly, reducing the osmolarity of the surrounding medium promoted the release of FITC-coupled dextrans (MW 72000) from preloaded inner medullary collecting duct kidney cells [38].

Because exocytosis, when exceeding the rate of endocytosis, leads to an increase in the plasma membrane surface area, membrane capacity measurements were performed by us and others to quantify exocytosis in response to hypotonic stimulation. In several different cell types, an increase in membrane capacity has been reported after hypotonic stimulation [39-42]. In our hands, attempts to monitor changes in membrane capacitance after hypo-osmotic stimulation of Intestine 407 cells were unreliable due to the large change in membrane conductance (ca. 1 nS in control versus 42 nS in osmotically stimulated cells [calculated from the results of Chapter 4]). The results obtained with the fluorescent membrane dye FM 1-43, however, clearly demonstrate that exocytosis is promoted by cell swelling, because 1) the area of plasma membrane with access to extracellular applied dye is rapidly increasing after hypo-osmotic stimulation, and 2) intracellular membranes labelled with FM 1-43 migrate to the plasma membrane upon osmotic cell swelling.

Like the hypotonicity-induced ATP release, the increase in total membrane area depended on an intact actin cytoskeleton, intracellular calcium levels and PLD activity. Furthermore, apyrase completely inhibited the hypotonic shock-induced FM 1-43 fluorescence, which suggests that extracellular ATP is required for the cell swelling-induced exocytosis. By this positive feedback loop, ATP seems to stimulate its own release. The mechanism of action of this feedback loop needs to be further investigated.

To conclude, unlike the release of ATP, the anion conductance triggered by osmotic cell swelling does not require an increased rate of exocytosis, arguing against a role for channel recruitment by vesicle fusion (cf. see Chapter 2).

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6

Osmotic cell swelling-induced endocytosis is regulated by ion channel activity and by extracellular ATP

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In preparation

ABSTRACT

Hypo-osmotic stimulation of Intestine 407 cells not only results in the activation of volume-regulated ion channels, but also triggers exocytosis and the subsequent release of ATP. Using exogeneously added TRITC-dextrans we investigated putative endocytotic processes triggered by cell swelling. Hypo-osmotic stimulation of Intestine 407 cells resulted in a robust (>100 fold) increase in the uptake of TRITC-dextran. Accumulation started after a distinct lag time of 2 - 3 minutes (i.e. after completion of the regulatory volume decrease) and lasted for approx. 10 - 15 minutes. Hypotonic stimulation was completely inhibited in the presence of 4-acetamido-4'-isothiocyanostilbene (SITS), suramin, genistein or millimolar concentrations of ATP, all substances known to block the activation of volume-sensitive Cl^--channels. In contrast, K^+-channel inhibitors like Gd^{3+} and quinidine, as well as high $[K^+]$ containing extracellular media potentiated both basal and osmotically promoted endocytosis. These results suggest that depolarization of the cell functions as a trigger to induce endocytosis. Unlike the cell swelling-induced exocytosis (Chapter 5), loading of the cells with BAPTA-AM or treatment with cytochalasin B did not affect the hypotonicity-induced endocytosis, indicating that both processes are not directly coupled. The osmotic swelling-induced uptake of TRITC-dextran was largely prevented in the presence of the ATP hydrolase apyrase. Exposure of the cells to micromolar concentrations of ATP under isotonic conditions however did not affect the TRITC-dextran uptake. In summary, the results indicate that cell swelling promotes an increase in the rate of endocytosis, independently of the hypotonicity-induced exocytosis, that is triggered by membrane depolarization and is dependent on extracellular ATP.

INTRODUCTION

In general, most mammalian cells respond to an increase in volume with the activation of separate K^+- and Cl^--selective ion channels. Through these channels, KCl moves out of the cell, thereby reducing the intracellular osmolarity. As a consequence, water will exit too and the original cell volume will be restored. In Intestine 407 cells, the volume-regulated K^+-channels are activated by a cell swelling-induced increase in $[Ca^{2+}]_i$ [1], while the opening of volume-sensitive Cl^--channels depends on the activation of tyrosine kinase(s) and the G-protein $p21^{rho}$ [2,3]. Recently, we observed that hypo-osmotic stimulation triggers exocytosis in Intestine 407 cells. Exocytosis was found to be the mechanism responsible for the release of endogenous ATP but was not involved in the activation of volume-regulated Cl^--channels (Chapter 5). In addition, the hypotonicity-induced release of ATP was found responsible for the activation of Erk-1/2 MAP kinases associated with osmotic cell swelling [4].

Because (increased) exo- and endocytosis often coincide [5,6], the hypotonicity-induced increase in exocytosis is expected to be accompanied by an increase in endocytosis. Indeed, for renal absorptive epithelial cells as well as for inner medullary collecting duct cells, an increase in endocytosis rate was found upon hypotonic stimulation [7,8]. Using membrane impermeable TRITC-dextrans as a marker for endocytosis, we investigated the occurrence of a similar increase in the rate of endocytosis in Intestine 407 cells, as well as its mode of regulation. We observed an increase in intracellular fluorescence after hypo-osmotic stimulation, starting after a lag of 2 - 3 min and lasting for at least 10 - 15 min. Furthermore, we found that hypotonicity-induced endocytosis is regulated independently of the osmotically-triggered exocytosis and requires membrane depolarization and extracellular ATP.

MATERIALS AND METHODS

Materials

Luciferin/luciferase reagent was obtained from Promega Corporation (Medison, WI). TRITC-dextran, FM 1-43 and jasplakinolide were from Molecular Probes (Eugene, OR). PD098059 and SB203580 were obtained from Calbiochem (La Jolla, CA, U.S.A.). 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 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.

TRITC-dextran uptake

Cells grown on coverslips (at 80 - 90% confluency) were incubated with 0.5 mg/ml TRITC-dextran (MW 10,000 Da) in isotonic (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) or hypotonic buffer (isotonic medium without mannitol). Thereafter, the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and fixed with a 2% formaldehyde solution for 20 min. Maximal images of 15 optical sections parallel to the substratum were constructed by confocal microscopy using a 63x oil immersion objective (Axiovert 135M; Zeiss, Oberkochen, Germany). The sections were each 1μm apart and the images consist of 512x512 pixels with a distance of 0.625 μm. Endocytosis was quantified using KS400 software (version 2.0; Contron, Oberkochen, Germany) by counting the number of fluorescent spots with a size between 3-75 pixels (representing endosomal vesicles), after subtraction of background fluorescence. Background fluorescence was set as measuring the mean pixel intensity of a circle drawn in the background increased by 5 times its standard deviation. Autofluorescence of the Intestine 407 cells was chosen as the background.

RESULTS

Hypotonic cell swelling induces endocytosis independently of hypotonicity- induced exocytosis

Hypotonic cell swelling dose-dependently induces an increase in the uptake of TRITC-dextran, a marker for endocytosis. Figure 1 shows confocal images of Intestine 407 monolayers incubated for 5 min with 0.5 mg/ml TRITC-dextran in media with differing osmolarity. A modest increase in intracellular trapping of TRITC-dextran was already observed after stimulation of the cells with mild hypotonic media (10 - 20%), whereas a massive uptake was found after a more severe challenge (40 - 50% reduction in tonicity). The time course of TRITC-dextran uptake of Intestine 407 cultures exposed to isotonic or a 40% hypotonic medium is shown in Figure 2. The cell swelling-induced increase in endocytosis shows a distinct lag-phase of approx. 2 - 3 min, i.e. starts only after completion of the RVD response [2], and levels off after approx. 10 - 15 min.

As shown previously, hypotonicity-induced exocytosis was almost completely inhibited by BAPTA-AM or cytochalasin B (see Chapter 5). To investigate whether endocytosis occurs as a direct result of an increase in exocytosis, we studied the effect of these treatments on hypotonicity-induced endocytosis. We found that the cell swelling-induced endocytosis was not sensitive to BAPTA-AM loading nor to cytochalasin B pretreatment (Table 1), suggesting that hypotonicity-induced endocytosis does not require an increase in $[Ca^{2+}]_i$ and is not sensitive to disruption of the actin cytoskeleton.

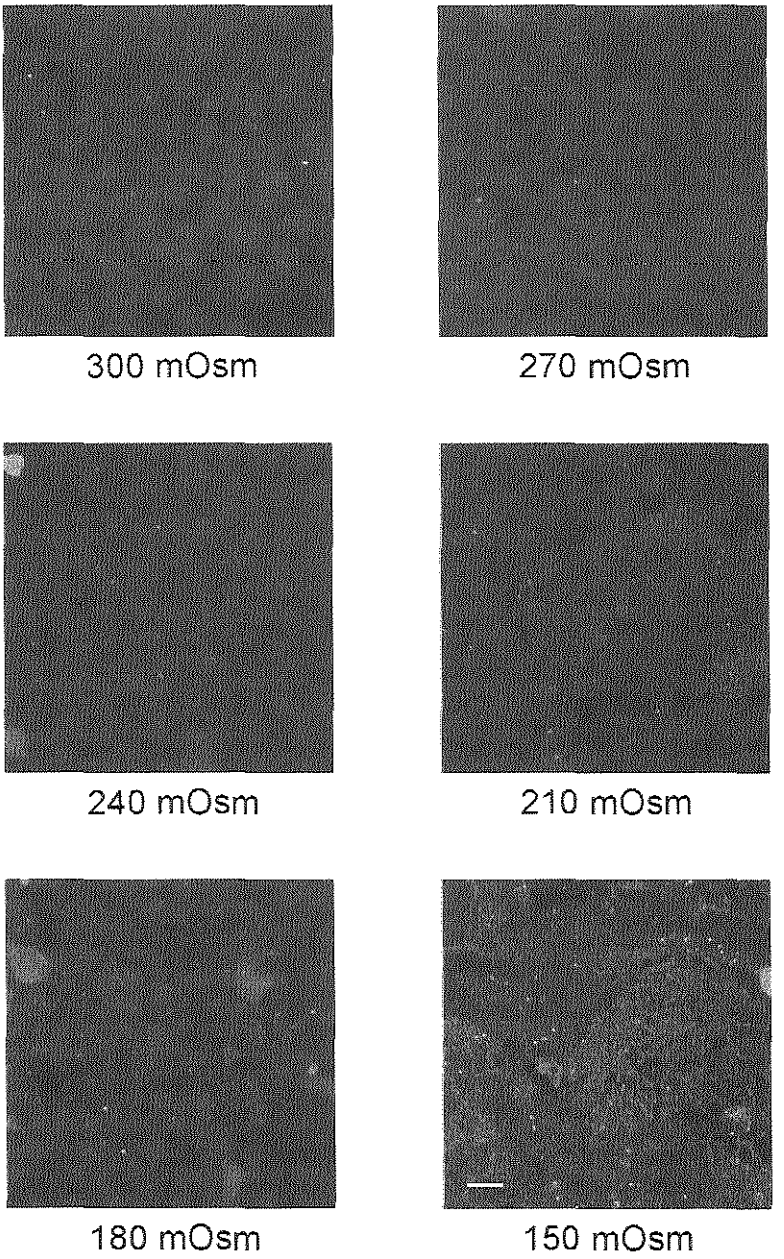

Figure 1. TRITC-dextran uptake induced by lowering medium osmolarity

Cells grown on coverslips were incubated for 5 min with TRITC-dextran containing medium (0.5 mg/ml) with different osmolarities as indicated in the figure. After fixation, images were constructed as indicated in Materials and Methods using CSLM. The images are representative of 10 images from 2 independent experiments (bar = 25 μm). **Full-color image on page 111.**

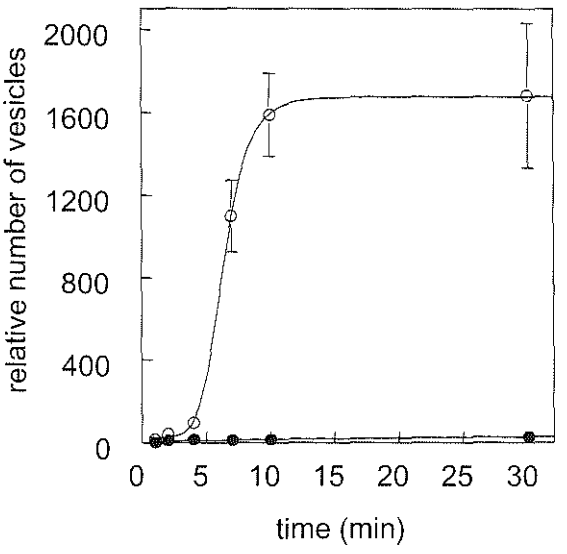

Figure 2. Time course of hypotonicity-induced TRITC-dextran uptake

Cells grown on coverslips were incubated for the times indicated with TRITC-dextran containing isotonic or 40% hypotonic medium (0.5 mg/ml). After fixation, images were constructed as indicated in Materials and Methods using CSLM. The number of vesicles is determined as described in Materials and Methods. Data are given as mean ± S.E.M. (n=5).

Table 1. Role of $[Ca^{2+}]_i$ and actin cytoskeleton in hyposhock-induced TRITC-dextran endocytosis

TRITC-dextran endocytosis was quantified in Intestine 407 cells from confocal images of cells exposed for 10 min to isotonic or 40% hypotonic medium containing 0.5 mg/ml TRITC-dextran with or without indicated agents. Data are expressed as mean ± S.E.M. (n). Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

	TRITC-dextran endocytosis (% of hypotonic control)	
Experimental condition (concentration, preincubation period)	Isotonic	Hypotonic
Control	6 ± 2 (10)	100 ± 6 (10)
BAPTA-AM (25μM, 1h)	3 ± 1 (7)	86 ± 19 (7)
A23187 (5μM)	2 ± 1 (5)	91 ± 26 (5)
Cytochalasin B (50μM, 30 min)	12 ± 3 (5)	109 ± 20 (5)
Jasplakinolide (100 nM, 1h)	6 ± 2 (5)	32 ± 7* (5)

In addition, in Intestine 407 cells, endocytosis could not be provoked by a major increase in $[Ca^{2+}]_i$ under isotonic conditions, because pretreatment of the cells with the calcium ionophore A23187 did not provoke TRITC-dextran uptake (Table 1). Unlike cytochalasin B, jasplakinolide, which induces actin stabilization and polymerization *in vitro* [9], partly inhibited osmosensitive endocytosis (Table 1). These results suggest that although endocytosis is not sensitive to disruption of the actin cytoskeleton, fixation of the cytoskeleton affects hypotonicity-induced endocytosis. Taken together, these results suggest the lack of a direct relationship between hypotonicity-induced endocytosis and exocytosis.

Depolarization induced by volume-sensitive Cl^--channel activity triggers hypotonicity-induced endocytosis

As summarized in Figure 3, hypotonicity-stimulated TRITC-dextran uptake is strongly inhibited by the volume-sensitive Cl^--channel inhibitors SITS and suramin as well as by genistein and millimolar concentrations of extracellular ATP that block the volume-sensitive activation of the ion conductance. In contrast, inhibition of K^+-channels by quinidine or Gd^{3+} potentiated both the basal and osmotic shock-induced endocytosis. As shown previously by Hazama and Okada [1], hypo-osmotic stimulation of Intestine 407 cells leads to a fast and transient hyperpolarization of the cells followed by a slower, more prolonged depolarization, due to the subsequent activation of K^+ and Cl^- channels. Because inhibition of Cl^--channels is likely to prevent membrane depolarization after hypo-osmotic stimulation, whereas K^+ channel blockers promote depolarization, it is attractive to propose a model in which the membrane potential regulates endocytosis. This notion is supported by our observation that in the presence of high extracellular $[K^+]$ (50 mM) hypotonicity-induced endocytosis in Intestine 407 cells is increased by approx. 2-fold (Figure 3).

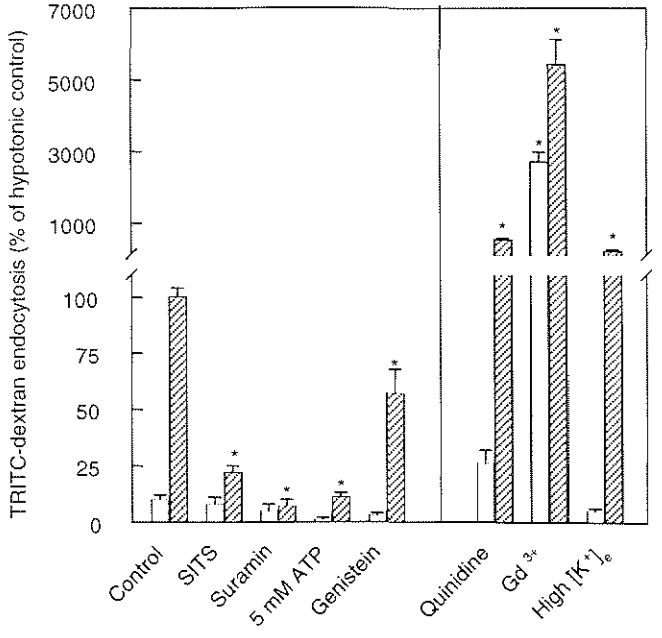

Figure 3. Effect of Cl^-- and K^+-channel blockers on hypotonicity-induced endocytosis

After preincubation of Intestine 407 cells in isotonic medium with or without SITS (100 μM), suramin (100 μM), ATP (5 mM), quinidine (500 μM), gadolinium (100 μM) or high $[K^+]$ (50 mM), cells were exposed for 10 min to isotonic (open bars) or 40% hypotonic (hatched bars) medium containing 0.5 mg/ml TRITC-dextran with or without indicated agents. After fixation, TRITC-dextran endocytosis was quantified from confocal images. Data are expressed as mean ± S.E.M. (n=5). Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

Extracellular apyrase inhibits the osmotic shock-induced endocytosis

In the presence of the ATP-hydrolase apyrase, the hypo-osmotic shock-induced increase in the uptake of TRITC-dextrans was almost completely abolished (Table 2), suggesting an essential role for extracellular ATP in cell swelling-induced endocytosis. However, addition of micromolar concentrations of ATP alone, giving rise to a limited increase in $[Ca^{2+}]_i$ [4], was not able to stimulate the endocytotic uptake of TRITC-dextrans, nor did it enhance the response to hypo-osmotic stimulation (Table 2). Taken together, the results suggest that a basal level of extracellular ATP is required but not sufficient for hypo-osmotic shock-induced endocytosis.

Table 2. Role of extracellular ATP and MAP kinases in hyposhock-induced TRITC-dextran endocytosis

TRITC-dextran endocytosis was quantified in Intestine 407 cells from confocal images of cells exposed for 10 min to isotonic or 40% hypotonic medium containing 0.5 mg/ml TRITC-dextran with or without indicated agents. Data are expressed as mean ± S.E.M. (n). Asterisk indicates a significant difference from the control ($p<0.05$; Student t-test).

	TRITC-dextran endocytosis (% of hypotonic control)	
Experimental condition (concentration, preincubation period)	Isotonic	Hypotonic
Control	5 ± 1 (6)	100 ± 3 (13)
Apyrase (3 U/ml, 2 min)	1 ± 1 (5)	11 ± 3* (5)
ATP (10 μM, 5 min)	7 ± 2 (5)	135 ± 20 (5)
PD098059 (50μM, 2h)	4 ± 1 (5)	45 ± 5* (10)
SB203580 (50 μM, 2h)	2 ± 1 (5)	45 ± 4* (9)

A regulatory role for MAP kinases in endocytosis

Release of endogenous ATP was shown to stimulate the MAP kinase Erk-1/2 through $P2Y_2$ purinoceptor activation [4]. Inhibition of Erk-1/2 with the MEK inhibitor PD098059 partially inhibited the hyposhock-induced endocytosis (Table 2), which may partly explain its dependence on extracellular ATP. In addition, inhibition of the MAP kinase p38 with the specific inhibitor SB203580 also diminished cell swelling-induced endocytosis (Table 2).

DISCUSSION

To evaluate whether osmotic cell swelling leads to an increased rate of endocytosis, we studied the uptake of TRITC-labelled dextran (MW 10,000) in response to hypotonic stimulation. A dose-dependent increase in dextran uptake was found in response to hypotonic stimulation with a characteristic lag time of approx. 2-3 min, i.e. starting only after completion of the

RVD response, and reaching its maximum after approx. 5 min. Thereafter, the rate of endocytosis returned to basal levels within another 5 to 10 min. Using bovine adrenal chromatin cells, Smith and Beets [10] found a similar time lag between the onset of exocytosis and endocytosis, after evoking massive and prolonged exocytosis by including 50 μM Ca^{2+} in the patch-pipette, and they suggested that endocytosis is triggered by exocytosis [10]. Furthermore, a negative correlation was observed between the increase in membrane tension and endocytosis, i.e. membrane expansion was found to increase endocytosis [11]. Taken together, this suggests that the delayed onset of endocytosis in response to cell swelling may be triggered by the preceding increase in exocytosis.

In case of hypo-osmotic cell swelling, it could be argued that the immediate increase of exocytosis is a necessary or essential mechanism to increase membrane surface, allowing cell swelling and preventing a deleterious increase in membrane tension. Exocytosis in combination with the RVD response will result in a low membrane tension, which may subsequently serve as a trigger for endocytosis. However, the results presented clearly demonstrate that exo- and endocytosis are regulated independently in hypo-osmotically stimulated Intestine 407 cells. Both cytochalasin B treatment and BAPTA-AM loading, which strongly inhibit cell swelling-induced exocytosis (Chapter 5), have no effect on the hypotonicity-induced endocytosis. In addition, exocytosis, but not endocytosis, can be triggered by raising $[Ca^{2+}]_i$ in the absence of osmotic stimulation. Although we cannot exclude that a reduction in membrane tension plays a role in activating endocytosis, the contribution of membrane insertion through exocytosis can only play a minor role.

In contrast, our results suggest that the cell swelling-induced endocytosis is triggered by cell membrane depolarization induced by the activation of volume-sensitive Cl^--channels, because inhibition of these channels by the Cl^- channel blocking agents SITS, suramin, millimolar concentrations of ATP and genistein, all inhibitors of VRAC activation [2,4], prevented the hypotonicity-induced endocytosis. Furthermore, enforced plasma membrane depolarization, using K^+-channel blockers (quinidin or Gd^{3+}), triggers endocytosis in the absence of osmotic stimulation and greatly potentiates the cell swelling-induced endocytosis. In addition, high extracellular $[K^+]$ increased the hypotonicity-induced endocytosis. This suggests that membrane depolarization rather than reduced membrane tension is the primary trigger to activate endocytosis. In line with our results, a role for depolarization in endocytosis has been reported for adrenal chromaffin cells [12].

Recently, Lazarowski et al. [13] reported a constitutive release of ATP and other nucleotides in several cell models, leading to a "purinergic tone" with potential physiological significance. Extracellular ATP, through purinoceptor activation, has been reported to be involved in membrane trafficking in a variety of different cell types. However, the mode of action as well as the signalling pathways used may differ significantly among cell types. In some cells, extracellular ATP promotes vesicle trafficking leading to net exocytosis and an increase in cell capacitance [14-19]. In contrast, in other cell types, extracellular ATP was found to suppress exocytosis

[20-23]. Both the stimulatory and the inhibitory effects of ATP have been ascribed to a modulation of $[Ca^{2+}]_i$; ATP has been reported to increase as well as to decrease $[Ca^{2+}]_i$ in selected cell models [14,20,21]. In addition, G-protein dependent, but Ca^{2+}-independent, mechanisms of ATP regulation of exocytosis, both potentiation and inhibition have been reported [14,22,23].

In Intestine 407 cells, apyrase was found to inhibit the hypo-osmotic shock-induced change in FM 1-43 fluorescence (Chapter 5) as well as in TRITC-dextran uptake almost completely. The effect of the purinoceptor inhibitor suramin on TRITC-dextran uptake is in line with this finding, however, no distinction can be made between the effect of suramin on Cl^--channel activity and purinoceptor inhibition. Addition of micromolar concentrations of ATP to the medium did not affect basal or hypotonicity-induced endocytosis. This suggests that a minimal level of extracellular ATP is required, but not sufficient, to promote endocytosis. Although ATP is able to raise $[Ca^{2+}]_i$ in Intestine 407 cells [4], the effect(s) of ATP on endocytosis is likely to be Ca^{2+} independent because treatment of the cells with A23187, leading to a massive and prolonged increase in $[Ca^{2+}]_i$, did not affect endocytosis.

Hypotonic stimulation leads to a rapid activation of the Erk-1/2 MAP kinases in all cell types studied to date (see Chapter 2). In Intestine 407 cells, Erk-1/2 activation is mediated through a $p21^{Ras}$/Raf pathway and involves ATP as an autocrine or paracrine factor [4,24]. Because treatment of the cells with the specific MEK inhibitor PD098059, leading to an inhibition of Erk-1/2, as well as with SB203580, an inhibitor of the p38 MAP kinase, partially inhibited both the uptake of TRITC-coupled dextran, both MAP kinases are likely to be involved, either directly or indirectly, in the activation of endocytosis is suggestive. Indeed, Nakashima et al. [25] recently reported that binding of EGF and insulin to their respective receptors lead to the endocytosis of the receptor-ligand complexes through a mechanism involving $p21^{Ras}$. A role for MAP kinases in regulation of membrane traffic is not unprecedented; Erk-1/2 has been reported to be involved in regulated insulin-receptor trafficking subsequent to the internalization process [26]. Furthermore, downregulation of Erk-1/2 activity resulted in inhibition of human polymorphonuclear leukocyte phagocytosis [27]. Other putative $p21^{Ras}$ effectors however, like the small GTP-binding protein Rab or Ral, might also be involved [25,28].

In conclusion, hypotonic stimulation results in an increase in the rate of endocytosis, triggered by depolarization due to the activation of volume-regulated Cl^--channel but independent of the swelling-induced increase in exocytosis. In addition, the presence of extracellular ATP is a prerequisite for endocytosis, possibly through its ability to promote the activation of MAP kinases.

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7

Visualizing membrane and cytokeletal dynamics in response to cell swelling

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In preparation

ABSTRACT

Osmotic swelling of Intestine 407 cells not only results in a rapid activation of volume-sensitive ion channels, but also triggers a robust increase in membrane traffic. Because of its limited resolution, dynamic processes at the plasma membrane of living cells are difficult to observe using conventional fluorescent microscopy techniques. In this report, we evaluated the perspectives of Atomic Force Microscopy (AFM), a novel technique which allows visualization of surface topology at ultrahigh resolution, to study the dynamics of membrane surface remodelling in living Intestine 407 cells. We found that contact mode AFM using soft cantilevers (spring constants of 0.01 - 0.02 N/m) is preferable to tapping mode AFM, performed with more stiff cantilevers (spring constant of 0.5 N/m), because the force applied to the cell in tapping mode AFM destroyed cellular surface structures. Using contact mode AFM with soft cantilevers, structures were visualized that closely resemble confocal images of the apical actin cytoskeleton obtained by staining F-actin with TRITC-conjugated phalloidin.

INTRODUCTION

Mammalian cells respond to osmotic cell swelling by activating volume regulatory mechanisms. In addition to activation of ion channels, leading to a decrease in intracellular osmotic pressure, Intestine 407 cells also respond to hypo-osmotic stress by a rapid alteration of

the organization of cellular F-actin and by an increase in membrane traffic (Chapter 5 and 6). In general, the cell membrane and the associated cytoskeleton are thought to be remodelled co-ordinately in response to mechanical stresses, such as osmotic cell swelling (for review see: [1]). Fluorescence and electron microscopy are routinely used to study the cell membrane and the associated cytoskeleton. These techniques, however, require chemical fixation or quickly frozen tissues and are therefore not very suitable for studying dynamic processes in living cells.

Atomic force microscopy (AFM) has been applied successfully as a tool to study native organic material and to obtain three-dimensional images at nanometer resolution. Several groups have now started to use the AFM to study surface topology of intact mammalian cells. During AFM scanning, a sharp tip attached to a soft cantilever moves over the surface of the cell and the deflection of the cantilever is recorded continuously. The deflection is determined from the reflection of a laser beam focused on the back of the reflecting cantilever.

Although AFM on living cells is still in an early developmental stage, several attempts have been made using AFM for studying changes in cell membrane topography. Images of living cells have been acquired using both contact mode AFM [2,3] and tapping mode AFM [4-7]. Using contact mode AFM, the probe is in continuous contact with the surface, whereas in tapping mode AFM, the cantilever oscillates at a high frequency and only makes intermittent cell surface contacts. These topographic studies have given information about dynamical membrane processes such as exocytosis [8].

In addition to surface topology, AFM has been shown to be very useful for analysing the elastic properties of the cell surface [9] and for quantifying the volume of cells adhered to a substrate [10]. The elastic properties of a cell surface can be studied by analysing force-distance curves, created by quantifying the indentation of the cell membrane in response to different forces applied [9]. Because of the large contribution of the cortical cytoskeleton to the stiffness of the apical membrane, information about the elastic properties of the membrane may reflect the cytoskeletal structure inside the cell [11-13]. Using the AFM, very precise cell volume estimations have been obtained by processing multiple images into a 3D structure of the cell [10]. Taken together, AFM is a novel, promising and attractive tool to investigate cell surface topology and dynamics as well as alterations in the elastic properties of the membrane and its associated cytoskeleton in intact cells.

MATERIALS AND METHODS

Cell culture

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

F-actin staining and visualization

Cells grown on coverslips were fixed for 20 min with 3% formaldehyde after 5 min of stimulation with isotonic or hypotonic media. After permeabilization with 0.2% Triton X-100 in PBS, cell were stained with TRITC-conjugated phalloidin (250 mg/ml in PBS; Sigma, St. Louis, MO, U.S.A). Confocal images were made with a x63 oil immersion objective.

Atomic force microscopy

A stand-alone AFM system integrated with an optical microscope was used as described in detail previously [14]. Intestine 407 cells were cultured in Petri dishes for several days until large islands of cells (>100 cells) were formed. Prior to the experiments, cells were washed several times with PBS and incubated in Hepes buffered medium (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). All experiments were performed at room temperature. Two modes of AFM were used in this study; the tapping mode and the contact mode. In contact mode AFM (DC mode), the feedback of the piezo tube keeps the force (and thereby the height) between the tip and the sample constant, with the deflection as a set point for the loop. Tapping mode AFM (AC mode) is based on amplitude detection of the oscillating tip. At the bottom of the cantilever swing, the tip touches the surface and the feedback loop keeps the amplitude decrease constant. Three types of Si_3N_4 cantilever tips were used which differ in stiffness: k_F=0.5 N/m, k_B=0.02 N/m or k_C=0.01 N/m. The cantilever was attached to a piezo tube which has three degrees of freedom (Figure 1). Scanning in the x,y direction was limited to an area of 20 μm x 20 μm, while the maximum z travel was 5 μm. The tip of the pyramidal formed cantilever had a typical radius of 20 nm and a height of 3 μm.

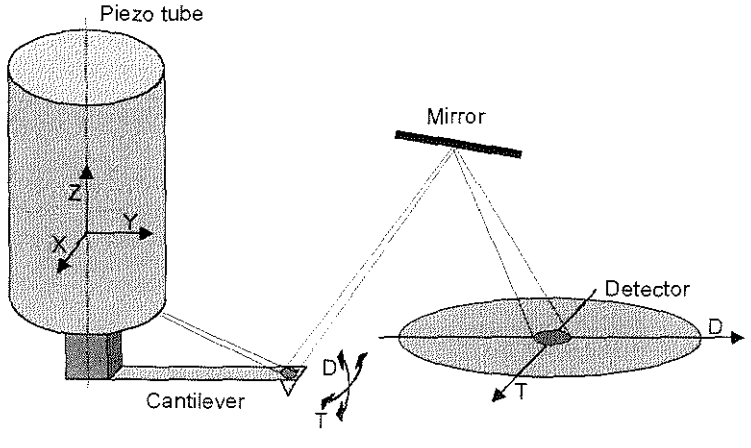

Figure 1. Atomic force microscope

Schematic representation of an AFM set-up. Indicated are the three degrees of freedom of the piezo tube and the two degrees of freedom of the cantilever. The x,y directions represent the scanning movement and the z direction is related to the topographical height. The deflection (D) of the cantilever gives the normal force transduction (adhesion, electrostatic-, magnetic-, van der Waals- and repulsion-forces) and the torsion (T) gives the lateral force transduction (friction force).

RESULTS

Cytoskeleton changes induced by hypotonic stimulation

As shown previously, hypotonic stimulation leads to a transient increase in total cellular F-actin, which returns to basal levels within 5 min of stimulation [15]. In addition, we found that the cellular distribution of F-actin was rapidly altered. A transient increase in stress fibre formation was observed at the basal part of the cell, whereas the amount of apical F-actin associated with the plasma membrane ruffles, was dramatically reduced [15]. Figure 2 shows confocal images of the apical, perinuclear and basal part of the cell after 60 seconds of hypo-osmotic stimulation (30% hypotonic solution); at that time-point the increase in total F-actin was maximal [15]. In addition to a clear increase in stress fibre formation and a reduction in apical F-actin (Figure 2A/B and 2E/F), a dramatic increase of F-actin was found at the perinuclear side to the cell (Figure 2C/D).

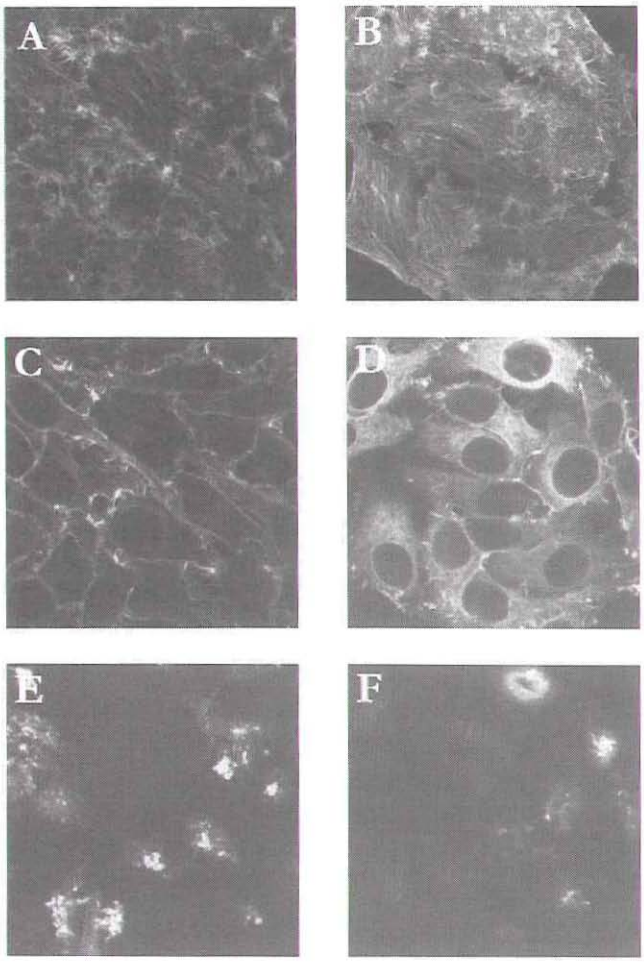

Figure 2. Hypotonic-shock induced F-actin cytoskeleton remodelling

Monolayers of Intestine 407 cells, grown on coverslips, were stimulated with isotonic (A, C and E) or 40% hypotonic medium (B, D and F) for 1 min. Thereafter, the cells were fixed and stained with TRITC-conjugated phalloidin as described under "Materials & Methods". Confocal images were constructed from the basal (A and B), the perinuclear (C and D) and the apical side parts of the cell (E and F). The images presented are representative out of 10 obtained from 2 independent experiments.

Using the AFM for studying membrane elasticity

Force-versus-distance curves give information about the contact point or region and about the sample viscoelasticity. In the case of hard surfaces, the contact is well defined and there is a visible transition between the attractive and the repulsive regime. For soft surfaces, the contact point is more difficult to define, since the surface will be deformed easily by the force of the tip (Nastasa and Van der Werf, unpublished results). The cantilever behaves as a second order harmonic oscillator, which can be regarded as a spring in case of low frequencies. By representing the elastic behaviour of a cantilever-sample system as two springs in a serie (Figure 3), the linear dependence of the cantilever deflection (Δd) to the tip-sample separation (Δz) is related directly to the stiffness of the sample by the following equation:

$$\frac{\Delta d}{\Delta z} = \frac{1}{\frac{k_{sample}}{k_{cantilever}} + 1}$$

where k represents the spring constant/stiffness of the sample and cantilever, respectively. Therefore, at a known value of the spring constant and the deflections of the cantilever, the stiffness of the cell (k_{sample}) can be determined. Experimental force curves were obtained by applying a triangular signal and by ramping the piezo tube up and down with constant speed at frequencies between 0.6 - 1.2 Hz. Figure 4 shows two force curves acquired by tapping on a soft surface (cell) or a hard surface (petri dish). In this particular example, the stiffness of the cell was 12.6 mN/m. For Intestine 407 cells, the membrane stiffness is about 8 - 14 mN/m. Typical cell membrane stiffnesses mentioned in literature are in the order of 10 mN/m) [16,17].

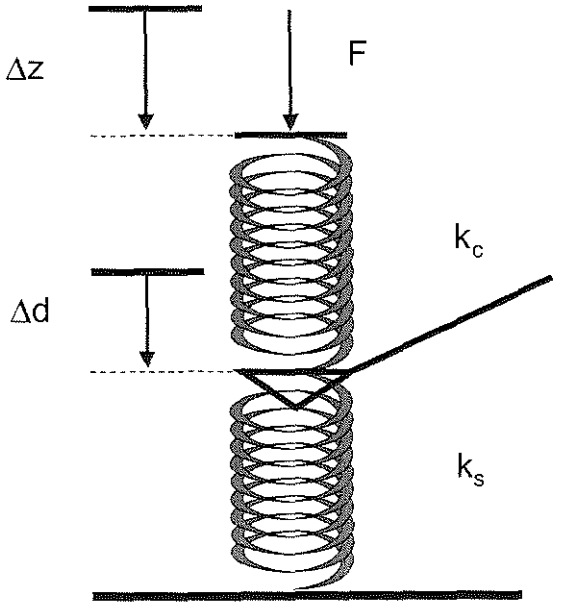

Figure 3. Spring-model of the tip-sample interaction

Model of the tip-sample interaction with the elastic behaviour approach. $F=k\Delta z$ is the applied force and Δd the total deflection measured.

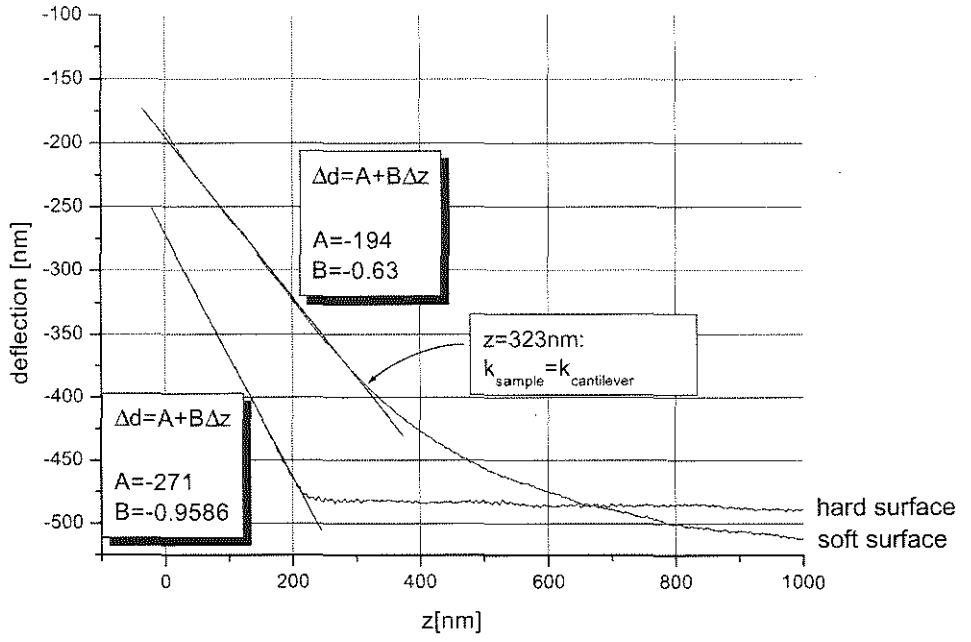

Figure 4. Force curves for hard (Petri dish) and soft (cell) surface

Stiffness can be determined from the slope of the equation: $y=A+Bx$; the slope is $B=\Delta d/\Delta z$. Stiffness is given by $S=k|\Delta d/\Delta z|=F/\Delta z$. Since $|S_{hard}|>|S_{soft}|$, the force curve will be steeper for hard surfaces. In this case $S_{cell}=20$ mN/m*0.63=12.6 mN/m. At z=323 nm, k_{cell} equals $k_{cantilever}$ (0.02 N/m). The cell stiffness presents a non-linear behaviour with indentation

Membrane topology of Intestine 407 cells as determined using the tapping or contact mode AFM

For tapping mode investigations, an F-type cantilever (k_F=0.5 N/m) was used. In liquid, this cantilever has a resonance frequency of 28 - 35 kHz. Figure 5 shows the topography (Figure 5A) and error signal (Figure 5B) of the surface of Intestine 407 cells, scanned with high indentations and small amplitudes (A_{free}=218.4 nm, $A_{setpoint}$=118.3 nm, z=50 nm and the applied force is F=25 nN). Fine structures are visible, but, due to the high-energy transfer during scanning, the cell surface changes dramatically during the scanning procedure and repeated scanning will eventually lead to cell death.

In contact mode, soft type cantilevers (type B and C; k_B=0.02 N/m and k_C=0.01 N/m) were used. A major disadvantage, most notably when larger scan forces are used, is a significant friction force. A high friction force may lead to a distorted image especially when soft objects like intact cells are studied. Therefore, only very weak scanning forces can be used. This is accomplished by finding a contact point with minimal deflection, corresponding to a force of 0.1 - 1 nN. In this situation, contact is maintained solely by the adhesion force between the cell and the tip. The major advantage of this type of surface analysis is its high resolution. This mode

of scanning results in a surface image showing highly detailed fibre-like structures that seem to originate from high density centres (Figure 6). The surface topology map constructed from this images shows a striking resemblance with the apical actin cytoskeleton as visualized using confocal laser microscopy (cf. see Figure 2).

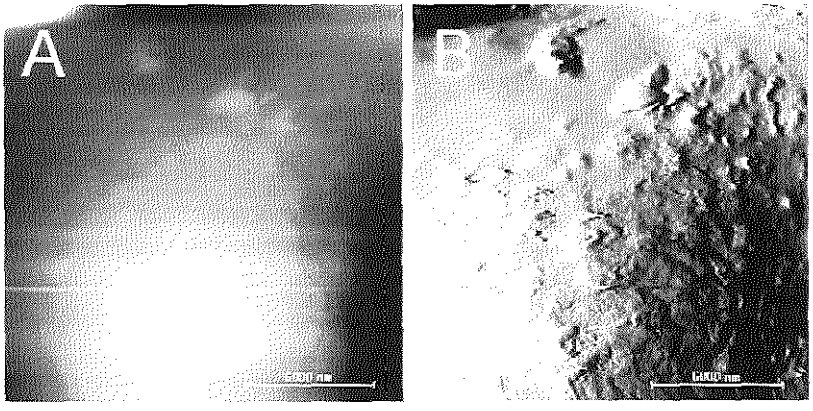

Figure 5. Cell surface topography in tapping mode AFM at high indentation

Shown is an AFM image of Intestine 407 cell surface scanned at high indentations and small amplitudes (A_{free}=218.4 nm, $A_{setpoint}$=118.3 nm, z=50 nm and the applied force is F=25 nN). A) Gives the topography and B) the error signal. Scan area is 18 µm x 18 µm. Image resolution is 300 points/line x 300 lines/images and the delay between two pixels is 1000 µs (1 kHz pixel frequency).

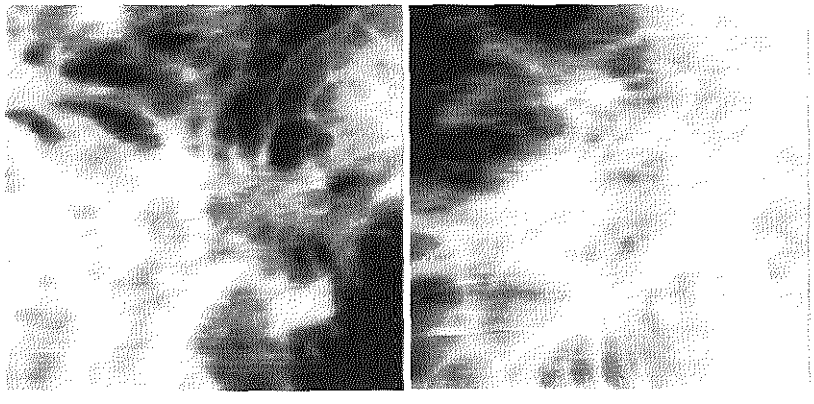

Figure 6. Cell surface topography in contact mode AFM at small indentation

Shown are AFM images of two cells scanned in the contact mode at a contact point that gives minimal deflection (applied force is F=0.1 - 1 nN). Scan area is 18 µm x 18 µm. Image resolution is 300 points/line x 300 lines/images and the delay between two pixels is 1000 µs (1 kHz pixel frequency).

DISCUSSION

In this preliminary study, we investigated the feasibility to construct high-resolution topological images of living Intestine 407 cells using the AFM. At least two properties of living mammalian cells make them rather difficult specimen for AFM studies; (1) the softness of the (membrane) surface and (2) the relatively large differences in height. Due to the softness of the cell surface, a contact point is more difficult to define and the membrane is damaged easily. Because of the vulnerability of the cell membrane, we experienced that scanning in contact mode with very soft cantilevers is preferred to scanning in the tapping mode, when the cell is subjected to relatively large forces. Substantial differences in height is a second major complication for studying intact cells with AFM, because the maximum z travel of the piezo tube is only 5 μm. Scanning soft preparations with relatively large differences in height can therefore cause deformation of the surface, leading to folding of cellular structures in the direction of the scan movement. The cantilever tip has a height of 3 μm with a shape that can be considered as pyramidal on the first 2.8 μm and conical on the last 0.2 μm. Applying AFM on preparations with large differences in height, the tip wall of the cantilever will start to contribute to the image, a phenomenon called "wall imaging".

Studying dynamic membrane processes, like exo- or endocytosis, remodelling of the cortical, membrane-associated cytoskeleton or changes in cellular volume, all require a high time-resolution since all these processes are expected to occur on a time scale of minutes or less. In our study, to acquire an image of 18 μm x 18 μm took about 4 min. The time resolution can be much improved by scanning much smaller membrane patches or by constructing simple line scans. In the apical membrane of pancreatic acinar cells, Schneider et al. [8] found so-called "pits" with a diameter of approx. 500 nm to 2000 nm, each containing multiple depressions ranging from 100 nm to 200 nm in size. A membrane patch of roughly 2 μm x 2 μm contains approx. 20 depressions. Stimulation of the cells with Mas7, thereby activating secretion, leads to an increase in the diameter of the depressions by approx. 35% after 5 minutes. Within 30 minutes after addition of Mas7, the size of the depressions returned toward basal conditions [8]. The enlargement of the depressions is suggested to be due to vesicle fusion at so-called "exocytotic fusion pores" [8]. Because Intestine 407 cells, unlike the synaptic terminals of neurones, are more likely to have a rather slow secretory machinery as observed in pancreatic aciner cells, with a time scale of minutes rather than seconds or less cells [18,19], a reduction of the scan area to 2 μm x 2 μm will meet both the time resolution requirements as well as the likelihood to capture exo- and endocytotic events. It must be stressed, however, that the time resolution described by Schneider et al. [8] is biased by the fact that the AFM experiments were performed at room temperature.

Using contact mode AFM and cantilevers with a low spring constant (0.01 - 0.02 N/m) detailed fibre-like structures were visible on the surface map. The resemblance between these AFM images and the distribution of F-actin at the apical side of the cell suggests that the cor-

tical cytoskeleton, just beneath the plasma membrane and perhaps interacting with the lipid bilayer, is visualized with this technique. The apical cytoskeleton gives a counter force when probed by the AFM tip. This mechanical tension influences the indentation of the cantilever and thereby gives the notion of altitude differences on the surface of the cell. Experiments with agents that interfere with the cytoskeletal network (cytochalasin B, jasplakinolide, nocodazol and paclitaxel) are necessary to verify that these structures are indeed involved.

Taken together, these initial studies show that high resolution AFM is a new and promising approach to study membrane topology in intact cells. To capture membrane remodelling efficiently, however, it is recommendable to improve the time resolution while keeping the scan area as large as possible. Furthermore, a large scan range is required for proper cell-volume measurements. If these requirements are met, AFM will be a powerful approach to study dynamic processes within plasma membranes in living cells. However, it should be noted that the interpretation of AFM images of living cells are complicated by the fact that the images are based on both a height map and a local stiffness map.

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8

Summary, concluding remarks and future perspectives

A major aim of the studies described in this thesis was to delineate the signal transduction pathways involved in the RVD response of Intestine 407 cells, with emphasis on their effects on VRAC activity. A major line of investigation was focused on the role and activation pathway of the MAP kinases Erk-1/2. Erk-1/2 is highly homologous to the yeast MAP kinase Mpk1p that is required for growth at low osmolarity [1]. In analogy to yeast, an important role for Erk-1/2, which are activated upon osmotic cell swelling in all mammalian cell types studied to date, in the volume regulatory response has been suggested previously. A second line of investigations was the role of endogenously released ATP in the RVD response. Extracellularly released ATP was suggested to be responsible for the activation of VRAC by an auto-/paracrine pathway in HTC hepatoma cells as well as in rat biliary epithelial cells [2,3]. Here we studied not only the mechanism by which ATP is released upon hypotonic stimulation, but also its effect on intracellular signalling as well as its role in volume regulatory responses in Intestine 407 cells.

REGULATION OF VRAC ACTIVITY IN INTESTINE 407 CELLS

Attempts to gain a better understanding of the regulation of compensatory responses to hypo-osmotic stress are hampered significantly by the lack of information about the molecular identity of the Cl^--channel involved. Several candidates have been proposed, but none of them have all the specific characteristics known to be associated with volume-sensitive anion channels (see **Chapter 1**). Members of established families of transport proteins such as the ABC-type of transporters and members of the ClC family might contribute to, but are not requi-

red for the volume-sensitive Cl^- current (**Chapter 1**). Furthermore, since most if not all cell types express volume-sensitive anion channels, it has been very difficult to identify potential channel proteins by expression cloning. In the very near future, however, mapping of the human genome will be completed (for review see: [4]). It is likely that many new potential ion channels will be discovered, one or more of them serving a function in cell volume control. Besides the lack of knowledge on channel identity, also very little is known about the osmo-receptor or osmo-sensor. In the yeast *Saccharomyces cerevisiae* as well as in the plant *Arabidopsis thaliana*, a transmembrane histidine kinase (a so-called two component system) functions as the osmo-sensor [5,6]. In contrast to prokaryotic, lower eukaryotic and plant cells, histidine kinases are very rare in mammalian cellular signal transduction. So far, only a few examples have been documented [7-9]. Although involvement of histidine kinases cannot be excluded yet, it is more likely that other proteins, e.g. of cytoskeletal origin, or perhaps the channel protein itself, might function as an osmo-sensor. This notion is supported by the observations that adjustment of cellular volume after osmotic swelling is much faster in mammalian cell types as compared to bacterial, yeast or plant cells.

The osmo-sensor may be sensitive to different aspects of hypo-osmotic stress; we can distinguish a chemical and a physical factor. The chemical factor includes the reduction in osmotic pressure, while the physical factor incorporates the mechanical stretch of the cell membrane and the attached cytoskeleton. Several groups have postulated that reduction of intracellular ionic strength regulates VRAC activity [10,11]. Cannon et al. [10] found that ionic strength only modulates but does not trigger VRAC activity; they demonstrated that positive pressure on the patch pipette, which forces the cell to swell without changing intracellular ion strength, activated VRAC. Voets et al. [11], however, showed that a reduction in intracellular ionic strength itself is the initial trigger for VRAC activity. They found that lowering ionic strength was sufficient to activate VRAC even when the volume was not increased. Furthermore, and in contrast to the findings of Cannon et al. [10], they reported that an increase in cellular volume without changing ionic strength did not induce VRAC activity [11]. In this light it will be interesting to investigate the sensitivity of VRAC and its modulators on intracellular ionic strength in Intestine 407 cells.

A potential candidate for being the osmo-sensor is the VRAC protein itself. Stretch-sensitivity or ion strength-sensitivity of VRAC, however, can be investigated only after isolation and reconstitution of the protein into artificial lipid bilayers. To date, only a few examples of stretch-activated channels in lipid bilayers have been reported, the bacterial MscL non-selective ion channels being the most extensively studied [12]. Crystallographic data and studies with mutant proteins resulted in a plausible model for the regulation of membrane-tension-gated MscL (for review see: [13]). The channel, which is suggested to provide an efflux pathway for solutes upon hypotonic stress, is a homopentamer with each subunit contributing to the pore. According to the so-called "mechanical wheel model", lateral membrane stress induces the helices to move and thereby increase the pore size [14]. To investigate whether or not VRAC itself

acts as a stretch-sensitive channel analogous to bacterial MscL, the isolation and purification of the protein is a prerequisite.

Alternatively, VRAC may be regulated by stretch- or ion strength-sensitive enzymes located near the channel. The folding of membrane proteins could be affected by the lateral pressure that lipids exert on protein domains. Activation of phospholipase A_2 by membrane tension has previously been reported [15]. The magnitude and direction of the lateral pressure depends on the lipid composition of the membrane [16]. Consequently, a change in membrane composition might influence protein folding and perhaps modulate its function. In a preliminary study, we reduced the cholesterol content of Intestine 407 cells and observed a two-fold increase in the hypotonicity-induced VRAC activity (unpublished results).

In **Chapter 2**, we already speculated about putative mechanisms by which VRAC modulating factors (cytoskeleton, $[Ca^{2+}]_i$ and tyrosine kinase phosphorylation) alter VRAC activity. We postulated three different ways by which the cytoskeleton might act on VRAC activity; (1) by a direct interaction between the F-actin cytoskeleton, or auxiliary proteins associated with the cytoskeleton, and the channel, (2) by promoting unfolding of the plasma membrane, thereby increasing the accessibility of channels, and (3) by recruitment of channels through regulated vesicle fusion. In the experiments described in **Chapter 5**, we indeed found strong evidence for the occurrence of a fast increase in exocytosis in response to hypotonic stimulation. Disruption of the F-actin filaments by cytochalasin B, however, which markedly potentiated VRAC activity, inhibited the volume-sensitive increase in exocytosis, making it very unlikely that a significant portion of the hypotonicity-induced anion current is brought about by the insertion of ion channels through vesicle fusion. In contrast, inhibition of exocytosis might promote the volume-sensitive anion efflux by preventing membrane insertion and thereby increasing membrane tension.

MAP KINASE AND AUTOCRINE ATP IN CELL VOLUME REGULATION

In Intestine 407 cells, Erk-1/2 is activated by the Ras/Raf/MEK pathway (**Chapter 3**); the "classical" signalling cascade through which tyrosine kinase-containing receptors induce Erk-1/2 activation. As summarized in Figure 1, our studies indicate that upstream of Ras, a $P2Y_2$-type purinoceptor is involved, activated by endogenously released ATP (**Chapter 4**). For a number of different cell models, it has been observed that activation of Erk-1/2 by G protein-coupled receptors requires a stimulation of clathrin-mediated endocytosis [17-20]. Pierce et al. [21] showed that Erk-1/2 activation stimulated by α(2A) adrenergic receptor activation is sensitive to inhibitors of the clathrin-mediated endocytosis, whereas the receptor itself is not internalized. They suggested that G protein-activated endocytosis leads to transactivation of the EGF receptor and subsequent stimulation of the Ras/Raf/MEK pathway, and that internalization of the EGF receptor or a downstream effector is an essential step in Erk-1/2 activation [21]. Whether

receptor-mediated endocytosis is also required for the activation of Erk-1/2 by hypo-osmotic cell swelling needs to be investigated further. This may be achieved by studying the effects of inhibitors of clathrin-mediated endocytosis (phenylarsine oxide [22], monensin [23] or dansylcadaverin [24]).

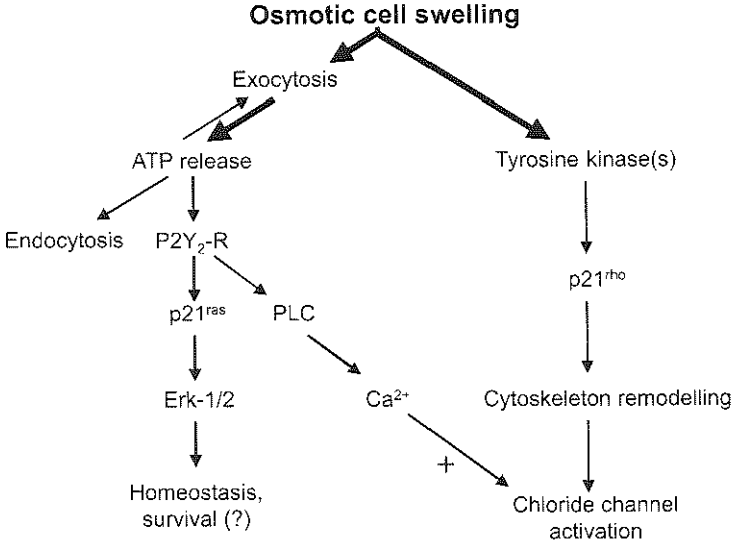

Figure 1. Cellular signalling in Intestine 407 cells induced by hypotonic stimulation

In Intestine 407 cells, hypotonic stimulation leads to the activation of VRACs, which involves tyrosine kinases(s), $p21^{rho}$ and the actin-cytoskeleton. In parallel, osmotic cell swelling induces an increase in exocytosis, which was found to be responsible for ATP release. Through an autocrine or paracrine mechanism, hypotonic shock-induced ATP release activates $P2Y_2$ purinoceptors. Besides the activation of Erk-1/2 through Ras/Raf signalling, $P2Y_2$ activation may modulate VRAC activity by increasing $[Ca^{2+}]_i$. Furthermore, the presence of extracellular ATP was required for both the hypotonicity-induced exo-and endocytosis.

In addition to Erk-1/2 activation, two other members of the MAP kinase family, p38 and JNK, were activated by hypotonic stimulation as well. Neither of these kinases, however, was involved in regulating volume-sensitive ion channels [**Chapter 3**; 25]. Because inhibitors of the Erk-1/2 and p38 MAP kinases were found to reduce hypotonicity-provoked endocytosis, these MAP kinases might play a role in cell swelling-induced membrane traffic (**Chapter 6**). In analogy, it is of interest that in yeast cells the MAP kinase Mpk1p is suggested to be involved in the increase in cell wall synthesis upon hypotonic stimulation [1], which involves exocytotic secretion of cell wall material. In addition, MAP kinases, through their putative role in vesicle trafficking, may also play a role in the insertion and/or retrieval of other transporters. During prolonged hypo-osmotic stimulation, the ionic balance/membrane potential must be restored while

keeping intracellular osmolarity low. This is attained during the late phase of the RVD response by the adjustment of metabolism and transport of organic osmolytes that do not perturb cellular protein function. It is tempting to speculate that vesicle transport regulated by MAP kinases is involved in the retrieval of these organic osmolyte transporters. Häussinger et al. [26] proposed that the fusion of bile salt transporter bearing vesicles with the canalicular membrane during hypo-osmotic stimulation depends on Erk-1/2 activation [27]. It is unclear, however, how MAP kinases do affect exo- and endocytosis. In liver cells, microtubule associated proteins downstream of Erk-1/2 are suggested to be involved [26,27]. In Intestine 407 cells, however, cell swelling-induced membrane traffic was found insensitive to microtubule disrupting agents (**Chapters 5 and 6**).

CELL SWELLING: MEMBRANE UNFOLDING OR INCREASE IN MEMBRANE AREA DUE TO EXOCYTOSIS

In addition to the above-mentioned role in receptor cycling and transporter insertion/retrieval and the involvement in the increased secretion of autocrine factors like ATP, exo- and endocytotic processes may serve as a mechanism to change the total plasma membrane area, necessary for allowing cell swelling and subsequent shrinkage. Whether an increase in cellular volume requires an increase in exocytosis may be verified by studying the effect of exocytosis inhibitors (like cytochalasin B or BAPTA-AM [**Chapter 5**]) on changes in cellular volume in response to hypotonic stimulation. Likewise, the role of endocytosis inhibitors (such as apyrase) on hypotonicity-induced volume changes will clarify a role for endocytosis in RVD.

Using electron microscopy, Pfaller et al. [28] found that the cell surface is not smoothened by hypotonic stimulation in rat hepatocytes. In fact, the cell membrane displays even more microvilli during osmotic cell swelling than in isotonic controls. Morphometric analysis revealed that hypotonic stimulation, which leads to an increase in cellular volume of 25%, nearly doubles the plasma membrane surface within 5 min of exposure [28]. Thereafter, the surface/volume ratio returns to control values after approx. 15 min [28]. These results suggest that hypotonic stimulation leads to an increase in exocytosis rather than unfolding of the plasma membrane. It has to be considered, however, that these studies were all performed on fixated preparations and not on living cells. In this thesis, we report the results of our preliminary attempts to study changes in the surface topology of Intestine 407 cells with AFM (**Chapter 7**). Although several improvements are required, the outcome suggests that AFM may indeed turn out to be a more suitable technique to study membrane surface and topology in living cells.

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Samenvatting

In dit proefschrift is onderzoek beschreven naar het mechanisme van signaal-overdracht betrokken bij de regulatie van het celvolume. Dit mechanisme (de 'regulatory volume decrease' of RVD) wordt geactiveerd na blootstelling van de cel aan een omgeving met een lage osmotische druk (hypotone stimulatie) of na een toename van de intracellulaire osmotische druk. Celvolume-regulatie voorkomt langdurige functie-bedreigende celzwelling. Een toename van de intracellulaire osmotische druk kan o.a optreden bij hormoon-gereguleerde metabole processen en opname van nutriënten door de cel. Een afname in extracellulaire osmotische druk is onder normale, fysiologische omstandigheden uitsluitend te verwachten bij nier- en darmepitheel. In dit proefschrift is de aandacht speciaal gevestigd op het chloride-kanaal dat betrokken is bij de regulatie van het celvolume: het zogenaamde volume-gevoelige anionkanaal of VRAC. Voor dit onderzoek is de humane epitheliale cellijn Intestine 407 als model gebruikt. Deze uit foetaal jejunum geïsoleerde cellijn is bij uitstek geschikt voor onderzoek naar de regulatie van VRAC, aangezien weinig andere chloride-kanalen, zoals calcium- en volt-geactiveerde chloride-kanalen, en geen CFTR-kanalen, in deze cellijn tot expressie komen.

In **hoofdstuk 2** wordt een overzicht gegeven van de veelheid aan signaaltransductieroutes die geactiveerd worden na hypotone stimulatie van zoogdiercellen. Alhoewel de betrokkenheid van sommige signaalmoleculen bij de celvolume-regulatie celtype-afhankelijk lijkt te zijn, blijken enkele signaalroutes meer universeel een rol te spelen. Kinases die in alle bestudeerde zoogdiercellen worden geactiveerd na hypotone stimulatie, zijn Erk-1/2 behorende tot de familie van de MAP kinases. In Intestine 407 cellen worden Erk-1/2 geactiveerd door de Ras/Raf/MEK route (**hoofdstuk 3**). Deze Ras/Raf/MEK/Erk-1/2 signaaltransductieroute blijkt echter niet betrokken te zijn bij de activering van volume-gereguleerde ionkanalen (**hoofdstuk 3**).

Zoals vele vormen van cellulaire stress leidt ook hypotone stimulatie tot het secreteren van ATP door zoogdiercellen. ATP kan als autocriene factor optreden door middel van activering van purinerge receptoren. We vonden dat extracellulair ATP tot een verhoging van de intracellulaire calciumconcentratie leidt, en via dit mechanisme de activiteit van het volume-gevoelige chloride-kanaal potentieert (**hoofdstuk 4**). Voorts bleek dat het bij de celzwelling vrijgekomen ATP verantwoordelijk is voor de activering van Erk-1/2 via binding aan en activering van de $P2Y_2$ receptor (**hoofdstuk 4**).

Het mechanisme van ATP-secretie als gevolg van osmotische celzwelling bleek afhankelijk te zijn van een intact cytoskelet, van een verhoging van de intracellulaire calciumconcentratie, en van de activiteit van SNARE-type fusie-eiwitten (eiwitten betrokken bij de fusie van vesicles met de plasmamembraan) en phospholipase D (**hoofdstuk 5**). Deze bevindingen sug-

gereren dat exocytose betrokken is bij het secretieproces. In overeenstemming met dit model vonden we dat hypotone stimulatie leidt tot een toename in oppervlak van het celmembraan, zoals gemeten met behulp van de fluorescerende membraan probe FM 1-43 (**hoofdstuk 5**). Voorts werd aangetoond dat osmotische celzwelling leidt tot een verhoogde snelheid van endocytose, welke echter optreedt met een vertraging van 2 - 3 minuten en na 10 - 15 min weer op controle niveau is teruggekeerd (**hoofdstuk 6**). We vonden dat de endocytose kritisch afhankelijk is van de toename in activiteit van het volume-gereguleerde chloride-kanaal, en mogelijk een gevolg is van de depolarisatie van de plasmamembraan veroorzaakt door een verhoogde efflux van chloride (**hoofdstuk 6**).

Autocrien werkend extracellulair ATP bleek cruciaal te zijn voor de door celzwelling geactiveerde exo- en endocytose. De gedeeltelijke remming van beide processen door remmers van Erk-1/2 activiteit suggereert dat een deel van de effecten van ATP wordt bewerkstelligd middels Erk-1/2 (**hoofdstuk 6**).

Tot slot hebben we, om de membraan dynamiek beter te kunnen bestuderen, een begin gemaakt met het in kaart brengen van het celoppervlak van levende Intestine 407 cellen met behulp van de atomic force microscoop. Hoewel het systeem nog verder geoptimaliseerd moet worden, zijn de eerste interpreteerbare resultaten verkregen (**hoofdstuk 7**).

Dankwoord

Nu dit proefschrift af is, wil ik hier nog enkele woorden wijden aan de mensen die mij (op welke wijze dan ook) hebben geholpen met de tot stand koming ervan. Eigenlijk zijn dat er teveel om op te noemen, want elk goed gesprek en bemoedigend woord is een fortuin waard bij het klaren van een klus waarbij vooral doorzettingsvermogen vereist is. Toch wil ik hier een paar mensen in het bijzonder bedanken.

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Curriculum vitae

Thea van der Wijk werd geboren op 19 juni 1973 te Heerenveen. Na het behalen van het VWO diploma aan de RSG te Heerenveen in 1991, begon zij haar studie Humane Voeding aan de Landbouwuniversiteit te Wageningen. Tijdens het afstudeervak Humane Voeding heeft ze mee gewerkt aan een onderzoek naar de effecten van onverteerbare vetten (sucrosepolyester) op de maagzuursecretie bij gezonde proefpersonen (Afdeling Gastroenterologie, Academisch Ziekenhuis Nijmegen). De effecten van verschillende groeifactoren en cytokines op intercellulaire communicatie en celproliferatie in het kader van het atherosclerose-onderzoek stonden centraal bij het afstudeervak Toxicologie (Vakgroep Toxicologie, Landbouwuniversiteit Wageningen). Gedurende haar stage bij TNO Voeding zijn de effecten van modulerende stoffen op de tight-junction permeabiliteit bestudeerd m.b.v. een in vitro model voor paracellulair darmepitheel transport. In 1996 is zij cum laude afgestudeerd met als specialisatie Voeding, voedsel en toxicologie. In juli 1996 begon zij haar promotie-onderzoek op de afdeling Biochemie van de Erasmusuniversiteit Rotterdam alwaar het in dit proefschrift beschreven onderzoek is uitgevoerd. Sinds 1 oktober 2000 is zij werkzaam als postdoc bij het Hubrecht Laboratorium te Utrecht.

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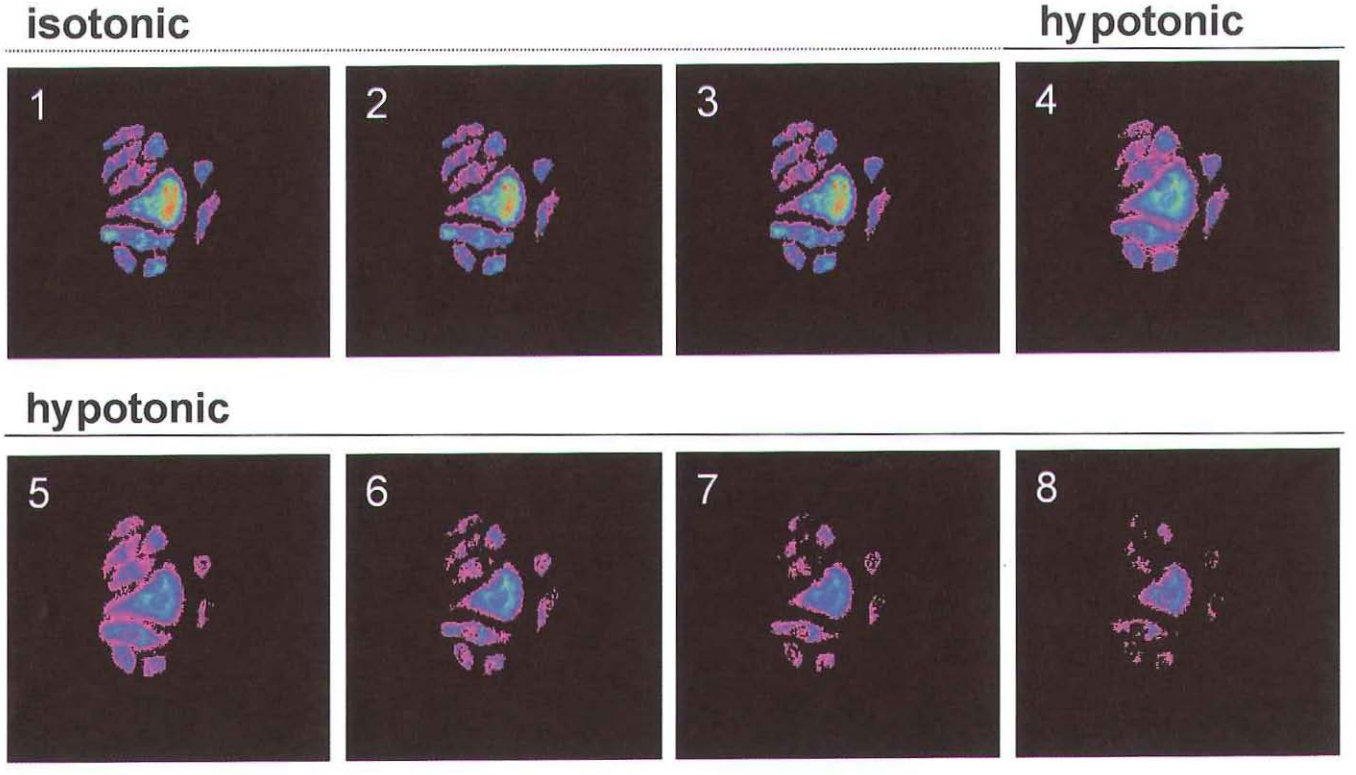

Figure 2A. Hypotonic stimulation leads to an increase in washout of preloaded FM 1-43; Fluorescence microscope images of a group of Intestine 407 cells

Groups of 8-10 Intestine 407 cells were preloaded for 2 h with FM 1-43 (1 μM). Thereafter, cells were continuously perfused (2 ml/min) using medium without FM 1-43 (washout). After a calibration period of 5 min, the experiment was started. Images were sampled every minute and show the fluorescence intensity. After 3 minutes, isotonic medium was replaced by a 40% hypotonic medium.

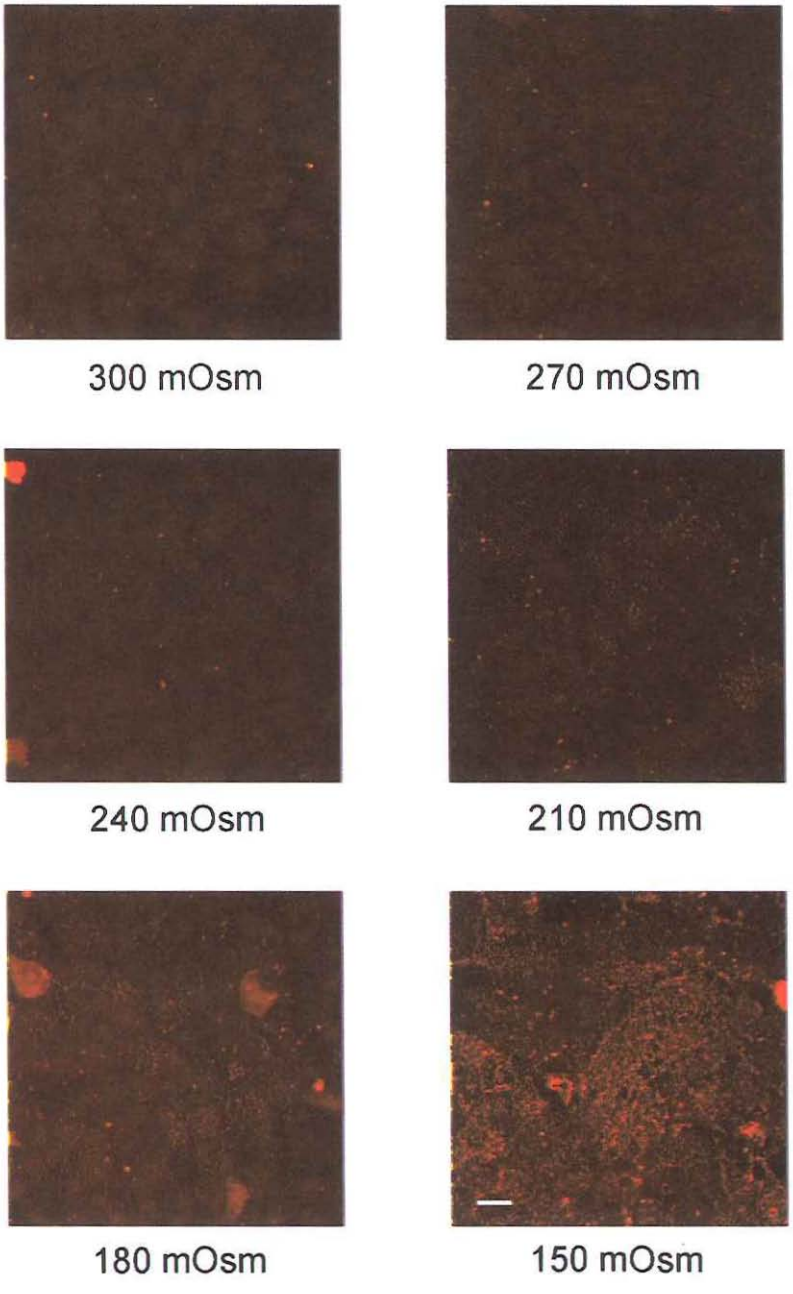

Figure 1. TRITC-dextran uptake induced by lowering medium osmolarity

Cells grown on coverslips were incubated for 5 min with TRITC-dextran containing medium (0.5 mg/ml) with different osmolarities as indicated in the figure. After fixation, images were constructed as indicated in Materials and Methods using CSLM. The images are representative of 10 images from 2 independent experiments (bar = 25 μm).