REVIEW

Regulatory Volume Decrease and P Receptor Signaling in Fish Cells: Mechanisms, Physiology, and Modeling Approaches

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ABSTRACT

For animal cell plasma membranes, the permeability of water is much higher than that of ions and other solutes, and exposure to hyposmotic conditions almost invariably causes rapid water influx and cell swelling. In this situation, cells deploy regulatory mechanisms to preserve membrane integrity and avoid lysis. The phenomenon of regulatory volume decrease, the partial or full restoration of cell volume following cell swelling, is well-studied in mammals, with uncountable investigations yielding details on the signaling network and the effector mechanisms involved in the process. In comparison, cells from other vertebrates and from invertebrates received little attention, despite of the fact that e.g. fish cells could present rewarding model systems given the diversity in ecology and lifestyle of this animal group that may be reflected by an equal diversity of physiological adaptive mechanisms, including those related to cell volume regulation. In this review, we therefore present an overview on the most relevant aspects known on hypotonic volume regulation presently known in fish, summarizing transporters and signaling pathways described so far, and then focus on an aspect we have particularly studied over the past years using fish cell models, i.e. the role of extracellular nucleotides in mediating cell volume recovery of swollen cells. We, furthermore, present diverse modeling approaches developed on the basis of data derived from studies with fish and other models and discuss their potential use for gaining insight into the theoretical framework of volume regulation. *J. Exp. Zool. 313A, 2011.* © 2011 Wiley-Liss, Inc.

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In most animal cells, a change of environmental osmotic conditions elicits a change in cell volume which must be kept within certain limits, as excessive swelling would eventually lead to cell bursting and excessive shrinkage would compromise proper cell metabolism, both of which ultimately results in cell killing. Therefore, most cells are equipped with mechanisms to counteract or prevent excessive volume changes. In the case where cells are swollen relative to isoosmotic conditions, they engage a response termed regulatory volume decrease (RVD), and if they are shrunken, a response called regulatory volume increase

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(RVI) is triggered. How exactly changes of relative osmolarity are sensed by cells, transmitted within the cellular signaling network, and ultimately lead to the activation of volume regulatory effector mechanisms, as well as the nature of these, has been amply studied over many years, and an excellent review summarizing our current knowledge has recently been delivered by Hoffmann et al. (2009). However, one thing that becomes clear from this review is that most of the studies that constitute the fundamentals of our present insight have been conducted on mammalian cell models. In comparison, relatively few studies have addressed volume regulation in nonmammalian cells. This is unfortunate, considering that e.g. teleost fish form an enormously heterogeneous group of animals with an extreme variability in ecology and lifestyle, which may be mirrored by an equally huge diversity of physiological adaptive mechanisms, presumably including those related to cell volume regulation where adequate. This becomes most obvious and is in principle true for any teleost fish, when e.g. gill cells are considered, which are in direct contact with the surrounding water and are thus directly confronted with any change in water osmolarity, as well as pH and temperature and any other physical parameter that may impact on cell volume regulatory mechanisms (Dascalu et al., '92; Riquelme et al., '98; Ahmed et al., 2006). Similar conditions may apply for the intestinal tract which in many euryhaline fish serves as the main osmoregulatory organ (Lionetto and Schettino, 2006). Given that fish may experience relatively large changes of environmental variables on a daily basis, e.g. in estuarine teleosts when changing between fresh water and brackish water for brief excursions for food (Marshall, 2003), one could expect that volume regulatory effector mechanisms have been developed which may either have no equivalent in mammals or which may not be observable in a comparable clarity in these. Other mechanisms that have evolved to deal with even larger changes of environmental osmolarity, when migrating between freshwater and seawater for reproductive purposes, may also ultimately impact on volume regulation, even if their primary use is in systemic osmoregulation.

In this review, we summarize some of the most important facets currently known on hypotonic volume regulation in fish (and other lower vertebrates, where appropriate), providing a brief overview of transporters for osmolytes described in these groups and some of the underlying signaling pathways. Subsequently, we will focus on the role of extracellular nucleotides as the main extracellular signaling factors mediating the RVD, highlighting findings on fish hepatocyte models we have established over the past few years. Finally, we will present some modeling approaches developed on the basis of data derived from studies with fish and other models, and discuss how they may be used to gain insight into the theoretical framework of volume regulation.

REGULATORY VOLUME DECREASE IN FISH CELLS

As outlined in more detail in the modeling section, cell volume regulation requires numerous components to interact in a coordinated fashion (Wehner et al., 2003). Of these, the effector mechanisms are experimentally clearly the most accessible level of volume regulation, and these are thus by far the best understood among these components. The role of these volume-regulatory transporters is to mediate net fluxes of osmolytes across the membrane so as to compensate for osmotic imbalances that have caused volume changes. Accordingly, the choice of suitable osmolytes is limited to such that are relatively abundant and for which a gradient across the cell membrane exists, so that their net flux will cause a sufficiently large flux of osmotically obliged water to affect cell volume. The available choice of osmolytes in nonmammals seems to be the same as in mammals, and thus includes inorganic ions K^+, Na^+, and Cl^-, as well as abundantly available amino acids, primarily taurine (Kirk, '97; Kirk and Strange, '98). Other osmolytes found with some abundance in lower vertebrates, such as urea, trimethylamine oxide, betaine (Goldstein and Kleinzeller, '87), or glutamine (Ip et al., 2009), are primarily involved in the regulation of long-term osmotic balance and are generally not considered important contributors to cell volume regulation (with exceptions, such as trimethylamine oxide in spiny dogfish *Squalus acanthias* red cells (Koomoa et al., 2001). In consequence, the mechanisms transporting osmolytes involved in volume regulation is—as far as we currently know—equally limited in mammals and nonmammals with hypotonic swelling of cells generally triggering efflux pathways for K^+, Cl^-, and taurine, whereas shrinkage leads to the uptake of Na^+ (Hoffmann et al., 2009).

Effector Mechanisms of Volume Regulation Identified in Fish

K^+-Cl^- Cotransport. Efflux pathways for $K^+$and Cl^- from swollen fish cells include K^+-Cl^- cotransporters (KCC) as well as channels for the individual ions. The first description of a fish KCC stimulated by swelling was published by Lauf ('82) for toadfish *Opsanus tau* erythrocytes. When these cells were exposed to hypotonic saline, swelling was followed by an RVD associated with a concurrent loss of K^+ and Cl^-, and both the volume recovery and the loss of K^+ were dependent on the presence of Cl^- and could be inhibited with the loop diuretic furosemide. Hypotonic activation of KCC with similar characteristics was also described in red cells from rainbow trout (Borgese et al., '87), brook trout (Marshall et al., '90), carp (Jensen, '95) and eel (Bursell and Kirk, '96), in hepatocytes from trout (Bianchini et al., '91), from the marine flatfish turbot, *Scophthalmus maximus* (Ollivier et al., 2006a), and from Gilthead sea breams, *Sparus aurata* (Faggio et al., 2011), as well as in proximal renal tubules from goldfish (Terreros et al., '90) and trout (Kanli and Norderhus, '98). Thus, a volume regulatory form of electroneutral KCC seems to be widely expressed in different fish cells, as is the case in mammals (Gamba, 2005). It should be pointed out here that, in fact, electroneutrality of the Cl^- dependent K^+ fluxes has very rarely been experimentally verified and numerous mathematical models, including that presented below, would become

much more complex if potential electrogenicity had to be taken into account.

Signaling molecules involved in KCC regulation include protein kinases and phosphatases, with phosphorylation of the transporter acting inhibitory and dephosphorylation leading to activation/deinhibition (Lauf et al., '92; Lauf and Adragna, 2000; Adragna et al., 2004). A reciprocal mode of activity control (i.e. activation by phosphorylation, inhibition by dephosphorylation) is generally observed for the shrinkage-activated Na^+-K^+-Cl^--cotransporter, suggesting the presence of an integrated and efficient control system. Although this model is mainly based on findings in mammalian cells, it seems also valid for fish red cells (Cossins et al., '94), frog, *Rana temporaria*, erythrocytes (Gusev and Agalakova, 2010), and presumably a number of other cell types from lower vertebrates for which significant effects of inhibitors of protein kinases and/or phosphatases have been reported (e.g. Ebner et al., 2006; Ollivier et al., 2006c), but for which no direct link to KCC activity has yet been established. A recent study investigating regulatory phosphorylation sites in KCCs reported that these were largely conserved from sea squirt over green puffer fish to humans (Rinehart et al., 2009), supporting the likely ubiquitous presence of this control mechanism.

The KCC-regulatory protein kinases and phosphatases identified in fish cells so far include serine/threonine phosphatases (Cossins et al., '94) and tyrosine kinases (Weaver and Cossins, '96) whereas, to our knowledge, no direct evidence has yet been presented for the involvement of WNK (with no lysine) kinases in fish cells. However, as WNK kinases were shown to act on the above mentioned conserved phosphorylation sites of KCCs (Rinehart et al., 2009), and as they are apparently expressed in fish (e.g. zebrafish protein: NCBI database accession number XP_689656; transcripts and protein sequences for *Fugu*, *Medaka fish*, three-spined stickleback, and the green spotter pufferfish are given in the Ensemble database at http://www.ensembl.org/), their contribution to KCC control seems likely.

A way of activating KCC of fish cells (and also mammalian cells), even under isotonic conditions, is by addition of the cysteine alkylating reagent N-ethylmaleimide. With this compound (almost) full activation of the transporter is possible, leading to relatively rapid cell shrinkage even in an isoosmotic setting both in erythrocytes (Bursell and Kirk, '96) and in hepatocytes from fish (Bianchini et al., '91; Espelt et al., 2003; Ollivier et al., 2006c; Krumschnabel et al., 2007).

A peculiarity described for KCC of red cells from fish and other vertebrates, but not for other cell types, is that it may be activated and inhibited by high and low oxygen tensions, respectively (Berenbrink et al., 2000, 2006). It was reported that this oxygen-sensitive regulation can not only contribute to hypotonic activation of the transporter, but may also be oxygen-dependently activated under isotonic conditions, leading to cell shrinkage (Nielsen et al., '92). How oxygen tension is perceived by the transporter is not known, although sensing by (bulk) hemoglobin seems unlikely as both pO_2- and pH-dependence of the sensor differ from that of hemoglobin (Berenbrink et al., 2000, 2006). It was also suggested that reactive oxygen species contribute to KCC regulation, presumably also through their action on the still unknown O_2-sensor (Bogdanova and Nikinmaa, 2001). In line, it was shown that antioxidants, such as reduced glutathione, tend to stabilize cell volume physiologically (Marshall et al., '90). A comprehensive review on the current knowledge on oxygen sensitive ion transporters in erythrocytes has been given by Bogdanova et al. (2009).

Channels for K^+ and Cl^- Transport. The release of K^+ and Cl^- through separate pathways—which may or may not be electrogenic (Garcia-Romeu et al., '91)—during hypotonic cell volume recovery is at least as widely observed as their electroneutral cotransport described above. In some cases, such as trout erythrocytes, it seems that the mechanism mediating RVD is specific for the nature of swelling elicited (Berenbrink et al., '97). Thus, isotonic swelling of trout red blood cells caused by adrenergic stimulation (leading to the uptake of Na^+ via enhanced Na^+-H^+ exchange, NHE; Nikinmaa and Huestis, '84) primarily activated KCC, whereas hypotonic swelling activated K^+ efflux through Cl^--independent pathways (Guizouarn and Motais, '99). Generally, however, both mechanism may be activated concurrently and contribute to RVD in concert (e.g. Ollivier et al., 2006c). For mammals, a wide variety of K^+ channels has been described to be activated upon cell swelling, being sensitive to the volume change or to membrane stretch as such, to associated alterations of membrane potential or to secondary changes, such as that of intracellular Ca^{2+} or lipid intermediates (Hoffmann et al., 2009). A similar diversity of K^+ channels seems to be activated in fish cells, at least judged from their identification based on the use of more or less specific inhibitors. Given that the specificity of these inhibitors has been mostly derived from studies with mammalian cells, it should be emphasized that any conclusions derived from their use needs to be considered with appropriate caution. Thus, the presence of voltage-sensitive K^+ efflux, inhibited e.g. by $BaCl_2$, was reported in goldfish renal proximal tubules (Terreros et al., '90), sea bass (*Dicentrarchus labrax*) gill cells (Duranton et al., 2000b), trout red cells (Egée et al., 2000), *Gobius niger* enterocytes (Trischitta et al., 2004), turbot hepatocytes (Ollivier et al., 2006c), and Gilthead sea bream hepatocytes (Faggio et al., 2011). In contrast, $BaCl_2$ was without effect on swelling-induced K^+ fluxes in rainbow trout hepatocytes (Bianchini et al., '88) and brown trout (*Salmo trutta*) proximal renal tubules (Kanli and Norderhus, '98), indicating not only cell-specific, but also species-specific differences in the expression of the transporter.

Ca^{2+}-sensitive K^+ channels, inferred from the effect of quinine and other more or less selective K^+ channel antagonists and also inhibited by $BaCl_2$, have been described, among other

examples, for renal proximal tubules from goldfish (Terreros et al., '90), brown trout (Kanli and Norderhus, '98), goldfish retinal ganglion cells (Edmonds and Koenig, '90), and eel (*Anguilla anguilla*) intestinal cells (Trischitta et al., 2005). A closer characterization of the Ca^{2+}-sensitive K^+ transporter in the eel intestinal epithelial cells indicated that it is a large conductance Ca^{2+}-activated K^+ channels with high sequence homology to other members of this family of so-called BK channels, which was sensitive to the specific BK channel inhibitor iberiotoxin, was localized to both apical and basolateral membranes of enterocytes in situ, and was activated upon hypotonic stress but silent in isotonic conditions (Lionetto et al., 2008). Later, in section 3.1.5, a mathematical model for volume regulation is described where swelling is associated with changes in cytosolic Ca^{2+}, which in turn activate Cl^- and K^+ channels, underscoring the importance of such channels in some cells. Hypotonic K^+ channel activity sensitive to charybdotoxin (another specific inhibitor of BK channels) and apparently primarily localized at the basolateral membrane of the cells was demonstrated in sea bass gill cells (Duranton et al., 2000a,b). As with voltage- sensitive K^+ channels, this type of channel is not uniformly expressed, as K^+ efflux seems to be independent of Ca^{2+} in red cells from trout (Garcia-Romeu et al., '91) and carp (Jensen, '95), as well hepatocytes from rainbow trout (Bianchini et al., '88).

Swelling-activated Cl^- channels are referred to in the literature with a number of different names and, in accordance with Hoffmann et al. (2009), we will address them here as volume-regulated anion channel (VRAC), to highlight the possibility that other ions may as well be transported via this pathway. As with K^+ channels, identification of VRACs in fish is heavily based on the use of pharmacological inhibition and other indirect approaches; but as the molecular identity of the channels is not even clear in mammals, this may not be too surprising. First experimental evidence for the existence of a swelling activated Cl^- channel in trout red cells was provided by Egée et al. ('97). Subsequent characterization of the channel showed that it was sensitive to 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), outwardly rectifying, silent under isotonic conditions, and that it accounted for about 50% of the RVD response in these cells (Egée et al., 2000). In trout proximal renal tubule cells, volume recovery was similarly affected by the anion channel blocker MK-196 but not by NPPB (Kanli and Norderhus, '98), whereas in turbot hepatocytes, NPPB and 9-anthracene carboxylate (9-AC) also inhibited up to 50% of RVD (Ollivier et al., 2006c). Almost complete inhibition of RVD by NPPB was observed in *G. niger* enterocytes (Trischitta et al., 2004) and in the rainbow trout hepatoma cell line RTH-149 (Krumschnabel et al., 2007). These quantitative differences may not actually reflect different contributions of Cl^- channels to RVD, as the concentrations at which the inhibitors were used differed between studies and concurrent inhibition of other transporters cannot be ruled out. This is also true for the inhibitory effect of DIDS. Although this compound inhibits Cl^- channels, it also blocks the Cl^-/HCO_3^- exchanger, which was suggested to contribute to RVD in *G. niger* enterocytes (Trischitta et al., 2007), turbot hepatocytes (Ollivier et al., 2006a), and RTH-149 hepatoma cells (Krumschnabel et al., 2007). Interestingly, although the trout anion exchanger largely preserved its transport properties when expressed in *Xenopus* oocytes, no comparable anion conductive activity was detected for the anion exchanger of skate and zebrafish (Guizouarn et al., 2005), indicating that the exchanger transport characteristics are specific to the trout lineage and underscoring the use of fish cells for the detection of potentially novel transport pathways.

Taurine Efflux Pathways. An important organic osmolyte contributing to RVD in numerous fish cell types is taurine, which may be the reason why fish cells were among the first vertebrate cells where the role of taurine was actually detected (Fugelli and Zachariassen, '76). The molecular entity mediating swelling-induced release of taurine is neither known in fish nor in mammals, and its detection is complicated by the fact that taurine may be released via not only one but also several pathways. For example, it was shown that in red cell of the little skate (*Raja erinacea*) taurine release occurs through the anion exchanger, skAE1 (Perlman and Goldstein, 2004). This fairly well-characterized release pathway is controlled through complex signaling events involving tyrosine kinase stimulated formation of a tetrameric channel created by interaction of anion exchanger dimers with cytoplasmic proteins, phosphatidylinositol 3-kinase mediated exocytic insertion of the transporter into the membrane (Puffer et al., 2006), and endocytic removal associated with monoubiquitination (Musch et al., 2008). In contrast, taurine release triggered in hyposmotic eel erythrocytes differed significantly from the anion exchanger with regard to inhibitor sensitivities, suggesting that a different yet also Cl^- permeable efflux pathway is used in these cells (Lewis et al., '96), which is also true for trout red blood cells (Garcia-Romeu et al., '91; Kiessling et al., 2000). Noteworthy, as mentioned above regarding Cl^- transport, ectopic expression of these transporters in *Xenopus* oocytes indicated that only trout anion exchanger but not that of zebrafish or skate led to increased permeability for taurine (Guizouarn et al., 2005). A hypothetical explanation for these discrepancies was that the anion exchanger may either not be the channel by itself but rather control an osmosensitive anion efflux pathway, or that it adopts a new conformation upon ectopic expression (Shennan, 2008). In trout erythrocytes, this anion exchanger may switch to a swelling-induced anion conductive pathway permeable to Cl^-, taurine, and possibly even K^+ and Na^+ (Guizouarn et al., 2001).

Taurine efflux was also found to be important in the RVD response of many other models, e.g. in teleost heart ventricular

cells (Vislie, '83), dogfish shark (*S. acanthias*) rectal gland cells (Ziyadeh et al., '88), trout hepatocytes (Michel et al., '94), goldfish renal proximal tubules (Fugelli et al., '95), winter flounder (*Pleuronectes americanus*) renal cells (Benyajati and Renfro, 2000), walking catfish (*Clarias batrachus*) liver (Goswami and Saha, 2006), and sea bass gill cells (Avella et al., 2009). Regulation of taurine release includes control by external Ca^{2+} (trout hepatocytes; gill cells, goldfish proximal renal tubules) and prostaglandin synthesis (Fugelli et al., '95), but further characterization of control mechanisms still awaits investigation.

Na^+, K^+-ATPase Activity and RVD. The impact of Na^+, K^+-ATPase (the Na^+-pump) on RVD has been little explored, but is supported by some experimental evidence (Andersson et al., 2004) and theoretical models (Hernandez and Cristina, '98). Accordingly, in brown trout renal proximal tubules RVD was at least partly inhibited by ouabain, indicating that Na^+, K^+-ATPase activity was involved early on in the process (Kanli and Norderhus, '98). This is not typically the case, as Na^+, K^+-ATPase is mostly active after cell volume restoration, reestablishing the transiently disturbed ion homeostasis (Jensen, '95). In line, in goldfish renal proximal tubules, hypotonic RVD was neither affected by addition of the selective Na^+, K^+-ATPase inhibitor ouabain (Terreros et al., '90) nor by either acutely lowering medium oxygen levels or by blocking mitochondrial ATP production with NaCN (Terreros and Kanli, '92), which in most cell types would compromise Na^+, K^+-ATPase activity (Krumschnabel et al., '96). As to the latter, it needs to be considered that goldfish and their cells are outstandingly hypoxia/anoxia tolerant, and enhanced glycolytic ATP production and preferential fuelling of the Na^+-pump—as observed in goldfish hepatocytes (Krumschnabel et al., 2001)—could have offset an effect on Na^+, K^+-ATPase activity. In *Xenopus* A6 kidney epithelial cells, metabolic inhibition impaired RVD owing to the associated intracellular acidification (Smets et al., 2002). An alternative explanation provided for the impact of Na^+, K^+-ATPase activity on RVD was that the local decline of ATP at the membrane caused by the ion pump could help activating ATP-sensitive K^+ channels (similar to the inward rectifier potassium channels that mediate insulin secretion of pancreatic cells) and thus enhance hypotonic K^+ release (Tsuchiya et al., '92; Andersson et al., 2004).

Some Signaling Pathways Involved in RVD

An overview of sensing and signaling pathways in fish cells activated in response to osmotic stress has been given by Fiol and Kültz (2007) and, not unexpectedly, these overlap with the pathways involved in sensing and signaling of acute hypotonic volume changes identified. Below, a brief account of signaling elements not yet discussed in the context of specific ion transporters—with no claim of comprehensiveness—shall be given.

Calcium. In addition to the above described evidence for a role of Ca^{2+} in the activation of specific channels, a number of studies have not directly addressed such links, but established dependence of RVD on changes in intracellular Ca^{2+} (Ca^{2+}i) as such. Generally, it seems that an elevation of Ca^{2+} is triggered upon hypotonic exposure in fish cells just as in mammals, although this response may be highly variable. For example, in trout hepatocytes, hypotonic exposure elicited Ca^{2+}i fluctuations in only 60% of the cells, and in these either a single robust elevation, a series of oscillatory increases, or moderate fluctuations around baseline values were noted (Krumschnabel et al., 2003). At the population level, these responses added up to a slowly increasing but persistent elevation of Ca^{2+}i (Krumschnabel et al., 2003; Ebner et al., 2005). Although the source of Ca^{2+} was not specifically examined in these studies, it seems that principally both external Ca^{2+} and intracellular stores may contribute and/or be important. Thus, it was reported that in goldfish proximal tubules omission of extracellular Ca^{2+} had no effect on hypotonic RVD, whereas depletion of internal stores, in particular of the ER, or buffering of cytoplasmic levels largely inhibited RVD (Terreros and Kanli, '92). The opposite was observed in freshly isolated and in cultured rainbow trout gill cells, where hypotonic swelling caused a biphasic increase of Ca^{2+}i, the second pulse of which was owing to Ca^{2+} influx. When this second pulse was abolished during hypotonic exposure in Ca^{2+} free saline, this concurrently also inhibited RVD (Leguen and Prunet, 2004). Finally, in eel enterocytes (Trischitta et al., 2005) and Gilthead sea bream hepatocytes (Faggio et al., 2011), both Ca^{2+} influx and release from intracellular stores were important, as inhibition of either pathway completely blocked RVD. Turbot hepatocytes also showed diminished RVD after depletion of Ca^{2+}, but no distinction was made between extra- and intracellular Ca^{2+} (Ollivier et al., 2006c).

Recently, it was suggested that the absence or presence of specific isoforms of aquaporins may underlie differences in Ca^{2+}-sensitivity of the RVD response between cell types (Ford et al., 2005; Galizia et al., 2008). Whether this can be generalized, and if this also may apply for fish cells with their apparent huge diversity of expressed aquaporins (Tingaud-Sequeira et al., 2010), remains to be tested.

Given that Ca^{2+}i elevation occurs during swelling and may contribute to controlling the RVD response, one might as well expect that conditions affecting Ca^{2+} homeostasis could impact on RVD in fish cells. However, although Cu^{2+} affected Ca^{2+} changes in trout gill cells, it had no impact on RVD (Leguen and Prunet, 2001); we observed a similar lack of effect of several hours of exposure to Cu^{2+}, which causes an elevation of Ca^{2+}i (Manzl et al., 2003), or to the Ca^{2+} ionophore ionomycin on RVD of primary trout hepatocytes, RTH-149 hepatoma cells, and

RTgill-W1 cells (G.K., unpublished observation). The elevation of $Ca^{2+}i$ may thus only be important when it occurs acutely during or following cell swelling, whereas prolonged $Ca^{2+}i$ increase is no longer perceived by otherwise sensitive mechanisms.

Mitogen-Activated Protein Kinases. Mitogen-activated protein kinases (MAPKs) are generally considered as regulators of cell survival, differentiation, and cell death, and as such they have been found to be involved in the adaptive response of *Fundulus heteroclitus* gill epithelium during the freshwater/sea water transitions (Kultz and Avila, 2001). Above this, however, MAPKs may also be responsive to acute cell volume changes and contribute to volume regulatory responses (Hoffmann et al., 2009). This is also true for fish cells, where it was seen that extracellular signal-regulated MAPK ERK and p38-MAPK are activated upon hypotonic exposure in primary hepatocytes, hepatoma cells, or a gill cell line from trout (Ebner et al., 2006; Krumschnabel et al., 2007), and where inhibition of these MAPKs, but not of JNK, significantly diminished RVD. Interestingly, although ERK shuttled between the cytosolic and the nuclear compartment upon hypotonic stimulation in the gill cell line, it remained strictly cytosolic in hepatocytes and hepatoma cells, indicating that it may acutely rather impact on cytosolic and/or membrane localized targets than acting as transcriptional activator (Ebner et al., 2007).

In turbot hepatocytes, only inhibition of p38 activation diminished RVD, whereas preventing ERK activation had no effect in these cells (Ollivier et al., 2006c) or in skate red blood cells (Hubert et al., 2000). Furthermore, it was reported that p38 and JNK are responsive to osmotic shock in opercular epithelium of the killifish and that p38 seems to be involved in the activation of Cl^- secretion in these cells (Marshall et al., 2005). In several of these studies, it appeared that PKC is also involved in the RVD response, either directly or through a crosstalk with MAPKs, suggesting that a complex regulatory network is yet to be unraveled.

Cytoskeleton Elements. A structure closely related to cell shape, cell volume, and membrane localized transporters, but also acting as relay for multiple signaling pathways, is the cytoskeleton, and as such it may obviously play an important role in cell volume regulation. An early model, where major rearrangements of cytoskeletal elements and specifically of actin filaments, have been documented upon hypotonic exposure is the shark rectal gland (Ziyadeh et al., '92; Henson et al., '97). A hypotonic stress-induced decrease of actin filaments has been reported for eel intestinal epithelium (Lionetto et al., 2002). Furthermore, in this model, it was also shown that agents disrupting either actin filaments or microtubules significantly affected electrophysiological changes associated with the swelling response, although no direct measurement of RVD was presented (Lionetto et al., 2002). Direct evidence of an impairment of RVD by disrupting actin filaments was given for both attached and suspended trout hepatocytes, but in these cells microtubule disruption was without effect (Ebner et al., 2005). Apart from our observation that Na^+,K^+-ATPase was significantly diminished, whereas the hypotonic $Ca^{2+}i$ elevation was unaltered (Ebner et al., 2005), the nature of the ion transporter and/or signaling molecules affected by actin filament disruption in fish cells has not been addressed so far.

Arachidonic Acid Metabolites. Among uncountable signaling pathways, other than those already mentioned, a few studies on fish cells addressed those involving arachidonic acid (AA) metabolites. For example, in goldfish proximal renal tubules the inhibition of 5-lipoxygenase, an enzyme converting AA to leukotrienes, significantly inhibited RVD (Fugelli et al., '95). Although there was no concurrent inhibition of taurine efflux detectable, the stimulation of which is generally considered a main effect of AA metabolites in hypotonic swelling, inhibition of cyclooxygenase, which forms prostaglandins from AA, did reduce taurine efflux. Similar observations with regard to RVD were made in trout proximal renal tubules, with inhibition of 5-lipoxygenase being more potent than that of cyclooxygenase (Kanli and Norderhus, '98), whereas in turbot hepatocytes only inhibition of 5-lipoxygenase but not of cyclooxygenase affected RVD (Ollivier et al., 2006c). In the latter cells, it was also reported that exocytosis is enhanced and may contribute to RVD, presumably by serving as a way to release ATP (see Section 2.2), and this process was shown to be affected by 5-lipoxygenase and possibly some other molecules acting in a related context (Ollivier et al., 2006c).

Lack of RVD in Some Cell Types—A Fishy Peculiarity?

It is generally agreed that most cell types do regulate their volume as a necessity, as specific excessive swelling could rapidly lead to bursting of the cell membrane and thus cell death. Nonetheless, the capability to induce an RVD response is obviously either not developed in some fish cells or, as discussed later, it may not easily be detectable in some cells. The former seems to be true for hagfish (*Myxine glutinosa*) erythrocytes, which showed no RVD response upon hypotonic exposure, whereas those of two different lamprey species did (Nikinmaa et al., '93). Although all examined species are members of a primitive vertebrate group, Agnatha, they differ with regard to their evolutionary history and lifestyle, and it was suggested that the absence of volume regulatory capacity in hagfish reflects the lack of an ancestor exposed to freshwater and thus hypotonicity (Nikinmaa et al., '93).

In goldfish intestinal mucosal strips, hypotonic exposure induced cell swelling that was maintained for more than 60 min and was fully reversible upon restoration of isotonic conditions (Groot, '81). It was suggested that such a lack of RVD might be of importance for the maintenance of epithelial barrier function. However, intestinal preparations from *G. niger* (Trischitta et al.,

2004) and eel (Lionetto et al., 2005) were capable of volume regulation, arguing against this. Similarly, we observed that goldfish hepatocytes do not display RVD in standard experimental settings, keeping their volume unregulated for at least 5 hr of exposure (Espelt et al., 2003). One might, therefore, suspect that the unusual capability of goldfish cells to withstand other various stress conditions (Krumschnabel et al., '96, '94) comes at the price of an apparent inability to volume regulate by its cells and tissues. However, proximal renal tubules from goldfish are clearly capable of an RVD response (Terreros et al., '90) and, as shown below (Section 2.6.), the apparent absence of volume regulation may in this case in fact only reflect incomplete/inappropriate experimental conditions. Basically, the same applies for human erythrocytes, which for more than six decades were thought to lack RVD mechanisms, until it was recently shown that activation of specific surface receptors (Section 2.6.) can trigger RVD in these cells (Pafundo et al., 2010).

EXTRACELLULAR NUCLEOTIDES AND P RECEPTORS SIGNALING

The numerous studies discussed so far showed that (most) fish cells can regulate their volume when exposed to conditions eliciting cell swelling, and that teleost fish use a vast array of different mechanisms and signaling pathways for this purpose. However, although there is abundant information regarding the intracellular mechanisms mediating volume regulation, there is relatively little on extracellular signaling events contributing to this process. Specifically, although RVD was shown to depend on extracellular nucleotides interacting with membrane-bound P receptors (i.e. receptors binding di- and trinucleotides and adenosine; Lazarowski et al., 2003) and ectonucleotidases (Pafundo et al., 2008), the issue has not been studied systematically in mammals or fish. Given that in some cases P receptor-mediated signaling induces RVD (see Section 2.6), understanding this pathway seems rather important. Thus, to understand nucleotide-dependent RVD, it is essential to analyze (1) how nucleotides and adenosine accumulate in the extracellular space (nucleotide transmembrane transport), (2) how nucleotides are interconverted by ectonucleotidases (nucleotide extracellular metabolism), and (3) how nucleotides activate different P receptors. Before we consider these questions in detail, a brief general introduction will be given on the subject of extracellular nucleotides and P receptors.

Ubiquity of Extracellular Nucleotides

Nucleosides and nucleotides are essential components of any biological system. They are part of the nucleic acids encoding our genetic information, they act as a source of molecules fuelling most metabolic processes, and they participate as cofactors in many biochemical reactions (Lazarowski et al., 2003). Particularly ATP, which very early in evolution was selected as one of the main coins of energy exchange, became an indispensable feature of life (Burnstock et al., '70).

According to their presumed primary role in cell metabolism, for decades nucleotides were thought to be strictly intracellular until 1970, when Geoffrey Burnstock demonstrated that ATP can be released from presynaptic neurons and act as a neurotransmitter (Burnstock et al., '70; Burnstock, '72). However, yet another 20 years had to pass until the full relevance of this finding became obvious and nucleotides were considered key factors of extracellular signaling in virtually all tissues and cell types (Burnstock, 2006). Accumulated evidence has since then shown that not only ATP but also other uridine and adenine di- and trinucleotides, as well as adenosine that accumulated in the extracellular space are able to modulate a broad range of cell and organ functions, such as cell swelling, vascular tone, immune responses, muscle contraction, cell proliferation, mucociliary clearance, platelet aggregation, and neurotransmission (Grygorczyk and Hanrahan, '97; Beigi et al., '99; Yegutkin et al., 2000; Sorensen y Novak, 2001). As mentioned above, all these effects are mediated via a series of nucleoside and nucleotide-selective P receptors.

Pathways of ATP Release

All cells can release ATP either by lysis or through regulated pathways. The regulated release of ATP has been identified in various cell types from lower and higher vertebrate species, where extracellular ATP can act as a potent autocrine/paracrine signal, including hepatocytes from goldfish, trout, and turbot (Pafundo et al., 2004; Ollivier et al., 2006b; Pafundo et al., 2008), vascular cells and erythrocytes from trout (Jensen et al., 2009), frog renal epithelia (Jans et al., 2002), and astrocytes, circulating lymphocytes, monocytes, and erythrocytes from mammalian systems (Neary et al., 2003; Hanson et al., 2010) as well as many transformed cell lines (Schöfl et al., '99; Tatur et al., 2007).

Irrespective of species or cell type, three general mechanisms for regulated ATP release have been proposed (Fig. 1) including (a) a conductive pore (channel), (b) a solute transporter, and (c) exocytic release of ATP-enriched vesicles (Fitz, 2007; Praetorius and Leipziger, 2009).

At physiological pH, most ATP molecules exist in anionic form (Smith et al., '91). On the other hand, in all vertebrate cells studied so far, the electrochemical gradient across the plasma membrane for the prevalent anion forms of ATP is at least three orders of magnitude (Sabirov and Okada, 2005). Therefore, it is possible that an anion channel or transporter can electrogenically transport ATP, thereby serving as a conductive pathway for ATP release.

Although various channels and transporters have been implicated in the process, many of them proved to be only indirect modulators of the actual—still not unambigously determined—ATP transporter. Among the current candidates for driving regulated ATP efflux are: ATP-binding cassette

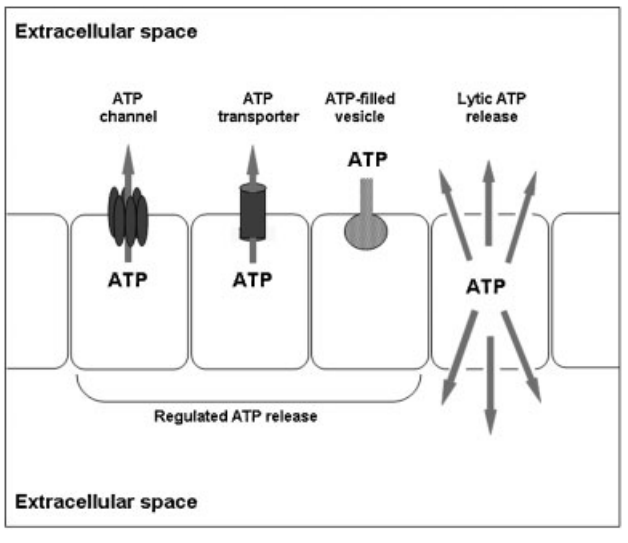

Figure 1. A simple scheme showing different mechanisms for ATP release from cells.

transporter proteins (such as the multidrug resistance proteins, cystic fibrosis transmembrane conductance regulator, and the multiple organic anion transporters) and plasma membrane variants of the voltage-dependent anion channels found in mitochondria. In addition, ATP release can be mediated by hemichannels composed of protein subunits of the connexin family (Cx) (Cotrina et al., '98; Dubyak, 2009). Although enhanced ATP release was observed in C6 glioma cells and epithelial HeLa cells overexpressing Cx43, *Xenopus* oocytes transfected with Cx43, Cx46 and Cx50 are known to form cation-selective but not anion-selective transporters. Also, connexin overexpression was reported to be associated with altered expression of other genes (McLachlan et al., 2006). Thus, it is possible that Cx expression upregulates the activity of an ATP-releasing anion channel which is distinct from the Cx-hemichannel (Sabirov and Okada, 2005). VRAC are found in virtually all cell types and could in principle also permeate ATP in certain experimental conditions. These channels are present in trout erythrocytes (Egée et al., 2000), frog (*Rana esculenta*) skin and urinary bladder and in oocytes, and A6 epithelial cells of *Xenopus* (Schmieder et al., 2002). However, pharmacological studies conducted in various human cell lines and Chinese hamster ovary cells argue against the actual involvement of this channel in ATP release (Sabirov and Okada, 2005). Similar conflicting data allow ruling out the cystic fibrosis transmembrane conductance regulator as a direct ATP transporter, although its function has been postulated to modulate ATP transport (Sprague et al., '98). Recently, pannexins have been postulated as potential ATP channels. These proteins share a membrane topology similar to the invertebrate gap junction channels innexins (an invertebrate ortholog) and the mammalian connexins. Pannexins, such as connexins, might function as nonselective channels to molecules of less than MW 1,000 (ATP molecular weight $\cong$507). Among the three pannexins found in mammals, pannexin-1 was identified as a protein associated to the ionotropic P receptor P2X7, which seems to either be a large pore itself or to be responsible for activation of a large pore capable of carrying ions and signaling molecules between the cytoplasm and the extracellular space (Dahl and Harris, 2009). As such, pannexin-1 is a candidate ATP release channel in various cell types, including erythrocytes, astrocytes, bronchial epithelial cells, and taste cells. In support of this notion, in situ hybridization and immunohistochemistry show that pannx1 is widely expressed in most cell types (Dahl and Harris, 2009). Furthermore, electrophysiological analysis showed mammalian pannexin-1 expressed in *Xenopus* oocytes to be highly permeable to ATP, suggesting that it could in vivo transport ATP down its transmembrane electrochemical gradient (Locovei et al., 2007). Irrespective of the pathway leading to ATP release, it has been observed that ATP and other nucleotides can induce the release of ATP, which in the case of ATP-induced ATP release constitutes a positive feedback loop.

The above-described findings show a still speculative picture of potential ATP transporting pathways of conductive ATP release, so that an unequivocal characterization of a plasma membrane ATP pore remains to be performed. This is one of the main reasons why, in the mathematical models for ATP kinetics presented within this review, the ATP release pathways will be considered as a black box whose properties are not based on solid molecular data, but are deduced from experiments in which the dynamics of extracellular ATP accumulation after stimulation of cells was studied.

Regarding the vesicular pathway in classically secreting cells, such as neurons and neuroendocrine cells, ample evidence indicates that ATP release occurs via exocytosis. More recently, vesicular transport of ATP has also been found in nonexcitatory tissues, such as endothelial, intestinal, ureter epithelium, and biliary cells (see Lazarowski et al., 2004). In hepatic cells, there is evidence for both, a conductive channel-mediated pathway and ATP efflux via exocytosis: stimuli that increases ATP efflux correlate with a strong increase of exocytosis, whereas inhibition of this process decreases regulated ATP release (Fitz, 2007; Feranchak et al., 2010). Such observations have also been reported for fish hepatocytes, where hypotonic swelling led to enhanced exocytosis and the release of ATP (Ollivier et al., 2006c), and both processes showed at least overlapping sensitivity toward various inhibitors of signaling pathways. Furthermore, not only conductive transport and exocytosis can be operative in the same cell type, but it has been hypothesized that exocytosis could also lead to insertion of ATP-permeable ion channels (Fitz, 2007).

In addition to the pathways of regulated ATP release described above, purine and pyrimidine nucleotides can also be released from dying cells, as has been demonstrated in swollen goldfish

hepatocytes (Pafundo et al., 2004). As discussed later, irrespective of the action of other nucleotides, the lytic release of ATP even from a very small subpopulation of dying cells may constitute a potent signaling factor modulating extracellular ATP (ATPe) dynamics and consequently RVD.

Immediately after release, ATP and other nucleotides are enzymatically degraded and interconverted by an extended family of ectonucleotidases (Zimmermann, 2006); this process is physiologically relevant, as ATP metabolites also act as signaling molecules of P receptors.

P Receptors

Nucleotides and adenosine act upon target cells through activation of metabotropic P1 receptors to adenosine and nucleotide receptors of the P2 family, the latter being further subdivided into P2Y metabotropic and P2X ionotropic subclasses (Lazarowski et al., 2003; Burnstock, 2007). This classification is mostly based on cloned receptors from mammalian species; although based on pharmacological studies, P receptors were found in all cell types of all vertebrates species studied so far (Burnstock and Verkhratsky, 2009).

To date, the P1 class includes four types of G protein-coupled adenosine receptors A1, A2A, A2B, and A3. These receptors are coupled with adenylate cyclase, with activation of the A1 and A3 receptors having an inhibitory effect, whereas A2A and A2B stimulate production of cyclic AMP (cAMP) (Burnstock, 2007).

The P2X class represents ionotropic receptors, which are classic cationic (Na^+, K^+, and Ca^{2+}) ATP-gated channels composed of seven major subunits dubbed P2X1 to P2X7 (Burnstock, 2007; Abbracchio et al., 2009).

P2Y ATP receptors are widely expressed in mammalian tissues and regulate a broad range of activities. Multiple subtypes of P2Y receptors have been identified and are distinguished both on a molecular basis and by pharmacologic substrate preference. P2Y receptors are nucleotide-sensitive G protein-coupled receptors, which act through second messenger systems of cAMP or inositol triphosphate (InsP3) (Abbracchio et al., 2009). In mammalian systems, these receptors couple predominantly to Gq (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11) which, therefore, activate phospholipase C-β and to Gi (P2Y11, P2Y12, P2Y13, and P2Y14), which inhibits adenylyl cyclase and regulates ion channels.

Except for some insects and roundworms, P2X receptors appeared with the first invertebrates, whereas P1 and P2Y receptors were evolved either at the same time or some time later (Burnstock and Verkhratsky, 2009). The teleost zebrafish *Danio rerio* exhibits both P2X and P2Y receptors. Seven zebrafish P2X-like genes, when expressed in HEK293 cells, expressed homomeric functional channels capable of generating cationic currents in the presence of exogenous ATP (Burnstock and Verkhratsky, 2009). Another zebrafish protein described displayed considerable homology to the mammalian P2X3 receptor, although the pharmacological profile was different. Also, in zebrafish genome, two orthologues for mammalian P2X4 and P2X5 receptors were identified.

Unlike the complex expression pattern of different P2Y receptors in different vertebrate species, in skate (*R. erinacea*) tissues a single P2Y receptor was cloned. When expressed in *Xenopus* oocytes, it showed a broad pharmacological selectivity related to the evolutionary forerunner of the P2Y1 receptor of higher vertebrates (Dranoff et al., 2000). When genomic DNAs of various animals were examined by dot blot, strong hybridization was seen to crab, skate, shark, and flounder, suggesting that P2Y receptor homologues are expressed in a range of organisms, with more closely related genes found in marine species (Dranoff et al., 2000).

In many nonmammalian vertebrates, P receptor-mediated effects of nucleotides/nucleosides are similar to those found in mammals. For example, in the heart of mammals, as in that of several amphibia, adenosine inhibits, whereas ATP stimulates the rate of heart beat. In the aorta of *R. temporaria*, P receptors were found with the pharmacological features of P2X (Knight and Burnstock, '96). Among fish, adenosine binding sites with characteristics of P1 receptors have been found in the brain and liver of goldfish, in the heart of carp, in the gills of the trout, and the cichlid *Oreochromis niloticus* (Burnstock, 2007; Pafundo et al., 2008). In zebrafish retina, a recent in vivo study demonstrated a crucial role for ADP as a paracrine signal in the repair of retinal tissue following injury (Battista et al., 2009). That is, extracellular ADP (but not ATP or adenosine) significantly enhances cell division. Intraocular injection of selective antagonists showed that the ADP effect was mediated by P2Y1 receptors.

Ectonucleotidases

As their name would suggest, ectonucleotidases are plasma membrane-associated nucleotidases that display their active site to the extracellular space. There are at least several different members in this group of enzymes, which hydrolyze ATP, ADP, and AMP to adenosine. These include E-NTPDases (ectonucleoside triphosphate diphosphohydrolase), a family of enzymes that hydrolyze nucleoside diphosphates and triphosphates, ecto-5′-nucleotidases that promote the dephosphorylation of monophosphate nucleotides ectoadenylate kinase, which converts two ADP to ATP and AMP, an ectonucleoside diphosphate kinase, which interconverts di- and trinucleotides, and ectoadenosine deaminase that converts adenosine to inosine (Zimmermann, 2006). Similar to P receptor signaling, most enzymes' action described takes place at the extracellular face of the cell membrane (Fig. 2).

Phylogenetic analyses have shown that members of the E-NTPDase family are conserved among vertebrates (Masse et al., 2006). Alleva et al. (2002) identified two different E-NTPDases in a microsomal fraction from isolated hepatocytes of goldfish

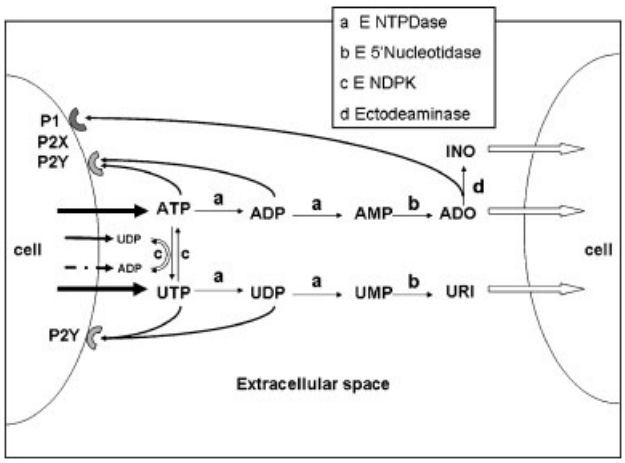

Figure 2. Metabolism and signaling of extracellular nucleotides. Di- and trinucleotides can be released from cells. In the extracellular space, nucleotides are substrates and products of different membrane bound ectonucleotidases. Except for nucleoside monophosphates, nucleotide metabolites can interact with different types of P receptors. Adenine and uridine nucleosides can be taken up by cells via specific transporters (not shown). All enzyme conversions and receptor signaling events illustrated in the scheme are occuring at the cell surface. E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; ADO, adenosine; URI, uridine; INO, inosine.

(*Carassius auratus* L.), whereas E-NTPDase activity and expression of E-NTPDases1, 2, and 3 have been reported in the nervous system of the zebrafish (Rico et al., 2003; Ricatti et al., 2009). Recently, an immunocytochemical localization of E-NTPDases 1 and 2 of zebrafish and mouse retina was reported (Ricatti et al., 2009). In mouse retina, E-NTPDase2 (an ectoATPase) was chiefly localized in Müller glia and ganglion cell processes. E-NTPDase1 (which hydrolyzes ATP and ADP with similar activity) was located on neurons as well, suggesting that nucleotides, such as ATP and ADP, can be hydrolyzed at the surface of these cells.

In several tissues, an ecto-5′-nucleotidase has been shown to hydrolyze extracellular AMP. Thus, in many cell types, such as goldfish and trout hepatocytes and in human airway epithelium, ATPe can be fully and sequentially dephosphorylated by the combined action of ENTPDases (which generate ADP and AMP from ATP), followed by ecto-5′-nucleotidase promoted conversion of AMP to adenosine. Also, membrane-enriched fractions of the catfish, *Rhamdia quelen*, were shown to exhibit E-NTPDase as well as ecto-5′-nucleotidase activities (Lermen et al., 2005).

Following its formation, extracellular adenosine may again be depleted from the cell surface by removal through either diffusive or perfusive fluxes and/or be taken up via either nucleoside transporters, acting as facilitated diffusion-limited channels or through the concentrative sodium/nucleoside cotransport (Hagberg et al., '87). Alternatively, adenosine can be directly inactivated on the cell surface via ectoadenosine deaminase activity with concomitant inosine formation.

RVD Induced by Nucleosides/Nucleotides

Diverse mechanical stimuli, such as shear stress, membrane stretch, media exchange, single cell micropipette stimulation, and hypotonic shock, are able to induce the release of ATP (Boudreault and Grygorczyk, 2004), with subsequent autocrine/paracrine activation of P receptors from endothelial and epithelial cells, from smooth muscle, fibroblasts, erythrocytes, and hepatocytes through unknown mechanisms. The notion that ATP release under hypotonic conditions, P receptors at the membrane, and the induction of volume regulatory processes may actually be related, and has first been suggested by Roman et al. ('96; Wang et al., '96). In this seminal study using murine hepatoma cells, a model has been proposed in which cell swelling triggers the release of ATP, leading to a localized increase of the nucleotide at the cell surface, and this ATPe then acts on P2 and thereby modulates volume regulatory processes (Fitz, 2007). Specifically, it has been suggested that P receptor activation by ATP triggered a signaling cascade which ultimately led to the efflux of osmolytes (e.g. an increase of Cl^- permeability). This efflux is accompanied by an osmotic loss of water and therefore constitutes an effector mechanism for RVD. Besides ATP, it is known that UTP and UDP can also be exported during an osmotic or mechanical challenge and may as well exert similar effects (Lazarowski et al., 2003).

The broader relevance of these findings has been confirmed in hepatocytes from several mammalian species (Lan et al., 2005), but also in hepatocytes from goldfish, trout (Pafundo et al., 2004), and the marine flatfish turbot (Ollivier et al., 2006b), where the removal of extracellular ATP (using nucleotide scavengers) or blockage of P2 receptors (using antagonists) inhibited osmolyte efflux and RVD. Moreover, a similar pattern was found in *Necturus* erythrocytes (Light et al., '99, 2001), extending the relevance to nonhepatic cells.

More recent reports allowed verifying and complementing this seminal model of nucleotide-induced RVD, with the following features:

(a) One important postulated role for ENTPDases is that they act to mediate a decrease by hydrolysis of di- and trinucleotides located at the cell surface (Robson et al., 2006) and thereby limit, in time and space, the action of extracellular nucleotides on P2-induced RVD (Pafundo et al., 2004).
(b) Ectonucleotidases not only degrade, but also interconvert nucleotides. Thus, another role attributed to the action of ectonucleotidases consists of their generation of natural P receptor agonists, such as ATP, ADP, adenosine, UTP, and UDP. The low submicromolar $K_{0.5}$ value for the interaction of most P receptors and their natural ligands is in the range of

the nucleotide/nucleoside concentrations; in fact, typically found in the extracellular space. In the case of ATP, the presence of less that 0.1% of their typical intracellular ATP content in the extracellular space is necessary to enable action as an extracellular signal, so that ATPe signaling does not pose an energetic burden for the cells (Schwarzbaum et al., '98; Joseph et al., 2003). Given the ubiquity of P receptors and ectonucleotidases in the animal kingdom, and the fact that ectonucleotidases are able to generate the above mentioned ligands, it is thus physiologically relevant to test the ability of nucleotides (other than ATP) and adenosine to modulate RVD.

(c) The most potent effects of extracellular nucleotides and adenosine are likely limited to specific extracellular microenvironments within tissues or in specific zones of the cell surface. These extracellular microenvironments may have a limited area of diffusion and a small volume (Schwiebert and Fitz, 2008). For example, in human astrocytes, P2 receptors seem to compete with ectonucleotidases for a limited ATP pool which is functionally segregated from the bulk extracellular compartment (Joseph et al., 2003). In this localized cell surface area, nucleotides could accumulate and be metabolized before diffusion into the bulk extracellular fluid. Plasma membrane subdomains may involve caveolae or lipid raft-based invaginations that enclose constrained extracellular spaces, and thereby retard rapid exchange with the bulk extracellular space (Joseph et al., 2003). For example, in caveolae of hepatoma and mammalian hepatocytes, a specific ectonucleotidase (ecto F1Fo-ATP) hydrolase (Martinez et al., 2003) promotes ATP hydrolysis. The extracellular ADP accumulated in this localized subdomain interacts with a $P2Y_{13}$ receptor to activate a specific cellular function, namely high density lipoprotein endocytosis (Jacquet et al., 2005).

(d) Studies on the action of ATPe on RVD have shown that the effective concentration of the nucleotide at the cell surface, and thus its action on P receptor-mediated RVD, depends on various processes acting simultaneously: ATP transport within the extracellular space, the rate of regulated ATP release, the kinetics of ectonucleotidases, and the rate of nucleotide release by cell lysis.

In Figure 2, we illustrate the pathways through which different nucleosides interact with transport proteins, ectonucleotidases, and P receptors. It can be seen that (1) cells may lose nucleotides by lysis or by one or more ways of regulated ATP transport; (2) several ectonucleotidases can degrade or interconvert nucleotides, generating multiple P receptor ligands whose concentrations vary with time. It is the complex interaction between nucleotide release, nucleotide metabolism, and nucleotide binding to P receptors that modulates a vast array of cellular responses. As we see later for goldfish hepatocytes under hypotonicity, one way to approach this complexity is through mathematical modeling, taking into consideration the experimental evidence gathered so far.

Nucleotide-Dependent RVD of Goldfish Hepatocytes

In the last 15 years, goldfish hepatocytes have developed into a model for studies of ionic balance and volume regulation, when cells are challenged by different physiologically relevant stimuli, such as hypoxia, chemical and physiological anoxia, hypothermia (Krumschnabel et al., '97, 2001; Mut et al., 2006), and relevant to this review osmotic transmembrane gradients (Espelt et al., 2003). These studies provided ample evidence that these cells are outstanding among vertebrate cells, as they are highly resistant toward energetic limitation, show considerable tolerance toward toxicants (Krumschnabel and Nawaz, 2004), and are an excellent, albeit unusual, model for the investigation of volume regulatory behavior (Pafundo et al., 2008).

With regard to volume regulation following anisotonic swelling of the cells, several aspects of nucleotide-dependent signaling were analyzed. An important initial observation with this cell model was that, when examined in standard experimental settings, goldfish hepatocytes exposed to anisotonic media were unable to trigger either RVD or RVI. The lack of volume regulation under hypotonicity appeared particularly striking, as in vitro cells remained swollen up to 1.7 times the isotonic reference volume for up to 5 hr without showing any sign of diminished cell viability. Moreover, swollen cells remained metabolically active, preserved Ca^{2+} and K^{+} homeostasis, and even increased their respiratory rate (Espelt et al., 2003).

Several other studies had hinted on the existence of functional P receptors coupled with Gq/phospholipase C/Ca^{2+}i in vertebrate hepatocytes. For example, in rat hepatocytes ATPe had been shown to bind to P2Y receptors, causing a rapid mobilization of Ca^{2+}i from intracellular Ca^{2+} stores via inositol trisphosphate (Dixon et al., 2005). Likewise, we had previously shown that in goldfish hepatocytes addition of low micromolar ATP was able to generate reversible increases in Ca^{2+}i, both in the presence and absence of extracellular calcium, suggesting the existence of P2Y receptors also in these cells (Schwarzbaum et al., '98). Furthermore, the observation that AMP-PCP, a nonhydrolyzable ATP analog, had no effect on Ca^{2+}i was consistent with its postulated action on P2X receptors, which are not linked to intracellular Ca^{2+} release. Thus, although P2Y receptors were neither purified nor cloned in goldfish hepatocytes, the pharmacological evidence pointed to the existence of P2Y receptors whose natural ligands are di- and trinucleotides of adenine and uridine.

Accordingly, when swollen cells—unable to execute an RVD response in standard conditions—were incubated in the presence of micromolar concentrations of ATP, UTP, and UDP, a significant RVD could be triggered. Moreover, experiments using

ATPγS (a slowly hydrolyzable ATP analog) showed that nucleotide induction of RVD was concentration-dependent and sensitive to blockage of P2 receptors (Pafundo et al., 2008)

Given that ectonucleotidases of goldfish hepatocytes can completely dephosphorylate ATP (Alleva et al., 2002; Pafundo et al., 2008) thereby generating extracellular adenosine, we wondered whether P1 receptor activation could also play a role in the regulation of RVD. Previous studies by our group on hepatocytes from trout had shown that activation of P1 receptors inhibited the late phase of RVD in these cells (Pafundo et al., 2004). Accordingly, we could confirm operation of an equivalent mechanism in the goldfish cells, where we observed that exogenous adenosine inhibited partially the RVD that had been induced by ATPγS, and that this inhibition could be blocked by P1 antagonists. Thus, in goldfish hepatocytes ATPe can activate RVD, and at the same time the ectonucleotidase dependent extracellular generation of adenosine will balance and control the RVD response.

The above-described experiments were mostly based on the use of exogenous nucleotides, and thus an important question remained whether goldfish hepatocytes can also be induced to trigger RVD by *endogenous* extracellular ATP, which we had not yet observed in our standard experimental conditions.

In order to tackle this problem, extracellular ATP was continuously assessed using a luminiscence method based on the ATP-dependent oxidation of luciferin (Pafundo et al., 2008). We observed that hypotonic exposure indeed caused a transient release of ATP from goldfish hepatocytes, leading to a nonlinear increase of ATPe to a maximum, followed by an exponential decrease of the nucleotide.

This again raised the question why, despite ATP release, no RVD could be observed. A thorough analysis of this suggested that, in principle, several reasons could underlie our apparently contradictive observations, some of which however were easily discarded:

A too high ectoATPase activity could degrade ATPe fast enough to prevent P2 activation—this is the principle behind the use of an excess of pure apyrase as a nucleotide scavenger that blocks RVD—but the measured ATPe kinetic showed a clear ATPe maximum, indicating that ATP release, at least transiently, clearly outpaced ATP degradation. Moreover, previous experiments had shown that even only a few minutes of exposure to exogenous ATP were sufficient to trigger RVD. Furthermore, even in the presence of AMP-PCP, which inhibited about 70% of ectoATPase activity and led to a significant increase in ATPe, no induction of RVD was observed.

Another possible reason for the lack of stimulation of RVD might be that the total mass of ATP released by the cells was insufficient to stimulate P2 receptors. However, experiments using the human hepatoma cells, which undergo nucleotide-dependent RVD, did not support this notion.

Both cell types released comparable ATP masses in similar hypotonic conditions (Pafundo et al., 2008). Finally, we considered the possibility that the affinity of P2 receptors for nucleotides might be too low to respond to the ATP released from the cells, which under standard experimental conditions was markedly diluted by the experimental saline. We, therefore, established experimental conditions in which the assay volume was decreased to such an extent that endogenous ATPe concentration matched the low micromolar exogenous ATP concentrations used to stimulate RVD. Indeed, this treatment induced RVD in the absence of extrinsic nucleotides, and this volume regulation response was completely blocked by P2 receptor antagonists, showing that in goldfish hepatocytes RVD can be induced by the interaction of P2 receptors with endogenous extracellular ATP. In the long term, ATPe is completely dephosphorylated to adenosine, and this may interact with P1 receptors to inhibit RVD (Fig. 3). Altogether, these results indicate that ATP released from goldfish hepatocytes exposed to hypotonic conditions is an important trigger of the RVD response when examined under appropriate conditions, and that the lack of RVD in a larger experimental volume is owing to excessive dilution of the nucleotides derived from the cells.

The information provided by the volumetric behavior of goldfish hepatocytes allows complementing Wang's seminal model of nucleotide-dependent RVD. A new hypothetical model includes not only P2, but also P1 receptors that control volume regulation, and ectonucleotidases that determine the effective

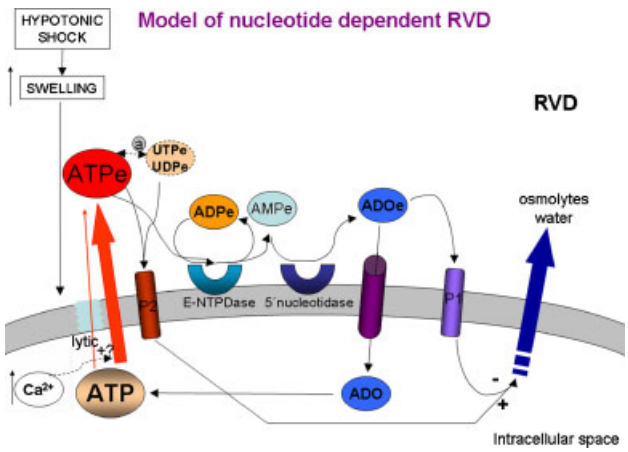

Figure 3. Scheme illustrating steps involved in RVD of goldfish hepatocytes. The term "a" denotes the reversible conversion of extracellular ATP (ATPe) into extracellular UTP (UTPe) and vice versa, promoted by ectonucleoside diphosphate kinase activity. The red arrow denotes pathways of regulated release of intracellular ATP (ATPi), whereas "lytic" accounts for ATP release by cell lysis. Activation of P1 and P2 leads to RVD inhibition (-) or activation (+). Increases in cytosolic free Ca^{2+} (Ca^{2+}i) activate regulated ATP release by an unindentified mechanism. Once in the extracellular medium, ATP can diffuse.

time-dependent concentration of the different nucleotides and adenosine.

As illustrated in Figure 3, in a constant hypotonic environment, cell swelling results in an increase of cytosolic $Ca^{2+}i$ together with regulated ATP release. In many but not all systems, this elevated $Ca^{2+}i$ is the main trigger for regulated ATP exit (Boudreault and Grygorczyk, 2004). When goldfish hepatocytes are highly diluted, a condition commonly seen in all experimental protocols, the relatively low ATPe concentration resulting from this ATP release, together with a hypothetical low affinity of P2 receptors, fails to induce RVD. However, low micromolar concentrations of exogenous nucleotides (ATP, UTP, UDP, and ATPγS) interact with P2 receptors to trigger the volume regulatory response. Similar to the response in *Necturus* erythrocytes (Light et al., 2001), in goldfish hepatocytes extrinsic ADP does not contribute to RVD (Pafundo et al., 2008).

In summary, we observed that when assay volume is reduced to a minimum, sufficient endogenous ATPe is accumulated near the surface of these cells to induce the loss of osmolytes and water, therefore triggering RVD. Extracellular ATP concentrations will change rapidly according to the rates of ATP release, ectoATPase activity, and ATPe transport within the extracellular space. In the long term, ATPe is completely dephosphorylated—by ectonucleotidases—to adenosine, and adenosine may interact with P1 receptors to inhibit RVD. So, clearly ectonucleotidases are as important as P receptors to determine the kinetics of volume regulation.

MODELING APPROACHES TO UNDERSTANDING HYPOTONIC CELL VOLUME REGULATION

Fish cells can be utilized as valuable models for the study of volume regulatory processes providing results of interest not only for understanding the biology of the fish cell, but also, more generally, for the biology of vertebrate cells. However, even the most detailed description of mechanisms and pathways will not enable us to understand how cell actually regulate their volume, as long as the interplay between all the components identified is not fully understood. A valuable approach to do so lies in the development of mathematical models. These can then be continuously refined and adjusted to new experimental data, e.g. indicate which components need to be experimentally investigated in more detail. Furthermore, mathematical models may lead to insights in which (experimental) conditions need to be considered to obtain meaningful data describing the in vivo situation experienced by cells. Altogether, numerous facets that may be useful or even imperative to truly understand cell volume regulation may be derived from the development of mathematical models. Over the past years, we have developed such mathematical models primarily based on our own experimental data and taking into account what we have found to be crucial for the assessment of cell volume regulation in our experimental cell systems, i.e. in hepatocytes from teleost fish. In the following paragraphs, we describe the basal considerations essential for the development of such a model and then focus on the aspect we have paid particular attention to in our research, the above described importance of extracellular nucleotides, and of P receptors for cell volume regulation.

Mathematical Models Simulating the Kinetics of Cell Volume Changes and Regulation

For the average scientist, mathematical models are difficult to grasp. This is because even a simple model usually includes the kinetic analysis of various processes that interrelate in a complex, nonlinear way. Thus, to describe volume regulation models, we make several simplifications and focus on the essential aspects that explain how cell volume can change in a hypotonic environment, and following hypotonic swelling, how swollen cells can downregulate their volume through RVD. Although cells use many chemically different osmolytes to induce volume changes (Hoffmann et al., 2009), here we only focus on ions as the sole osmolytes that can drive the loss of water during downregulation of cell volume.

In a first minimal model. the cell membrane behaves as an osmometer where only water is permeable. In the absence of volume regulatory mechanisms, a hypotonic challenge leads to a net water influx until a new equilibrium is achieved.

Model 1: Cell Swelling in the Absence of RVD. We first assume a nonpolarized spherical cell (Fig. 4A) where water can move across the cell membrane through lipid bilayer or water channels (aquaporins; Ishibashi et al., 2009) The net flux of water can be described according to the phenomenological law of osmosis (Pickard, 2008).

$$J_V = P_f A \sum_i \left(\frac{m_i \sigma_i}{V} - C_{ei} \sigma_i \right) \tag{1a}$$

where J_V, P_f, and A are the water flux across plasma membrane, the permeability coefficient for water, and the membrane area, respectively. The intracellular osmotic activity represents the sum of all the number of moles corresponding to the major contributing solutes (m_i) divided by volume (V) times the reflection coefficient[1] for that solute (σ_i) and the extracellular osmotic activity is the concentrations of the major solutes (C_{ei}) times the same reflection coefficient. Because the reflection coefficients are assumed symmetrical, this simplifies to:

$$J_V = P_f A \sum_i \sigma_i \left(\frac{m_i}{V} - C_{ei} \right) \tag{1b}$$

[1] The reflection coefficient is a measure of the osmotic response across a membrane for a given solute in relation to the response generated in the same system due to a completely impermeable solute. Then, it depends on the solute permeability. It is equal to 1 only in the case that solute permeability is zero, with the membrane being completely impermeable to the solute. A reflection coefficient of one thus implies that all the solute is reflected "back" from the membrane. When this coefficient is zero, the membrane does not retain the solute in one compartment. In other words, the contribution of osmotic pressure due to this solute in this last case is negligible.

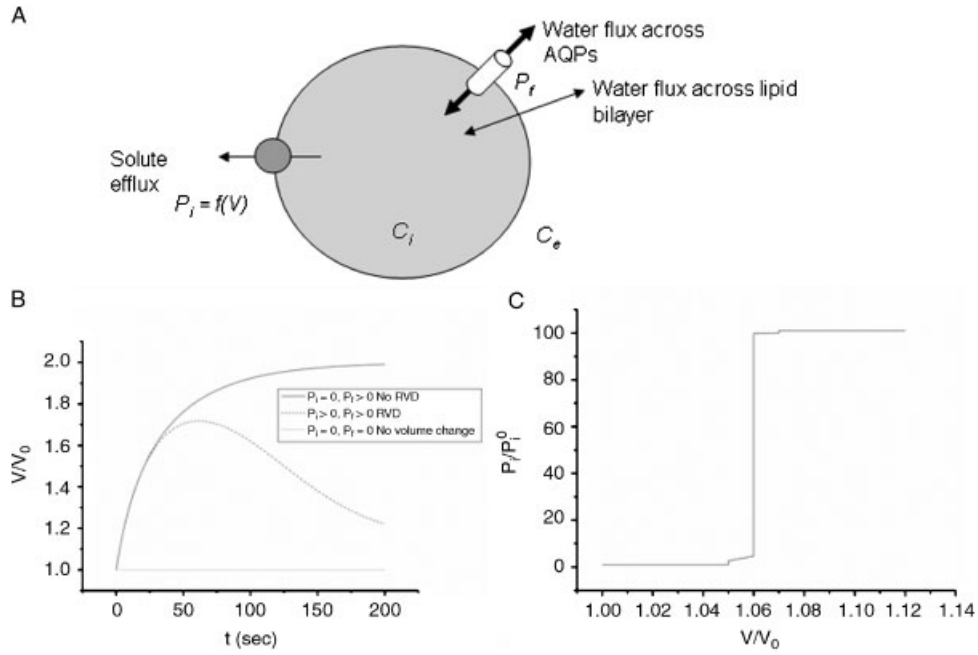

Figure 4. Model of transport mechanisms involved in changes of cell volume and RVD during hypotony. (**A**) Water can transverse the membrane through aquaporins (AQPs) and the lipid bilayer, with P_f being the osmotic permeability coefficient accounting for both modes of water transport. In the absence of net solute transport ($P_i = 0$), there is no RVD. In contrast, RVD is initiated when the increase in cell volume of swollen cells triggers net solute efflux ($P_i > 0$) accompanied by osmotically driven water ($P_f > 0$). C_i and C_e denote intracellular and extracellular osmolarities, respectively. (**B**) Time course of relative cell volume (V/V_0) for a cell impermeable to both water ($P_f = 0$) and solutes ($P_i = 0$), resulting in no volume change (dotted curve), a cell permeable to water and impermeable to solutes causing unopposed swelling (continuous curve), and for a cell showing permeability to water and to solutes and thus displaying volume regulation (dashed curve). (**C**) Activation of relative solute permeability (P_i/P_i^0) by alterations of relative cell volume (V/V_0) simulated according to the model reported by Strieter et al. ('90). Both parameters (P_i and V) were normalized by their initial values (i.e. P_i^0 and V_0).

In practical cases, Equation (1b) could simplify further, if there are fewer solutes involved and if they are effectively impermeable, and for most solutes σ_i could approach 1.0.

If the rate of change of cell volume (dV/dt) is only affected by the water flux, then:

$$J_V = \frac{dV}{dt} \tag{1c}$$

so that

$$J_V = \frac{dV}{dt} = P_f A \sum_i \sigma_i \left(\frac{m_i}{V} - C_{ei}\right) \tag{1d}$$

Equation (1d) allows modeling cell volume changes in the absence of volume regulation. Thus, in isotonic medium, the osmotic activities at both sides of membranes are equal, so that

$$\sum_i \sigma_i \left(\frac{m_i}{V} - C_{ei}\right) = 0$$

and J_v is zero, so that cell volume remains unchanged (Fig. 4B). However, when extracellular osmolarity is reduced,

$$\sum_i \sigma_i \left(\frac{m_i}{V} - C_{ei}\right) > 0,$$

J_v turns positive (i.e. a water influx is turned on) leading to cell swelling (Fig. 4B) until water equilibrates in the intra- and extracellular compartments

$$\sum_i \sigma_i \left(\frac{m_i}{V} - C_{ei}\right) = 0,$$

where again J_v is zero. In this way, the model works as a negative feedback system where changes in J_v oppose the osmotic gradient, leading the system to osmotic equilibrium.

Unlike in this minimal model, in a real cell, ions are indeed able to permeate the cell membrane, in which case the model should include an expression describing the passive flux of an ion in terms of the chemical and electrical forces present at the cell membrane (Goldman, '43), as modified by Hodgkin and

Katz ('49):

$$J_i = P_i u \left(\frac{C_{ei} - \frac{m_i}{V} e^{-u}}{1 - e^{-u}} \right) \quad (1e)$$

The electrical forces are included in the term "u," whereas the difference in extra- (C_{ei}) and intracellular (m_i/V) concentrations of a given ion—weighted by a factor dependent on u—account for the electrochemical gradient. For the sake of simplicity, the expression of only a single ion permeability (P_i) is displayed; a more realistic model would require the flux expression for each ion.

However, in isotonic steady state, these movements do not contribute to volume changes. This is because the influx of each permeable ion (Na^+, K^+, Cl^-, Ca^{2+}, etc) is balanced with its corresponding efflux, so that in steady state, irrespective of minor fluctuations, the net flux of each ion species is 0 and the ion concentrations at both sides of the cell membrane remain unaltered. A detailed description of the processes allowing a cell in steady state to balance the transmembrane fluxes of permeable ions can be seen in Hernandez and Cristina ('98). A different situation occurs during exposure to anisotonic media, where models should account for a net transport of water driven by a net flux of osmolytes. In principle, to account for net water transport through the membrane, one should consider not only *Jv*, as defined in Equation 1b, but also the contribution of solvent drag by net solute transport. However, the latter can be considered negligible, because in most animals the majority of solvent flow is via aquaporin channels that are quite selective for water (Parisi et al., 2007).

Given that, as explained above, cells do display basal ion permeabilities in isotonic medium, one could simulate volume regulation by assuming a change in ion permeability during the transition from isotonic to anisotonic conditions. Such volume regulation models include membrane-bound channels and/or transporters allowing net fluxes of osmotically active ions.

Model 2: RVD Induced by Changes in Solute Permeability. To account for RVD, we consider the magnitude of ion permeability P_i to change during the isotonic–hypotonic transition (Fig. 4B and C). In this respect, Hviid Larsen and Kristensen ('78) suggested for toad skin that chloride permeability could be variable, and Ussing proposed that in hypotonically exposed frog skin, the net loss of K^+ and Cl^- is mediated by an increase in the number of functional channels and electroneutral cotransporters (Ussing, '82). Strieter et al. ('90) considered this idea in a mathematical model explaining volume changes in a tight epithelium. Here, ion permeability (in this case, for chloride) was a function of cell volume (V) as follows:

$$P_i = P_i^0 \left(1 + \frac{M_i}{1 + \exp(-\xi(V - V_{1/2}))} \right) \quad (2)$$

where P_i^0 is the basal isotonic permeability, M_i is the maximum factor by which permeability may increase, $V_{1/2}$ is the cell volume at which the change in permeability is half of its maximum value, and ξ is the compliance (inverse of stiffness) of the basolateral membrane.

As can be seen in Figure 4C, following swelling P_i is described by a sigmoidal function of cell volume. This permeability expression is compatible with a steplike saturable activation of channels which, in the case of tight epithelium modeled by Strieter et al. ('90), correlates with basolateral K^+ and Cl^- channels that are activated by mechanical forces acting during swelling. The idea of variable permeability was further applied in models describing volume changes in airway epithelia (Novotny and Jakobsson, '96), in cardiac cells (Terashima et al., 2006) and, more recently, in cells from renal cyst (Gin et al., 2010).

Model 3: A Volume Sensor Triggers Activation of an RVD Effector Mechanism. Hernandez and Cristina ('98) proposed that RVD can be triggered by an ion cotransport mechanism which is activated by a cell volume sensor, but remains silent during isotonic conditions. Accordingly, in a hypotonic environment where cells rapidly swell, they propose basal ion permeabilities to remain fixed, whereas a previously dormant cotransport mechanism becomes activated, allowing the simultaneous loss of one anion per one cation, in this case represented by KCl, the net loss of which leads to RVD (Fig. 5A).

That is, in addition to basal ion fluxes (J_i in Equation (1e)), there is an electroneutral flux of K^+ and Cl^- ($Q_{K:Cl}$) which is activated after cell volume (V) reaches a value equal to that where a volume sensor (given by the parameter v^+) is triggered ($V \geq v^+$) (Hernandez and Cristina, '98). Thus, $Q_{K:Cl}$ is given by

$$Q_{K:Cl} = Q^*_{KCl} \left(\frac{v^+ - V}{v^+} \right) \left(C^e_{K^+} C^e_{Cl^-} - \frac{m_{K^+} m_{Cl^-}}{V^2} \right) \quad (3)$$

where $Q_{K:Cl}$ is a function of cell volume, and $Q^*_{K:Cl}$. is a proportionality constant related to the effective concentration of functional transporter units mediating net KCl loss during cotransport. C^e denotes extracellular concentrations and "m" the intracellular number of moles corresponding to the implicated osmolytes.

As seen in Figure 5B, in hypotonic medium there is a steep increase in cell volume (V). When V achieves a threshold volume called v^+, KCl cotransport is activated.

In this model, Q_{KCl} remains silent until $V \geq v^+$ (see Fig. 5C). A close examination of Equation 3 shows that RVD can be blocked when $Q^*_{KCl} = 0$ (i.e. transporters are absent or inactivated), if $V < v^+$ and/or if either K^+ or Cl are absent.

In vivo, a time lag ranging from a few seconds to several minutes is to be expected between the moment at which there is a volume change and activation of the RVD effector mechanism driven by the efflux of solutes.

Thus, in terms of the model, when $V = v^+$, a lag time (τ) is introduced before $Q_{K:Cl}$ can be activated, so that the onset of $Q_{K:Cl}$

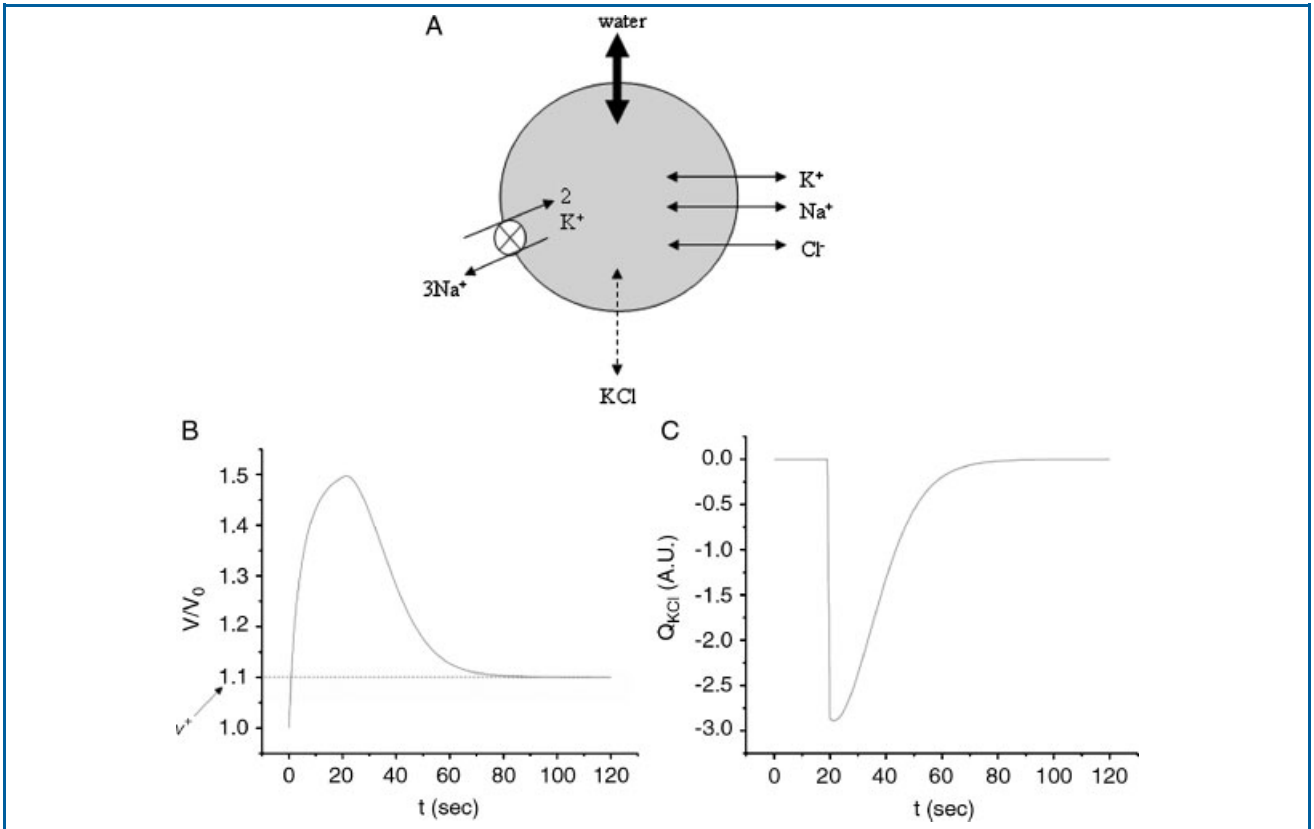

Figure 5. Model of a cell with RVD triggered by solute co-transport. (**A**) Cartoon of a cell showing diffusive pathways for Cl^-, Na^+, and K^+ (continuous double-headed arrows), the Na^+-K^+ pump (crossed circle), and cotransport fluxes of KCl (KCl, double-headed arrow dashed line). According to Hernandez and Cristina ('98), in many cell types, including red cells and hepatocytes from fish, KCl cotransport is the main effector mechanism activating the RVD. (**B**) Time course of relative cell volume (V/V_0) for a cell showing volume regulation. v^+ denotes the threshold volume at which an electroneutral KCl flux (see "c") is triggered. (**C**) Kinetics of KCl cotransport responsible for cell volume regulation. By convention, KCl flux (Q_{KCl} in arbitrary units, A.U.) is negative indicating solute efflux. By comparing the kinetics of cell volume (b) and KCl cotransport flux (c), it can be seen that Q_{KCl} activation is right shifted—i.e. delayed—compared with the time at which cell volume equals v^+. As expected, RVD is triggered almost simultaneously with Q_{KCl} activation.

(see Fig. 5C) is right shifted compared with the moment at which $V = v^+$ (Fig. 5B).

In line with this model, a pharmacological characterization of ion transport processes showed that swelling of trout hepatocytes activated an otherwise dormant cotransport mechanism that promoted KCl loss and RVD (Bianchini et al., '91). Moreover, a mathematical model considering the basic features of model 3 was successfully applied to characterize volume regulation in hepatocytes from rat, trout, and goldfish (Espelt et al., 2008). As seen in Figure 6, in trout and rat hepatocytes exposed to hypotonicity, the model simulates a steep net efflux of K^+, which according to the model proposed is accompanied by efflux of Cl and water. The loss of water, in turn, allows RVD to take place. The dotted lines of Figure 6A and C illustrate the hypothetical case where RVD is blocked.

Model 4: Adjusting the Extent of RVD (to Account for Generally Incomplete Volume Recovery). Assuming that $\sigma_i = 1$ for each solute i and that the total intracellular number of moles corresponding to solutes as $m_s = \sum_i m_i$, Equation (1d) can now be expressed as:

$$\frac{dV}{dt} = P_f \, AV_w \left(\frac{m_s}{V} - C_e\right) \tag{4a}$$

To account for the passive fluxes of both water and solutes, Lucio et al. (2003; Warren et al., 2009) modeled the kinetic of volume changes where time dependent changes in m_s are governed by a generic solute (in our considerations, ion) flux J_s:

$$J_s = \frac{dm_s}{dt} = -\alpha\left(\frac{V - V_R}{V_0}\right) \tag{4b}$$

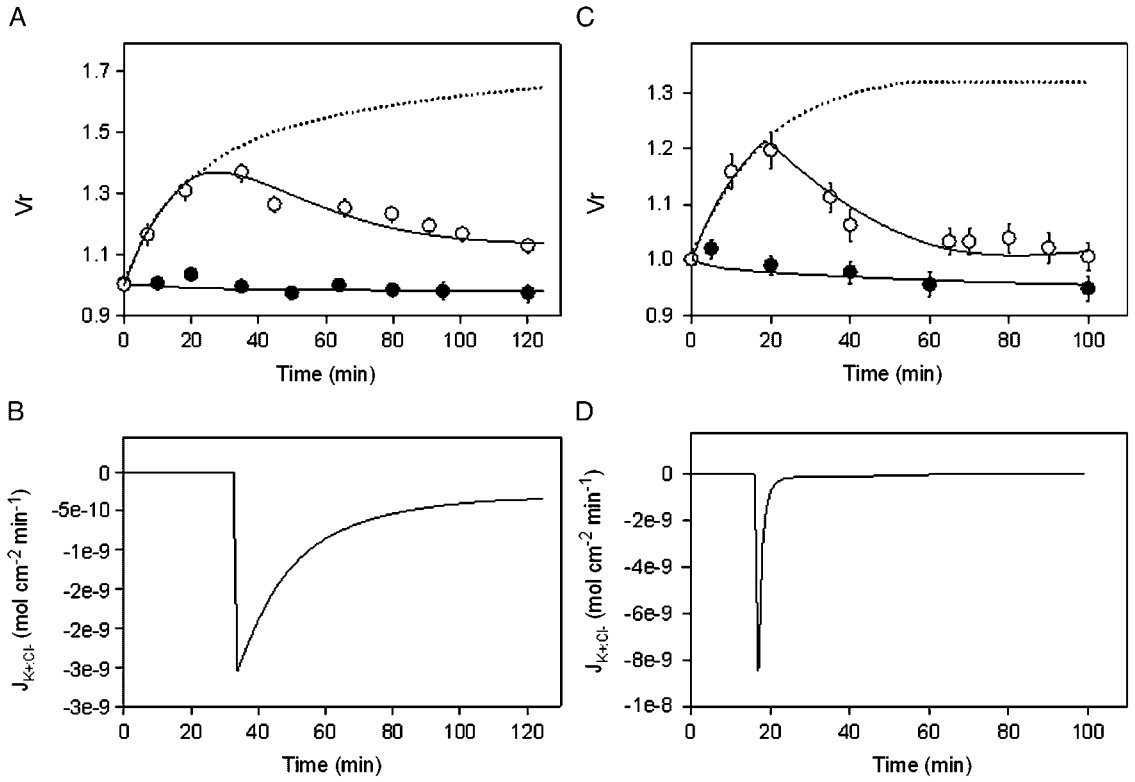

Figure 6. Experimental and theoretical results of rat (A, B) and trout (C, D) hepatocytes exposed to hypo-(180 mOsm) and isotonic media. A and C: time course of Vr of cells exposed to iso- (closed circles) and hypotonic (open circles) media. Values are means ± SEM of four independent experiments. The continuous and dotted lines represent predictions made by the mathematical model of the hypotonically induced volume change in the presence (continuous lines) and in the absence (dotted lines) of vse (volume sensor). B, D: Simulation of a potassium chloride flux ($J_{K:Cl}$) allowing the RVD response observed in A and C. *Reproduced with permission from Comparative Biochemistry and Physiology, Part B 150(2008):103–111.*

where α is a proportionality constant, V_O is the initial isotonic volume (before the anisotonic exposure), and V_R is the final volume obtained after regulation has finished and water distributes again in osmotic equilibrium. Then, this model is simpler than the previous one, because the dynamics of all solutes is lumped in a single expression.

Assuming hypotonic swelling, the rate of net ion loss (J_s) will vary in direct proportion to the extent of volume regulation given by $(V - V_R/V_0)$. The model successfully fitted to experimental data on volume regulation of the renal cell line MDCK (Lucio et al., 2003).

Model 5: Refining Conditions to a Limited and/or Variable Extracellular Compartment. Up to this point, the basic idea behind the RVD is that ion loss, accompanied by water, allows swollen cells to downregulate their volume. In model 2, this is achieved by including an expression that allows ion permeability to increase as cells swell, with ion permeabilities being dependent on cell volume changes. Alternatively, swelling could alter ion permeability indirectly, e.g. via a second messenger whose concentration is varying during the volumetric response. This strategy was used by Warren et al. (2009) to model volume regulation of airway epithelia bathed by periciliary liquid (Fig. 7A). In particular, the model describes how fluid secretion can be elicited after a rise in cytosolic free calcium ($Ca^{2+}i$).

Two features are clearly novel compared with the previously described models: (1) the volume of the extracellular compartment (the periciliary liquid) is small and varies according to water transmembrane transport and the rate of evaporation (or condensation), so that, unlike in the other models, extracellular osmolarity is not constant; (2) alterations in $Ca^{2+}i$ can modulate calcium-activated chloride channels (CaCC) and calcium-activated potassium channels (CaKC) whose open probability follows a Hill function (Fig. 7B):

$$P_i^{\text{open}} = \frac{1}{1+(K_i/[Ca^{2+}]_i)^{\eta_i}} \quad (5)$$

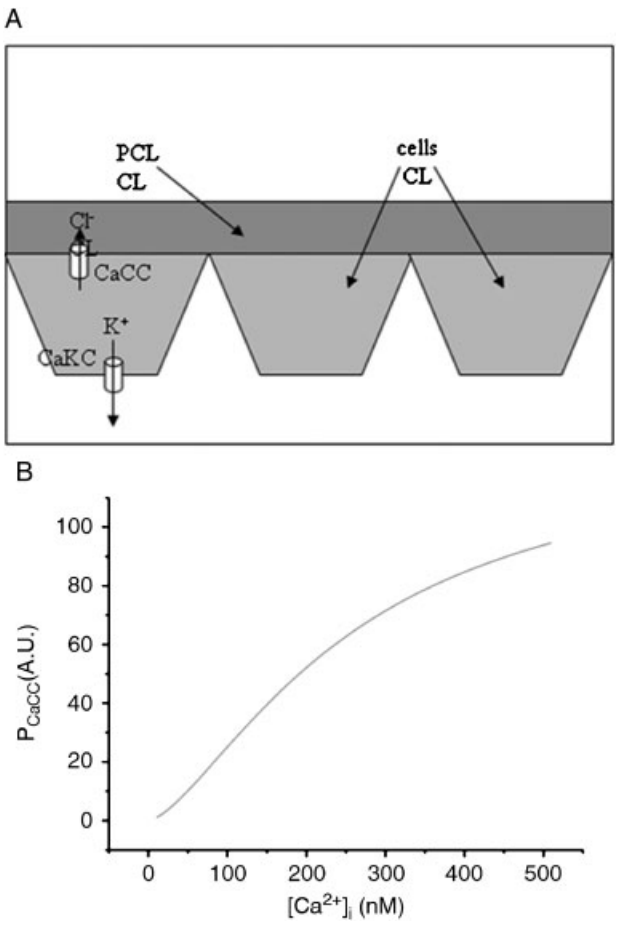

Figure 7. Model of RVD in airway epithelia. (A) The scheme shows the pericellular (PCL) and the cell compartments, as well as the calcium-dependent chloride channel (CaCC) in the apical membrane and the calcium-dependent potassium channel (CaKC) in the basolateral membrane. (B) Permeability of CaCC (P_{CaCC} in arbitrary units, A.U.) as a function of cytosolic Ca^{2+} concentration (Ca^{2+}i), calculated according to Warren et al. (2009).

where K_i is the apparent dissociation constant for ion–channel interaction and η_i the Hill coefficient. *i* denotes CaCC or CaKC. As ion permeability (P_i) is proportional to the open permeability for an ion, then changes in Ca^{2+}i will vary P_i^{open}, then alter P_i which in turn will modify K^+ and Cl^- fluxes.

The assumption of a small variable extracellular volume is novel and distinguishes the model from all previous models described above, where cells bathed in isotonic medium are assumed to be exposed to a hypotonic medium whose osmolarity and composition remain constant during the whole volumetric response. In many instances, the assumption of a constant extracellular volume is reasonable considering that in most experimental settings assay volume is much higher than the volume occupied by all the cells being tested. However, modeling an extracellular compartment where the volumetric response can affect the extracellular composition is an interesting approach to many in vivo situations where *cells are packed so tightly that the intracellular cytosol is relatively larger in volume than the restricted intercellular spaces.*

Studies on airway epithelia provided useful experimental information on hypotonically induced changes in cell volume, periciliary liquid depth (representing the extracellular compartment), and Ca^{2+}i (Okada et al., 2006) that were used to test the model. Under these conditions, the model predicts an RVD response that closely matches experimental data. Moreover, the model was also able to predict the experimentally observed increase in RVD with the concentration of Ca^{2+}i. Thus, experimental results are consistent with a model where swelling triggers the activation of K^+ and Cl^- efflux via CaCC and CaKC.

Three Elements of a Recipe to Model RVD. Altamirano et al. ('98) proposed that the RVD response exhibits three main elements: a sensor of cell volume, an effector mechanism, and a signal coupling the sensor to the effector.

In model 2, the cell volume sensor could be located in Equation (2), specifically in the $V_{1/2}$ parameter, which represents the cell volume at which the change in ion permeability is half maximal. If cell volume is lower than $V_{1/2}$, the cell is impermeable to the primary volume regulatory solutes (e.g. K^+ or Cl^-, assuming that only K^+ or Cl^- channels are operative). As cell osmotically swells, ion permeability remains zero (which could be interpreted in terms of closed or silent channels) until cell volume equals $V_{1/2}$ and the cell becomes permeable to K^+/Cl^-. Then, the $V_{1/2}$ parameter represents a threshold volume that determines when, during the volumetric response, volume sensitive K^+/Cl^- channels are functional. The effectors allowing RVD are included in the model as the factors M_i and ξ. These parameters transform the difference between actual volume and $V_{1/2}$ in changes in chloride permeability. Although the model lacks any explicit expression evoking signaling mechanisms, Equation (2) implies that the cell must suffer significant volume changes before K^+/Cl^- permeability can increase. This creates a time delay between the sensor and the effector—allowing intracellular signaling—that is similar to the lag period proposed in model 3.

In model 3, by assuming that swelling leads to membrane stretch, we could consider that the difference between the actual cell volume and the threshold value (i.e. $V - v^+$) is related to the degree of membrane stretch. Thus, in this case, v^+ would represent the volume sensor and Q_{KCl} a transport mechanism activated by membrane stretch, with Q^*_{KCl} being the density of mechanosensitive channels (i.e. the effector) which can be coupled to mechanoreceptor complexes. The higher the volume, the higher the stretch force, leading to increases of Q_{KCl} and concomitant leakage of KCl with associated water loss. Because the only requirement for mechanical sensitivity is that the

channels change shape between closed and open states and that membrane stress can reach the channels (Sachs, 2010), these may also be activated by forces transmitted via the extracellular matrix and/or the cytoskeleton (Maroto and Hamill, 2001).

As mentioned above, a signaling mechanism is included as a lag period (τ). By applying model 3 to study the volumetric behavior of hepatocytes from three vertebrate species, time lags ranging from 17 to 34 min were observed (Espelt et al., 2008).

In the simplified model 4 (Lucio et al., 2003), mechanisms for cell volume regulation were lumped into constants α, V_0, and V_R. Then, the model maintains two of the three elements characterizing a cell regulatory volume process. V_R represents the sensor, which is comparable to v^+ of model 3. The α parameter works as the density of membrane structures (channels or transports), i.e. an effector similar to

$$Q^*_{\mathrm{KCl}}\left(C^e_{K^+}C^e_{Cl^-} - \frac{m_{K^+}m_{Cl^-}}{V^2}\right)$$

that enables osmolyte efflux. Considering the absence of a lag period, this model would apply to cell systems where signaling events are fast.

In principle model 5 by Warren et al. (2009) does not include any explicit expression for the three basic elements that enable RVD. However, under hypotonicity the low assay volume imposed as well as the intracellular calcium-dependent channels activity will lead to changes in ion fluxes (J_i of Equation (1e)), which will ultimately account for an effector mechanism. Moreover, this effector will also be affected by the signaling events driving the changes in the concentration of $Ca^{2+}i$.

Different Features of Models Describing the Kinetics of $[ATP]_e$

In part 2 of this review, we have seen that in many cell types RVD is activated by ATPe in a concentration-dependent manner. As during the hypotonic challenge, ATPe accumulates nonlinearly; studying the factors governing the dynamics of $[ATP]_e$ is important to characterize the nucleotide-dependent RVD response. ATP release has been demonstrated in response to different stimuli (including osmotic and mechanical stress), but comparatively little is known regarding the molecular mechanisms mediating regulated ATP efflux (see section Pathways of ATP release). Although characterization of anionic pores, transporters and exocytosis pathways of ATP release are under way (Fitz, 2007; Dolovcak et al., 2009; Qiu and Dahl, 2009), parallel efforts have been made to explain the kinetics of ATPe by means of mathematical models.

In general, these models take into account the sources of ATP given by empirical fluxes (in A6 epithelia: (Gheorghiu and Van Driessche, 2004), in goldfish hepatocytes: (Pafundo et al., 2008; Chara et al., 2009)) and sinks of ATP where the concentration of ATPe decreases by extracellular diffusion in A6 epithelia: (Gheorghiu and Van Driessche, 2004), in retinal astrocytes and Mueller cells (Newman, 2001), and/or by ectonucleotidases activity (Choi et al., 2007; Pafundo et al., 2008; Zuo et al., 2008; Chara et al., 2009, 2010). Particularly in goldfish hepatocytes exposed to hypotonicity, our group presented models accounting for the various fluxes that produce a specific profile of ATPe accumulation.

Modeling the Kinetics of ATPe of Goldfish Hepatocytes Challenged by Hypotonicity. Swollen goldfish hepatocytes can trigger RVD in the presence of exogenous as well as endogenous ATPe. Two important features of this cell system are essential to understand why studying the kinetics of [ATP]e under hypotonicity is relevant to characterize the RVD response: (1) the degree of RVD is a function of [ATP]e; (2) in hypotonic medium, ATPe accumulates nonlinearly; it increases acutely to a maximum, followed by a slower exponential decay (Pafundo et al., 2008). Thus, there is a continuous change in [ATP]e generating a continuous modulation of RVD.

Previous experimental evidence had shown that, in goldfish hepatocytes, the hypotonically induced nonlinear accumulation of ATPe is consistent with simultaneous lytic and nonlyticATP release, together with ATP depletion by ectonucleotidases present at the cell surface.

In a first attempt to understand the relative importance of the various processes affecting the kinetic of [ATP]e, a model with two compartments (Fig. 8) was built (Chara et al., 2009), so that ATP can be located either in an intracellular compartment (denoted as *i*) or in an extracellular compartment (e). The concentration of ATPe is given by:

$$[\mathrm{ATP}]_e = J_{\mathrm{NL}} + J_L - J_{\mathrm{ecto}} \qquad (6)$$

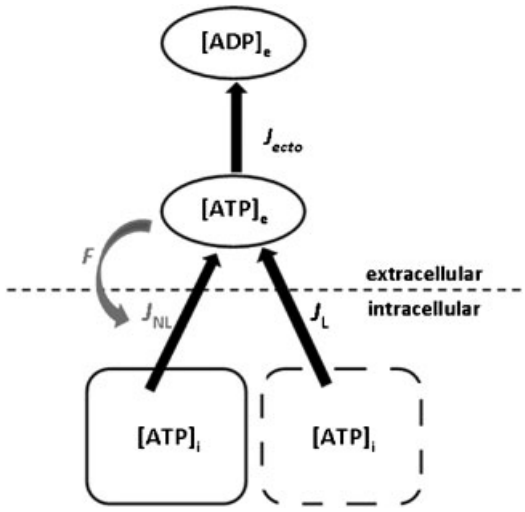

Figure 8. Schematic summary of a mathematical model describing the release of ATP in goldfish hepatocytes. The terms *i* and *e* denote the extra- and the intracellular media, respectively. *F*, J_{NL}, J_L, and J_{ecto} stand for the positive feedback mechanism, nonlytic and lytic ATP fluxes, and the ATPe consumption flux governed by EctoATPase activity (i.e. ATPe-ADPe conversion), respectively.

where J_L and J_{NL} accounts for lytic and nonlytic release of ATP respectively, and J_{ecto} is a flux of ATPe consumption by ectoATPase activity.

The term J_{NL} was assumed to be composed of a specific time function (J_R) modulated by a factor denoted as F. Given that the mechanisms responsible for the nonlytic release of ATP remain unknown, different expressions accounting for J_R were tested, so as to check which one allows the model to display the best fit to the experimentally observed ATPe kinetic: (i) a constant J_R (J_R does not vary with time), (ii) a step function in which J_R is zero until it becomes activated and remains constant thereafter, (iii) an impulse function, where J_R has the form of a rectangular pulse that can be triggered and shut off at variable times, and (iv) a log-normal function that includes a nonlinear fast increase to a maximum, followed by a relatively slowly nonlinear decrease.

Experimental data showed that in swollen goldfish hepatocytes, ATPe can activate a nonlytic ATP efflux (Pafundo et al., 2008). In terms of the model, a factor "F" accounts for a positive feedback process whereby ATPe can amplify J_R, as follows:

$$F = a[\mathrm{ATP}]_e + b \tag{7}$$

where the values of a and b were obtained by fitting a linear function to experimental data (Pafundo et al., 2008).

In addition to this ATP efflux of viable cells, a lytic flux of ATP (J_L) describes the release of intracellular ATP from dead cells. This was estimated by using experimental data on cell volume, cell death, and intracellular ATP concentration of hepatocytes under hypotonic conditions. That is, the intracellular compartment contains the total mass of intracellular ATP for any given number of cells, a mass which, according to the model, is released and diluted instantly into extracellular medium following cell death. This is important, as in most cell systems a certain degree of cell death is unavoidable during anisosmotic exposure. Moreover, because the intracellular ATP concentration is at least three orders of magnitude higher than that of ATPe, even a minor proportion of cell death might produce significant increases in $[\mathrm{ATP}]_e$.

The term J_{ecto} accounts for ectoATPase activity. Goldfish hepatocytes exhibit significant ATP hydrolysis at the cell surface (Schwarzbaum et al., '98) that is mediated by at least two E-NTPDases (Alleva et al., 2002). Experimental evidence suggests that ectoATPase activity in these cells is a hyperbolic function of [ATP]e (Pafundo et al., 2008). However, because the model was used to simulate ATPe in a concentration range which is at least 300 times lower than the apparent K_m, under this condition, J_{ecto} can be included in the model as a linear function of $[\mathrm{ATP}]_e$.

Interestingly, the model successfully fitted the experimental data (Fig. 9A) and allowed to analyze the specific contribution of each process controlling the kinetic of $[\mathrm{ATP}]_e$. Thus, according to the model, during the first minute of hypotonicity the concentration of ATPe is mainly governed by both lytic and nonlytic ATP efflux, whereas ecto-ATPase activity (Fig. 9B) becomes important in defining the time dependent decay of ATPe levels.

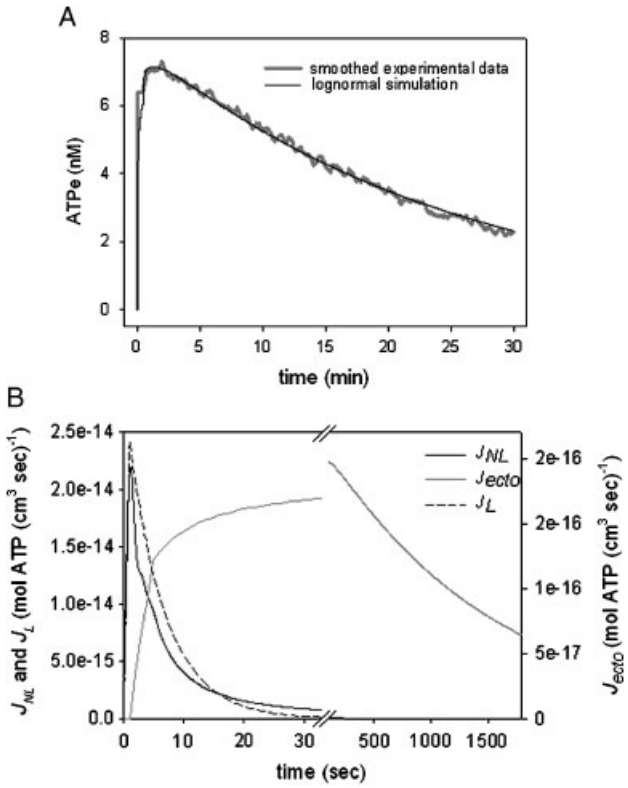

Figure 9. Fitting the mathematical model to experimental data. (A) Time course of simulated [ATP]e (i.e. model dependent fit; black) fitted to the smoothed experimentally observed values of ATPe (gray). Experimental data were computed from Pafundo et al. (2008). (B) Time course of J_{NL}, J_L, and J_{ecto} predicted by the mathematical model. J_{NL} is plotted in a continuous black line, J_{ecto} in a continuous gray line, and J_L in a dashed black line. The simulated data was extracted from Chara et al. (2009), used with permission.

Regarding the nonlytic release of ATP, model dependent fit showed that J_R is best described by assuming a log normal function accounting for the asymetrical nonlinear rise and decay phases of the experimentally observed $[\mathrm{ATP}]_e$ profile. To provide physical meaning to the function describing ATP release, we also tested a model where J_R was given by an equation describing ATP diffusion across the hepatocyte cell membrane, so that, unlike in the case of the lognormal function, the rate of ATP efflux depended now on the concentrations of ATP at both sides of the membrane. Results showed that the nonlytic ATP release can be driven by the steep electrochemical gradient of ATP across the plasma membrane.

In a further development, we tested the extent to which the extracellular diffusion of ATP can affect ATPe kinetic. This was

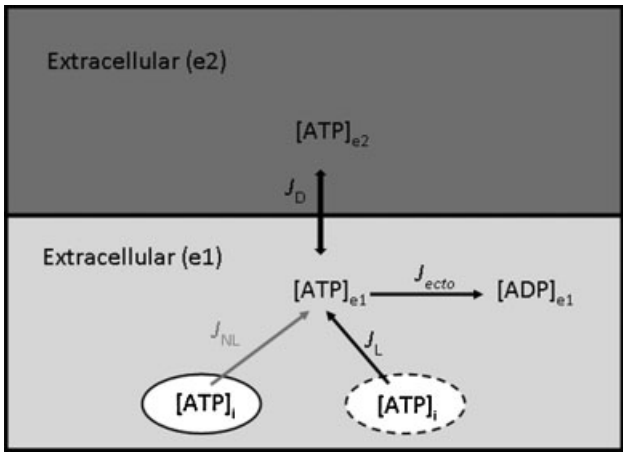

Figure 10. ATPe kinetic model with extracellular diffusion of the nucleotide. The terms *i*, e_1, e_2 denote the intra- and two extracellular media, respectively. J_D stands for ATPe diffusive flux between the two extracellular media. The meaning of *F*, J_{NL}, J_L, and J_{ecto} are given in Figure 8.

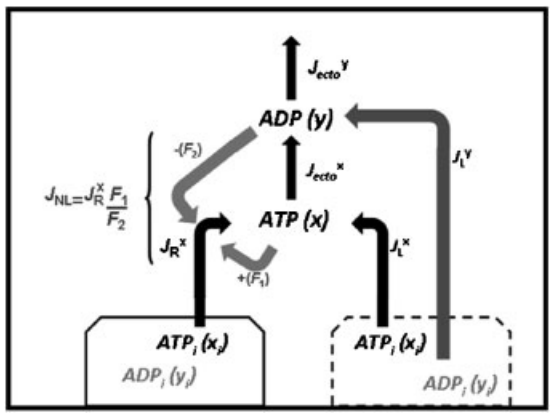

Figure 11. ATPe kinetic model with positive and negative feedback loops on regulated ATP release. J_{NL} and J_L are nonlytic and lytic fluxes of ATP (*x*) and ADP (*y*), respectively. J_{ecto} stands for nucleotide fluxes owing to ectoATPase activity (J^x_{ecto}) or ectoADPase activity (J^y_{ecto}). F_1 and F_2 correspond to positive and negative feedback mechanisms. *x*, *y*, x_i, and y_i denote the concentrations of extracellular ATP, extracellular ADP, intracellular ATP, and intracellular ADP, respectively.

important, as in some experimental settings, ATPe accumulation at the cell surface differs considerably from bulk extracellular ATP. Thus, we included in the goldfish hepatocyte model an expression describing ATPe diffusion (see Fig. 10; Pafundo et al., 2008; Chara et al., 2009). The extracellular medium was modeled as two volumes, with a small volume (*e*1) contacting the cell surface and a second outer extracellular volume *e*2. Both ectoATPase activity and the feedback mechanism F were assumed to be functional in *e*1 only, as they depend on proteins bound to the membrane surface. A diffusion flux J_D allowed ATPe to be exchanged between *e*1 and *e*2. The concentrations of ATPe in *e*1 and *e*2 were described by:

$$[\mathrm{ATP}]_{e1} = J^{e1}_{\mathrm{NL}} + J^{e1}_{L} - J^{e1}_{\mathrm{ecto}} - J^{e1-e2}_{D} \quad (8)$$

$$[\mathrm{ATP}]_{e2} = +J^{e2-e1}_{D} \quad (9)$$

A statistical comparison of the models with and without J_D showed that ATPe diffusion does not significantly affect the experimentally observed ATPe kinetic of goldfish hepatocytes (Chara et al., 2009). This means that, at least under the experimental conditions where the assay volume is relatively low—as in the case of ATPe measurements with goldfish hepatocytes—the measured bulk ATPe is a good estimate of the ATPe concentration at the cell surface.

In a last development, we used modeling to test if extracellular ADP accumulation (generated by ectoATPase activity) can affect the ATPe kinetic (Fig. 11). Although in models above acute ATPe increase was partly compensated by ectoATPase activity, a recent report using erythrocytes suggests that extracellular ADP, acting on specific P2Y receptors might initiate a negative feedback loop to oppose the ATPe-induced ATP release (Wang et al., '96). Taking into account that goldfish hepatocytes exhibit both a significant ectoATPase activity capable of generating ADP from ATP (Schwarzbaum et al., '98) and an active P2Y system capable of mediating RVD (Pafundo et al., 2008), we built a new mathematical model where ADP accumulates in the extracellular space and nonlytic ATP release is modulated by an ATP-driven positive feedback loop and a negative feedback system governed by ADPe.

In this new model, time dependent accumulation of ATPe was controlled by a lytic ATP flux, a nonlytic ATP flux, and ecto-ATPase activity. The kinetic of ADPe was governed by a lytic ADP flux and both ecto-ATPase activity (generating ADPe) and ecto-ADPase activity (an ADPe sink). Nonlytic ATPe release was included as a diffusion equation (i.e. ATP transport through the plasma membrane) modulated by ATPe activation (positive feedback) and ADPe inhibition (negative feedback). This model successfully fitted the experimental data and showed that while nonlytic ATP efflux can be activated by ATPe and inhibited by ADPe, the latter mechanism prevails. This is partly owing to the fact that, in the concentration range at which ATPe is present, the positive feedback mechanism—unlike the negative feedback by ADPe—is weak. The immediate prediction from this analysis is that in the absence of ADPe, the modeled system would release ATP explosively. It is worth emphasizing that, owing to the scarce information regarding the molecular identification of nonlytic ATP transport pathways (see Sabirov and Okada, 2005), equations describing ATP efflux can—up to date—not be confronted with structural information. Nevertheless, mathematical

modeling and model dependent fit to experimental data are useful in that they allow determining several kinetic properties that putative ATP transporters must fulfill.

What Should be the Next Step?

According to the hypothesis put forward by Roman et al. ('96), ATPe is an essential factor mediating RVD. Given that numerous models analyzed in previous studies seemed to have worked well even without considering a potential contribution of extracellular nucleosides, this may seem unlikely at first glimpse. However, in most studies, this contribution may have gone unnoticed as experimental conditions were not selected to account for this possibility. Furthermore, in some models, basal RVD may only be enhanced in the presence of extracellular nucleosides, an effect that may well remain undetected if not specifically sought for. Altogether, we thus strongly believe that ATPe is indeed an essential factor for the RVD response in many cells. Although the model by Roman et al. was very important in linking volume regulation to the world of P receptor signaling and ectonucleotidases, its qualitative nature precludes a quantitative estimation of how important ATPe—and other nucleotides—is for driving RVD. Thus, dealing with the entire process of cell volume regulation requires a quantitative RVD model describing not only the dynamics of intracellular ions, diffusible water, and membrane potential, but also the dynamics of ATPe. This, in turn, requires an analysis of the complex interactions between ATPe (and other nucleotides), P receptors, ectonucleotidases, and the lytic and nonlytic transport of nucleotides to the extracellular medium. Future studies should deal with joining intra- and extracellular factors modulating volume regulation of cells exposed to anisotonic media. Given the suitability of fish cells for the elucidation of RVD-related processes, we believe that the use of such cells will help to achieve this goal.

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LITERATURE CITED

Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. 2009. Purinergic signalling in the nervous system: an overview. Trends Neurosci 32:19–29.

Adragna NC, Di Fulvio M, Lauf PK. 2004. Regulation of K-Cl cotransport: from function to genes. J Membr Biol 201:109–137.

Ahmed KH, Pelster B, Krumschnabel G. 2006. Signalling pathways involved in hypertonicity- and acidification-induced activation of Na^+/H^+exchange in trout hepatocytes. J Exp Biol 209: 3101–3113.

Alleva KE, Espelt MV, Krumschnabel G, Schwarzbaum PJ. 2002. Identification of two distinct E-NTPDases in liver of goldfish (*Carassius auratus* L.). Comp Biochem Physiol B Biochem Mol Biol 131:725–731.

Altamirano J, Brodwick MS, Alvarez-Leefmans FJ. 1998. Regulatory volume decrease and intracellular Ca2+in murine neuroblastoma cells studied with fluorescent probes. J Gen Physiol 112:145–160.

Andersson RM, Aizman O, Aperia A, Brismar H. 2004. Modulation of Na^+,K^+-ATPase activity is of importance for RVD. Acta Physiol Scand 180:329–334.

Avella M, Ducoudret O, Pisani DF, Poujeol P. 2009. Swelling-activated transport of taurine in cultured gill cells of sea bass: physiological adaptation and pavement cell plasticity. Am J Physiol Regul Integr Comp Physiol 296:R1149–R1160.

Battista AG, Ricatti MJ, Pafundo DE, Gautier MA, Faillace MP. 2009. Extracellular ADP regulates lesion-induced in vivo cell proliferation and death in the zebrafish retina. J Neurochem 111:600–613.

Beigi R, Kobatake E, Aizawa M, Dubyak GR. 1999. Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am J Physiol 276:C267–C278.

Benyajati S, Renfro JL. 2000. Taurine secretion in primary monolayer cultures of flounder renal epithelium: stimulation by low osmolality. Am J Physiol Regul Integr Comp Physiol 279:R704–R712.

Berenbrink M, Weaver YR, Cossins AR. 1997. Defining the volume dependence of multiple K flux pathways of trout red blood cells. Am J Physiol 272:C1099–C1111.

Berenbrink M, Volkel S, Heisler N, Nikinmaa M. 2000. O(2)-dependent K(+) fluxes in trout red blood cells: the nature of O(2) sensing revealed by the O(2) affinity, cooperativity and pH dependence of transport. J Physiol 526:69–80.

Berenbrink M, Volkel S, Koldkjaer P, Heisler N, Nikinmaa M. 2006. Two different oxygen sensors regulate oxygen-sensitive K^+ transport in crucian carp red blood cells. J Physiol 575:37–48.

Bianchini L, Fossat B, Porthe-Nibelle J, Ellory JC, Lahlou B. 1988. Effects of hyposmotic shock on ion fluxes in isolated trout hepatocytes. J Exp Biol 137:303–318.

Bianchini L, Fossat B, Porthe-Nibelle J, Lahlou B. 1991. Activation by N-ethylmaleimide of a Cl^--dependent K^+ flux in isolated trout hepatocytes. J Exp Biol 157:335–348.

Bogdanova AY, Nikinmaa M. 2001. Reactive oxygen species regulate oxygen-sensitive potassium flux in rainbow trout erythrocytes. J Gen Physiol 117:181–190.

Bogdanova A, Berenbrink M, Nikinmaa M. 2009. Oxygen-dependent ion transport in erythrocytes. Acta Physiol (Oxf) 195:305–319.

Borgese F, Garcia-Romeu F, Motais R. 1987. Control of cell volume and ion transport by beta-adrenergic catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. J Physiol 382: 123–144.

Boudreault F, Grygorczyk R. 2004. Evaluation of rapid volume changes of substrate-adherent cells by conventional microscopy 3D imaging. J Microsc 215:302–312.

Burnstock G. 1972. Purinergic nerves. Pharmacol Rev 24:509–581.

Burnstock G. 2006. Purinergic signalling—an overview. Novartis Found Symp 276:26–48; discussion 48–57, 275–281.

Burnstock G. 2007. Purine and pyrimidine receptors. Cell Mol Life Sci 64:1471–1483.

Burnstock G, Verkhratsky A. 2009. Evolutionary origins of the purinergic signalling system. Acta Physiol (Oxf) 195:415–447.

Burnstock G, Campbell G, Satchell D, Smythe A. 1970. Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. Br J Pharmacol 40:668–688.

Bursell JD, Kirk K. 1996. Swelling-activated K^+ transport via two functionally distinct pathways in eel erythrocytes. Am J Physiol 270:R61–R70.

Chara O, Pafundo DE, Schwarzbaum PJ. 2009. Kinetics of extracellular ATP from goldfish hepatocytes: a lesson from mathematical modeling. Bull Math Biol 71:1025–1047.

Chara O, Pafundo DE, Schwarzbaum PJ. 2010. Negative feedback of extracellular ADP on ATP release in goldfish hepatocytes: a theoretical study. J Theor Biol 264:1147–1158.

Choi HW, Ferrara KW, Barakat AI. 2007. Modulation of ATP/ADP concentration at the endothelial surface by shear stress: effect of flow recirculation. Ann Biomed Eng 35:505–516.

Cossins AR, Weaver YR, Lykkeboe G, Nielsen OB. 1994. Role of protein phosphorylation in control of K flux pathways of trout red blood cells. Am J Physiol 267:C1641–1650.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M. 1998. Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci USA 95: 15735–15740.

Dahl G, Harris AL. 2009. Pannexins or connexins? Biomed Life Sciences 1:287–301.

Dascalu A, Nevo Z, Korenstein R. 1992. Hyperosmotic activation of the Na(+)-H+exchanger in a rat bone cell line: temperature dependence and activation pathways. J Physiol 456:503–518.

Dixon CJ, White PJ, Hall JF, Kingston S, Boarder MR. 2005. Regulation of human hepatocytes by P2Y receptors: control of glycogen phosphorylase, Ca2+, and mitogen-activated protein kinases. J Pharmacol Exp Ther 313:1305–1313.

Dolovcak S, Waldrop SL, Fitz JG, Kilic G. 2009. 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) stimulates cellular ATP release through exocytosis of ATP-enriched vesicles. J Biol Chem 284:33894–33903.

Dranoff JA, O'Neill AF, Franco AM, Cai SY, Connolly GC, Ballatori N, Boyer JL, Nathanson MH. 2000. A primitive ATP receptor from the little skate *Raja erinacea*. J Biol Chem 275: 30701–30706.

Dubyak GR. 2009. Both sides now: multiple interactions of ATP with pannexin-1 hemichannels. Focus on "A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP." Am J Physiol Cell Physiol 296:C235–C241.

Duranton C, Mikulovic E, Tauc M, Avella M, Poujeol P. 2000a. Potassium channels in primary cultures of seawater fish gill cells. I. Stretch-activated K(+) channels. Am J Physiol Regul Integr Comp Physiol 279:R1647–R1658.

Duranton C, Mikulovic E, Tauc M, Avella M, Poujeol P. 2000b. Potassium channels in primary cultures of seawater fish gill cells. II. Channel activation by hypotonic shock. Am J Physiol Regul Integr Comp Physiol 279:R1659–R1670.

Ebner HL, Cordas A, Pafundo DE, Schwarzbaum PJ, Pelster B, Krumschnabel G. 2005. Importance of cytoskeletal elements in volume regulatory responses of trout hepatocytes. Am J Physiol Regul Integr Comp Physiol 289:R877–R890.

Ebner HL, Fiechtner B, Pelster B, Krumschnabel G. 2006. Extracellular signal regulated MAP-kinase signalling in osmotically stressed trout hepatocytes. Biochim Biophys Acta 1760:941–950.

Ebner HL, Blatzer M, Nawaz M, Krumschnabel G. 2007. Activation and nuclear translocation of ERK in response to ligand-dependent and -independent stimuli in liver and gill cells from rainbow trout. J Exp Biol 210:1036–1045.

Edmonds BT, Koenig E. 1990. Volume regulation in response to hypo-osmotic stress in goldfish retinal ganglion cell axons regenerating in vitro. Brain Res 520:159–165.

Egée S, Harvey BJ, Thomas S. 1997. Volume-activated DIDS-sensitive whole-cell chloride currents in trout red blood cells. J Physiol 504: 57–63.

Egée S, Lapaix F, Cossins AR, Thomas SL. 2000. The role of anion and cation channels in volume regulatory responses in trout red blood cells. Bioelectrochemistry 52:133–149.

Espelt MV, Mut PN, Amodeo G, Krumschnabel G, Schwarzbaum PJ. 2003. Volumetric and ionic responses of goldfish hepatocytes to anisotonic exposure and energetic limitation. J Exp Biol 206:513–522.

Espelt MV, Alleva K, Amodeo G, Krumschnabel G, Rossi RC, Schwarzbaum PJ. 2008. Volumetric response of vertebrate hepatocytes challenged by osmotic gradients: a theoretical approach. Comp Biochem Physiol B Biochem Mol Biol 150:103–111.

Faggio C, Torre A, Pelle E, Raffa F, Villari V, Trischitta F. 2011. Cell volume regulation following hypotonic shock in hepatocytes isolated from *Sparus aurata*. Comp Biochem Physiol A Mol Integr Physiol 158:143–149.

Feranchak AP, Lewis MA, Kresge C, Sathe M, Bugde A, Luby-Phelps K, Antich PP, Fitz JG. 2010. Initiation of purinergic signaling by exocytosis of ATP-containing vesicles in liver epithelium. J Biol Chem 285:8138–8147.

Fiol DF, Kultz D. 2007. Osmotic stress sensing and signaling in fishes. FEBS J 274:5790–5798.

Fitz JG. 2007. Regulation of cellular ATP release. Trans Am Clin Climatol Assoc 118:199–208.

Ford P, Rivarola V, Chara O, Blot-Chabaud M, Cluzeaud F, Farman N, Parisi M, Capurro C. 2005. Volume regulation in cortical collecting duct cells: role of AQP2. Biol Cell 97:687–697.

Fugelli K, Zachariassen KE. 1976. The distribution of taurine, gamma-aminobutyric acid and inorganic ions between plasma and erythrocytes in flounder (*Platichthys flesus*) at different plasma osmolalities. Comp Biochem Physiol A Comp Physiol 55:173–177.

Fugelli K, Kanli H, Terreros DA. 1995. Taurine efflux is a cell volume regulatory process in proximal renal tubules from the teleost *Carassius auratus*. Acta Physiol Scand 155:223–232.

Galizia L, Flamenco MP, Rivarola V, Capurro C, Ford P. 2008. Role of AQP2 in activation of calcium entry by hypotonicity: implications in cell volume regulation. Am J Physiol Renal Physiol 294:F582–F590.

Gamba G. 2005. Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters. Physiol Rev 85:423–493.

Garcia-Romeu F, Cossins AR, Motais R. 1991. Cell volume regulation by trout erythrocytes: characteristics of the transport systems activated by hypotonic swelling. J Physiol 440:547–567.

Gheorghiu M, Van Driessche W. 2004. Modeling of basolateral ATP release induced by hypotonic treatment in A6 cells. Eur Biophys J 33:412–420.

Gin E, Tanaka EM, Brusch L. 2010. A model for cyst lumen expansion and size regulation via fluid secretion. J Theor Biol 264:1077–1088.

Goldman DE. 1943. Potential, Impedance, and Rectification in Membranes. J Gen Physiol 27:37–60.

Goldstein L, Kleinzeller A. 1987. Cell volume regulation in lower vertebrates. CurrTopics Membr Transport 30:181–203.

Goswami C, Saha N. 2006. Cell volume regulation in the perfused liver of a freshwater air-breathing cat fish *Clarias batrachus* under aniso-osmotic conditions: roles of inorganic ions and taurine. J Biosci 31:589–598.

Groot JA. 1981. Cell volume regulation in goldfish intestinal mucosa. Pflugers Arch 392:57–66.

Grygorczyk R, Hanrahan JW. 1997. CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. Am J Physiol 272:C1058–C1066.

Guizouarn H, Motais R. 1999. Swelling activation of transport pathways in erythrocytes: effects of Cl-, ionic strength, and volume changes. Am J Physiol 276:C210–C220.

Guizouarn H, Gabillat N, Motais R, Borgese F. 2001. Multiple transport functions of a red blood cell anion exchanger, tAE1: its role in cell volume regulation. J Physiol 535:497–506.

Guizouarn H, Christen R, Borgese F. 2005. Phylogeny of anion exchangers: could trout AE1 conductive properties be shared by other members of the gene family? Biochim Biophys Acta 1726:244–250.

Gusev GP, Agalakova NI. 2010. Regulation of K-Cl cotransport in erythrocytes of frog *Rana temporaria* by commonly used protein kinase and protein phosphatase inhibitors. J Comp Physiol 180:385–391.

Hagberg H, Andersson P, Lacarewicz J, Jacobson I, Butcher S, Sandberg M. 1987. Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. J Neurochem 49:227–231.

Henson JH, Roesener CD, Gaetano CJ, Mendola RJ, Forrest Jr JN, Holy J, Kleinzeller A. 1997. Confocal microscopic observation of cytoskeletal reorganizations in cultured shark rectal gland cells following treatment with hypotonic shock and high external K^+. J Exp Zool (Mol Dev Evol) 279:415–424.

Hanson MS, Stephenson AH, Bowles EA, Sprague RS. 2010. Insulin inhibits human erythrocyte cAMP accumulation and ATP release: role of phosphodiesterase 3 and phosphoinositide 3-kinase. Exp Biol Med (Maywood) 235:256–262.

Hernandez JA, Cristina E. 1998. Modeling cell volume regulation in nonexcitable cells: the roles of the Na+pump and of cotransport systems. Am J Physiol 275:C1067–C1080.

Hodgkin AL, Katz B. 1949. The effect of sodium ions on the electrical activity of giant axon of the squid. J Physiol 108:37–77.

Hoffmann EK, Lambert IH, Pedersen SF. 2009. Physiology of cell volume regulation in vertebrates. Physiol Rev 89:193–277.

Hubert EM, Musch MW, Goldstein L. 2000. Inhibition of volume-stimulated taurine efflux and tyrosine kinase activity in the skate red blood cell. Pflugers Arch 440:132–139.

Hviid Larsen E, Kristensen P. 1978. Properties of a conductive cellular chloride pathway in the skin of the toad (*Bufo bufo*). Acta Physiol Scand 102:1–21.

Ip YK, Loong AM, Ching B, Tham GH, Wong WP, Chew SF. 2009. The freshwater Amazonian stingray, *Potamotrygon motoro*, up-regulates glutamine synthetase activity and protein abundance, and accumulates glutamine when exposed to brackish (15 per thousand) water. J Exp Biol 212:3828–3836.

Ishibashi K, Hara S, Kondo S. 2009. Aquaporin water channels in mammals. Clin Exp Nephrol 13:107–117.

Jacquet S, Malaval C, Martinez LO, Sak K, Rolland C, Perez C, Nauze M, Champagne E, Terce F, Gachet C, Perret B, Collet X, Boeynaems JM, Barbaras R. 2005. The nucleotide receptor P2Y13 is a key regulator of hepatic high-density lipoprotein (HDL) endocytosis. Cell Mol Life Sci 62:2508–2515.

Jans D, Srinivas SP, Waelkens E, Segal A, Lariviere E, Simaels J, Van Driessche W. 2002. Hypotonic treatment evokes biphasic ATP release across the basolateral membrane of cultured renal epithelia (A6). J Physiol 545:543–555.

Jensen F. 1995. Regulatory volume decrease in carp red blood cells: mechanisms and oxygenation-dependency of volume-activated potassium and amino acid transport. J Exp Biol 198:155–165.

Jensen FB, Agnisola C, Novak I. 2009. ATP release and extracellular nucleotidase activity in erythrocytes and coronary circulation of rainbow trout. Comp Biochem Physiol A Mol Integr Physiol 152: 351–356.

Joseph SM, Buchakjian MR, Dubyak GR. 2003. Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. J Biol Chem 278:23331–23342.

Kanli H, Norderhus E. 1998. Cell volume regulation in proximal renal tubules from trout (*Salmo trutta*). J Exp Biol 201:1405–1419.

Kiessling K, Ellory JC, Cossins AR. 2000. The relationship between hypotonically induced taurine and K fluxes in trout red blood cells. Pflugers Arch 440:467–475.

Kirk K. 1997. Swelling-activated organic osmolyte channels. J Membr Biol 158:1–16.

Kirk K, Strange K. 1998. Functional properties and physiological roles of organic solute channels. Annu Rev Physiol 60:719–739.

Knight GE, Burnstock G. 1996. The effects of purine compounds on the isolated aorta of the frog *Rana temporaria*. Br J Pharmacol 117: 873–878.

Koomoa DL, Musch MW, MacLean AV, Goldstein L. 2001. Volume-activated trimethylamine oxide efflux in red blood cells of spiny dogfish (*Squalus acanthias*). Am J Physiol Regul Integr Comp Physiol 281:R803–R810.

Krumschnabel G, Malle S, Schwarzbaum PJ, Wieser W. 1994. Glycolytic function in goldfish hepatocytes at various temperatures: effects on Na^+-pump and protein synthesis. J Exp Biol 192: 285–290.

Krumschnabel G, Nawaz M. 2004. Acute toxicity of hexavalent chromium in isolated teleost hepatocytes. Aquat Toxicol 70:159–167.

Krumschnabel G, Biasi C, Schwarzbaum PJ, Wieser W. 1996. Membrane–metabolic coupling and ion homeostasis in anoxia-tolerant and anoxia-intolerant hepatocytes. Am J Physiol 270: R614–R620.

Krumschnabel G, Biasi C, Schwarzbaum PJ, Wieser W. 1997. Acute and chronic effects of temperature, and of nutritional state, on ion homeostasis and energy metabolism in teleost hepatocytes. J Comp Physiol B 167:280–286.

Krumschnabel G, Manzl C, Schwarzbaum PJ. 2001. Importance of glycolysis for the energetics of anoxia-tolerant and anoxia-intolerant teleost hepatocytes. Physiol Biochem Zool 74:413–419.

Krumschnabel G, Gstir R, Manzl C, Prem C, Pafundo D, Schwarzbaum PJ. 2003. Metabolic and ionic responses of trout hepatocytes to anisosmotic exposure. J Exp Biol 206:1799–1808.

Krumschnabel G, Maehr T, Nawaz M, Schwarzbaum PJ, Manzl C. 2007. Staurosporine-induced cell death in salmonid cells: the role of apoptotic volume decrease, ion fluxes and MAP kinase signaling. Apoptosis 12:1755–1768.

Kultz D, Avila K. 2001. Mitogen-activated protein kinases are in vivo transducers of osmosensory signals in fish gill cells. Comp Biochem Physiol B Biochem Mol Biol 129:821–829.

Lan WZ, Abbas H, Lam HD, Lemay AM, Hill CE. 2005. Contribution of a time-dependent and hyperpolarization-activated chloride conductance to currents of resting and hypotonically shocked rat hepatocytes. Am J Physiol Gastrointest Liver Physiol 288: G221–G229.

Lauf PK. 1982. Evidence for chloride dependent potassium and water transport induced by hyposmotic stress in erythrocytes of the marine teleost, *Opsanus tau*. J Comp Physiol B 146:9–16.

Lauf PK, Adragna NC. 2000. K-Cl cotransport: properties and molecular mechanism. Cell Physiol Biochem 10:341–354.

Lauf PK, Bauer J, Adragna NC, Fujise H, Zade-Oppen AM, Ryu KH, Delpire E. 1992. Erythrocyte K-Cl cotransport: properties and regulation. Am J Physiol 263:C917–C932.

Lazarowski ER, Boucher RC, Harden TK. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. Mol Pharmacol 64:785–795.

Lazarowski ER, Tarran R, Grubb BR, van Heusden CA, Okada S, Boucher RC. 2004. Nucleotide release provides a mechanism for airway surface liquid homeostasis. J Biol Chem 279: 36855–36864.

Leguen I, Prunet P. 2001. In vitro effect of various xenobiotics on trout gill cell volume regulation after hypotonic shock. Aquat Toxicol 53: 201–214.

Leguen I, Prunet P. 2004. Effect of hypotonic shock on cultured pavement cells from freshwater or seawater rainbow trout gills. Comp Biochem Physiol A Mol Integr Physiol 137:259–269.

Lermen CL, Vieira VLP, Schetinger MRC, Lappe R, Gioda CR, Crestani MR, Baldisserotto B, Morsch VM. 2005. Regulation of cytosolic free calcium concentration by extracellular nucleotides in human hepatocytes. Fish Physiol Biochem 31:295–301.

Lewis RA, Bursell JD, Kirk K. 1996. Anion-selectivity of the swelling-activated osmolyte channel in eel erythrocytes. J Membr Biol 149: 103–111.

Light DB, Capes TL, Gronau RT, Adler MR. 1999. Extracellular ATP stimulates volume decrease in Necturus red blood cells. Am J Physiol 277:C480–C491.

Light DB, Dahlstrom PK, Gronau RT, Baumann NL. 2001. Extracellular ATP activates a P2 receptor in necturus erythrocytes during hypotonic swelling. J Membr Biol 182:193–202.

Lionetto MG, Schettino T. 2006. The Na^+-K^+-$2Cl^-$ cotransporter and the osmotic stress response in a model salt transport epithelium. Acta Physiol (Oxf) 187:115–124.

Lionetto MG, Pedersen SF, Hoffmann EK, Giordano ME, Schettino T. 2002. Roles of the cytoskeleton and of protein phosphorylation events in the osmotic stress response in eel intestinal epithelium. Cell Physiol Biochem 12:163–178.

Lionetto MG, Giordano ME, De Nuccio F, Nicolardi G, Hoffmann EK, Schettino T. 2005. Hypotonicity induced $K^+$and anion conductive pathways activation in eel intestinal epithelium. J Exp Biol 208: 749–760.

Lionetto MG, Rizzello A, Giordano ME, Maffia M, De Nuccio F, Nicolardi G, Hoffmann EK, Schettino T. 2008. Molecular and functional expression of high conductance Ca 2^+ activated K^+ channels in the eel intestinal epithelium. Cell Physiol Biochem 21: 373–384.

Locovei S, Scemes E, Qiu F, Spray DC, Dahl G. 2007. Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. FEBS Lett 581:483–488.

Lucio AD, Santos RA, Mesquita ON. 2003. Measurements and modeling of water transport and osmoregulation in a single kidney cell using optical tweezers and videomicroscopy. Phys Rev E Stat Nonlin Soft Matter Phys 68:041906.

Manzl C, Ebner H, Kock G, Dallinger R, Krumschnabel G. 2003. Copper, but not cadmium, is acutely toxic for trout hepatocytes: short-term effects on energetics and ion homeostasis. Toxicol Appl Pharmacol 191:235–244.

Maroto R, Hamill OP. 2001. Brefeldin A block of integrin-dependent mechanosensitive ATP release from Xenopus oocytes reveals a

novel mechanism of mechanotransduction. J Biol Chem 276: 23867–23872.

Marshall WS. 2003. Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures. Biochim Biophys Acta 1618:95–105.

Marshall WS, Bryson SE, Sapp MM. 1990. Volume regulation in glutathione-treated brook trout (*Salvelinus fontinalis*) erythrocytes. Fish Physiol Biochem 8:19–28.

Marshall WS, Ossum CG, Hoffmann EK. 2005. Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. J Exp Biol 208:1063–1077.

Martinez LO, Jacquet S, Esteve JP, Rolland C, Cabezon E, Champagne E, Pineau T, Georgeaud V, Walker JE, Terce F, Collet X, Perret B, Barbaras R. 2003. Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. Nature 421:75–79.

Masse K, Eason R, Bhamra S, Dale N, Jones EA. 2006. Comparative genomic and expression analysis of the conserved NTPDase gene family in *Xenopus*. Genomics 87:366–381.

McLachlan E, Shao Q, Wang HL, Langlois S, Laird DW. 2006. Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis. Cancer Res 66:9886–9894.

Michel F, Fossat B, Porthe-Nibelle J, Lahlou B, Saint-Marc P. 1994. Effects of hyposmotic shock on taurine transport in isolated trout hepatocytes. Exp Physiol 79:983–995.

Musch MW, Puffer AB, Goldstein L. 2008. Volume expansion stimulates monoubiquitination and endocytosis of surface-expressed skate anion-exchanger isoform. Am J Physiol Regul Integr Comp Physiol 294:R1657–R1665.

Mut PN, Espelt MV, Krumschnabel G, Schwarzbaum PJ. 2006. Potassium transmembrane fluxes in anoxic hepatocytes from goldfish (*Carassius auratus* L.). Comp Biochem Physiol C Toxicol Pharmacol 142:205–211.

Neary JT, Kang Y, Willoughby KA, Ellis EF. 2003. Activation of extracellular signal-regulated kinase by stretch-induced injury in astrocytes involves extracellular ATP and P2 purinergic receptors. J Neurosci 23:2348–2356.

Newman EA. 2001. Propagation of intercellular calcium waves in retinal astrocytes and Muller cells. J Neurosci 21: 2215–2223.

Nielsen OB, Lykkeboe G, Cossins AR. 1992. Oxygenation-activated K fluxes in trout red blood cells. Am J Physiol 263:C1057–C1064.

Nikinmaa M, Huestis WH. 1984. Adrenergic swelling of nucleated erythrocytes: cellular mechanisms in a bird, domestic goose, and two teleosts, striped bass and rainbow trout. J Exp Biol 113: 215–224.

Nikinmaa M, Tufts BL, Boutilier RG. 1993. Volume and pH regulation in agnathan erythrocytes: comparisons between the hagfish, *Myxine glutinosa*, and the lampreys, Petromyzon marinus and Lampetra fluviatilis. J Comp Physiol 163:6.

Novotny JA, Jakobsson E. 1996. Computational studies of ion-water flux coupling in the airway epithelium. II. Role of specific transport mechanisms. Am J Physiol 270:C1764–C1772.

Okada SF, Nicholas RA, Kreda SM, Lazarowski ER, Boucher RC. 2006. Physiological regulation of ATP release at the apical surface of human airway epithelia. J Biol Chem 281:22992–23002.

Ollivier H, Pichavant-Rafini K, Puill-Stephan E, Calves P, Nonnotte L, Nonnotte G. 2006a. Volume regulation following hyposmotic shock in isolated turbot (*Scophthalmus maximus*) hepatocytes. J Comp Physiol B 176:393–403.

Ollivier H, Pichavant-Rafini K, Puill-Stephan E, Calves P, Nonnotte L, Nonnotte G. 2006b. Effects of hypo-osmotic stress on ATP release in isolated turbot (*Scophthalmus maximus*) hepatocytes. Biol Cell 98:427–437.

Ollivier H, Pichavant-Rafini K, Puill-Stephan E, Calves P, Nonnotte L, Nonnotte G. 2006c. Effects of hyposmotic stress on exocytosis in isolated turbot, *Scophthalmus maximus*, hepatocytes. J Comp Physiol B 176:643–652.

Pafundo DE, Mut P, Perez Recalde M, Gonzalez-Lebrero RM, Fachino V, Krumschnabel G, Schwarzbaum PJ. 2004. Effects of extracellular nucleotides and their hydrolysis products on regulatory volume decrease of trout hepatocytes. Am J Physiol Regul Integr Comp Physiol 287:R833–R843.

Pafundo DE, Chara O, Faillace MP, Krumschnabel G, Schwarzbaum PJ. 2008. Kinetics of ATP release and cell volume regulation of hyposmotically challenged goldfish hepatocytes. Am J Physiol Regul Integr Comp Physiol 294:R220–R233.

Pafundo DE, Alvarez CL, Krumschnabel G, Schwarzbaum PJ. 2010. A volume regulatory response can be triggered by nucleosides in human erythrocytes, a perfect osmometer no longer. J Biol Chem 285:6134–6144.

Parisi M, Dorr RA, Ozu M, Toriano R. 2007. From membrane pores to aquaporins: 50 years measuring water fluxes. J Biol Phys 33: 331–343.

Perlman DF, Goldstein L. 2004. The anion exchanger as an osmolyte channel in the skate erythrocyte. Neurochem Res 29: 9–15.

Pickard WF. 2008. Modelling the swelling assay for aquaporin expression. J Math Biol 57:883–903.

Praetorius HA, Leipziger J. 2009. ATP release from non-excitable cells. Purinergic Signal 5:433–446.

Puffer AB, Meschter EE, Musch MW, Goldstein L. 2006. Membrane trafficking factors are involved in the hypotonic activation of the taurine channel in the little skate (*Raja erinacea*) red blood cell. J Exp Zool A Comp Exp Biol 305:594–601.

Qiu F, Dahl G. 2009. A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP. Am J Physiol Cell Physiol 296:C250–C255.

Ricatti MJ, Alfie LD, Lavoie EG, Sevigny J, Schwarzbaum PJ, Faillace MP. 2009. Immunocytochemical localization of NTPDases1 and 2 in the neural retina of mouse and zebrafish. Synapse 63: 291–307.

Rico EP, Senger MR, Fauth Mda G, Dias RD, Bogo MR, Bonan CD. 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). Life Sci 73:2071–2082.

Rinehart J, Maksimova YD, Tanis JE, Stone KL, Hodson CA, Zhang J, Risinger M, Pan W, Wu D, Colangelo CM, Forbush B, Joiner CH, Gulcicek EE, Gallagher PG, Lifton RP. 2009. Sites of regulated phosphorylation that control K-Cl cotransporter activity. Cell 138: 525–536.

Riquelme G, Sepúlveda FV, Jørgensen F, Pedersen S, Hoffmann EK. 1998. Swelling-activated potassium currents of Ehrlich ascites tumour cells. Biochim Biophys Acta 1371:101–106.

Robson SC, Sevigny J, Zimmermann H. 2006. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2: 409–430.

Roman RM, Wang Y, Fitz JG. 1996. Regulation of cell volume in a human biliary cell line: activation of K+and Cl- currents. Am J Physiol 271:G239–G248.

Sabirov RZ, Okada Y. 2005. ATP release via anion channels. Purinergic Signal 1:311–328.

Sachs F. 2010. Stretch-activated ion channels: what are they? Physiology (Bethesda) 25:50–56.

Schmieder S, Lindenthal S, Ehrenfeld J. 2002. Cloning and characterisation of amphibian ClC-3 and ClC-5 chloride channels. Biochim Biophys Acta 1566:55–66.

Schöfl C, Ponczek M, Mader T, Waring M, Benecke H, von zur Mühlen A, Mix H, Cornberg M, Böker KH, Manns MP, Wagner S. 1999. Regulation of cytosolic free calcium concentration by extracellular nucleotides in human hepatocytes. Am J Physiol 276:G164–G172.

Schwarzbaum PJ, Frischmann ME, Krumschnabel G, Rossi RC, Wieser W. 1998. Functional role of ecto-ATPase activity in goldfish hepatocytes. Am J Physiol 274:R1031–R1038.

Schwiebert EM, Fitz JG. 2008. Purinergic signaling microenvironments: an introduction. Purinergic Signal 4:89–92.

Shennan DB. 2008. Swelling-induced taurine transport: relationship with chloride channels, anion-exchangers and other swelling-activated transport pathways. Cell Physiol Biochem 21:15–28.

Smets I, Ameloot M, Steels P, Van Driessche W. 2002. Loss of cell volume regulation during metabolic inhibition in renal epithelial cells (A6): role of intracellular pH. Am J Physiol Cell Physiol 283: C535–C544.

Smith RM, Martell AE, Chen Y. 1991. Critical evaluation of stability constants for nucleotide complexes with protons and metal ions and the Accompanying Enthalpy Changes. Pure & Appl Chem 53: 1015–1080.

Sorensen CE, Novak I. 2001. Visualization of ATP release in Pancreatic Acini in response to Cholinergic stimulus. Use of fluorescent probes and confocal microscopy. J Biol Chem 276:32925–32932.

Sprague RS, Ellsworth ML, Stephenson AH, Kleinhenz ME, Lonigro AJ. 1998. Deformation-induced ATP release from red blood cells requires CFTR activity. Am J Physiol 275:H1726–H1732.

Strieter J, Stephenson JL, Palmer LG, Weinstein AM. 1990. Volume-activated chloride permeability can mediate cell volume regulation in a mathematical model of a tight epithelium. J Gen Physiol 96: 319–344.

Tatur S, Groulx N, Orlov SN, Grygorczyk R. 2007. Ca^{2+}-dependent ATP release from A549 cells involves synergistic autocrine stimulation by coreleased uridine nucleotides. J Physiol 584:419–435.

Terashima K, Takeuchi A, Sarai N, Matsuoka S, Shim EB, Leem CH, Noma A. 2006. Modelling Cl- homeostasis and volume regulation of the cardiac cell. Philos Transact A Math Phys Eng Sci 364:1245–1265.

Terreros DA, Kanli H. 1992. Role of intracellular calcium in renal proximal tubule cell volume regulation. Am J Physiol 263:R1086–R1092.

Terreros D, Kanli H, Coombs J. 1990. Possible role of basolateral cell membrane in proximal renal tubule osmoregulation. Am J Physiol 258:R1022–R1033.

Tingaud-Sequeira A, Calusinska M, Finn RN, Chauvigne F, Lozano J, Cerda J. 2010. The zebrafish genome encodes the largest vertebrate repertoire of functional aquaporins with dual paralogy and substrate specificities similar to mammals. BMC Evol Biol 10:38.

Trischitta F, Denaro MG, Faggio C. 2004. Ion transport in the intestine of *Gobius niger* in both isotonic and hypotonic conditions. J Exp Zool A Comp Exp Biol 301:49–62.

Trischitta F, Denaro MG, Faggio C. 2005. Cell volume regulation following hypotonic stress in the intestine of the eel, *Anguilla anguilla*, is Ca^{2+}-dependent. Comp Biochem Physiol B Biochem Mol Biol 140:359–367.

Trischitta F, Pidala P, Faggio C. 2007. Nitric oxide modulates ionic transport in the isolated intestine of the eel, *Anguilla anguilla*. Comp Biochem Physiol A Mol Integr Physiol 148:368–373.

Tsuchiya K, Wang W, Giebisch G, Welling PA. 1992. ATP is a coupling modulator of parallel Na, K-ATPase-KChannel activity in the renal proximal tuble. Proc Natl Acad Sci 89:6418–6422.

Ussing HH. 1982. Volume regulation of frog skin epithelium. Acta Physiol Scand 114:363–369.

Vislie T. 1983. Cell volume regulation in fish heart ventricles with special reference to taurine. Comp Biochem Physiol A Comp Physiol 76:507–514.

Wang Y, Roman R, Lidofsky SD, Fitz JG. 1996. Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. Proc Natl Acad Sci USA 93: 12020–12025.

Warren NJ, Tawhai MH, Crampin EJ. 2009. A mathematical model of calcium-induced fluid secretion in airway epithelium. J Theor Biol 259:837–849.

Weaver YR, Cossins AR. 1996. Protein tyrosine phosphorylation and the regulation of KCl cotransport in trout erythrocytes. Pflugers Arch 432:727–734.

Wehner F, Olsen H, Tinel H, Kinne-Saffran E, Kinne RK. 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Rev Physiol Biochem Pharmacol 148:1–80.

Yegutkin G, Bodin P, Burnstock G. 2000. Effect of shear stress on the release of soluble ecto-enzymes ATPase and 5′-nucleotidase along

with endogenous ATP from vascular endothelial cells. Br J Pharmacol 129:921–926.

Zimmermann H. 2006. Ectonucleotidases in the nervous system. Novartis Found Symp 276:113–128.

Ziyadeh FN, Feldman GM, Booz GW, Kleinzeller A. 1988. Taurine and cell volume maintenance in the shark rectal gland: cellular fluxes and kinetics. Biochim Biophys Acta 943:43–52.

Ziyadeh FN, Mills JW, Kleinzeller A. 1992. Hypotonicity and cell volume regulation in shark rectal gland: role of organic osmolytes and F-actin. Am J Physiol 262:F468–F479.

Zuo P, Picher M, Okada SF, Lazarowski ER, Button B, Boucher RC, Elston TC. 2008. Mathematical model of nucleotide regulation on airway epithelia. Implications for airway homeostasis. J Biol Chem 283:26805–26819.