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ORIGINAL PAPER

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Volume regulation following hyposmotic shock in isolated turbot (*Scophthalmus maximus*) hepatocytes

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Abstract Regulatory volume decrease (RVD) following hyposmotic stimulation was studied in isolated turbot, *Scophthalmus maximus*, hepatocytes. Exposed to a reduced osmolality (from 320 to 240 mosm kg^{-1}), cells first swelled and then exhibited a RVD. Volume regulation was significantly inhibited in presence of NPPB, 9-AC, acetazolamide, DIDS and barium. Taken together, these results could suggest that RVD operated via separate K^+ and Cl^- channels and probably Cl^-/HCO_3^- exchanger in turbot hepatocytes. The K^+/Cl^- cotransporter could also be involved as furosemide and DIOA strongly inhibited the process whereas NEM, a K^+/Cl^- cotransporter activator, added under isosmotic conditions, led to cell shrinkage. RVD in turbot hepatocytes appeared also to depend on proteins p38 MAP kinase and tyrosine kinase but not on proteins ERK 1/2. Arachidonic acid and leukotrienes could also be involved since inhibition of synthesis of both these compounds by quinacrine and NDGA, respectively, inhibited the volume regulation. Likewise, Ca^{2+} has been proved to be an essential messenger as RVD was prevented in absence of Ca^{2+}. Finally, this work provides bases for novel studies on cell volume regulation in marine teleosteans.

Keywords Hyposmotic stress · Regulatory Volume Decrease · Fish hepatocytes · Turbot

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Volume constancy constitutes a permanent challenge for survival and normal functioning of cells. To counteract osmotic stress and subsequent flow of water across their membrane, most animal cells have the fundamental ability to regulate their volume by activation of a great diversity of transport systems and intracellular signalling events (Lang et al. 1998; Wehner et al. 2003). Hyposmotic cell swelling leads to activation of a complex volume regulatory mechanism, termed regulatory volume decrease (RVD), accomplished by intracellular osmolytes and osmotically obligated water efflux.

To date, research on cell volume regulation have been extensively reviewed in a variety of mammalian cells types events (Lang et al. 1998; Wehner et al. 2003). In contrast, informations about cell volume regulation process in other species are more scarce. For instance, only few investigations have been dedicated to fish cells, essentially conducted on fresh water fish such as trout (Cooper et al. 2001). To our knowledge, volume regulation mechanisms in marine teleost cell types have not been documented yet. Accordingly, studies of RVD process in marine teleost hepatocytes is of great interest, both in a general physiological context and also from a comparative point of view. Turbot (*Scophthalmus maximus*), a marine teleostean flat fish, represents a relevant model for physiological regulatory responses studies as it can resist large fluctuations of environmental parameters by triggering efficient adaptative mechanisms. In particular, this fish has been reported to tolerate wide fluctuations of ambient salinity (Gaumet et al. 1994). It could be hypothesized that such an ability may arise at the cellular level and that adaptative strategies to prevent damages due to cell swelling and restore original volume may be distinctive and efficient.

Strategies reviewed, although differing between species and cell types (Lang et al. 1998; Wehner et al. 2003), typically imply the K^+ and Cl^- coordinated efflux for RVD progress. This loss of K^+ and Cl^- could occur via

separate K^+ and Cl^- channels, via electroneutral K^+/Cl^- cotransporter or via K^+/H^+ and Cl^-/HCO_3^- exchangers. Moreover, a wide range of factors have been demonstrated to be involved in the signal transduction mechanisms underlying the sensing of cell volume perturbations and the modulation of regulatory processes (Jakab et al. 2002). These include rearrangements of cytoskeletal components, membrane stretch, vesicular trafficking, changes in intracellular pH, release of ATP, generation of lipid messengers or increase in intracellular calcium. Arachidonic acid metabolism, mediated by the action of phospholipases upon cell swelling, has also been widely postulated to play a role in transducing mechanism (Jorgensen et al. 1996; Light et al. 1997; Basavappa et al. 1998). Besides, activation of several protein kinases has been correlated with altered cell volume and may thus contribute to cell volume regulation (Light et al. 1998; Niisato et al. 1999; Koyama et al. 2001; Chiri et al. 2004).

The aim of the present study was to investigate the physiological responses of a marine teleost cell type suddenly exposed to acute hyposmotic stress. Using a video-imaging technique of morphometry, several cellular and molecular aspects of the regulatory volume process from swelling, including Cl^- and K^+ efflux and putative pathways, membrane stretch, potential role of eicosanoids and calcium and involvement of protein kinases, were examined in turbot hepatocytes.

Materials and methods

Cell isolation technique

Turbots (*S. maximus*, 200–400 g) obtained from France Turbot, Trédarzec (France), were kept in 400-l tanks with flowing 17°C sea water and fed with dry fish pellets. Fish were killed by a blow to the head. Then, turbot hepatocytes were isolated by collagenase digestion method adapted from Braunbeck and Segner (2001). The liver was removed and perfused via a cannula inserted into the hepatic portal vein with a 320 mosm kg^{-1} standard isosmotic saline solution (in mmol l^{-1}: 152 NaCl, 3.4 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5 $NaHCO_3$, 10 Hepes; pH 7.63; 5 ml min^{-1}; 30 min). Liver was then perfused with a collagenase (Type IV, Sigma ref. C5138) solution (1 mg ml^{-1} in 20 ml of standard isosmotic saline; 0.5 ml min^{-1}), minced using scalpel blades and filtered through 200 , 85 and 48 μm nylon mesh screens. Cells were repeatedly washed by centrifugation (500 rpm/4 min/4°C) and then resuspended in the standard isosmotic solution supplemented with 5 mM glucose and 1.5 mM $CaCl_2$. Cell populations in which viability, as determined by LDH leakage (Legrand et al. 1992), was less than 85% post-isolation were discarded. Hepatocytes were maintained under slight agitation at least for 1 h at 17°C before experimentation in order to restore ionic concentrations on either side of the cell membrane.

Cell volume estimation

Turbot hepatocytes were fixed to the bottom of a glass experimental chamber coated with poly-L-lysine (0.01% w/v, Sigma) and located on the thermostated stage (17°C) of an inverted microscope (Axiovert 25 Zeiss). The chamber was continuously flushed with the solutions by means of a peristaltic pump. Isosmotic conditions were obtained with hepatocytes exposed for 60 min to the 320 mosm kg^{-1} standard isosmotic saline supplemented with glucose and $CaCl_2$ (Table 1). Hyposmotic control conditions were obtained with hepatocytes exposed for 10 min to the 320 mosm kg^{-1} standard isosmotic saline supplemented with glucose and $CaCl_2$, then for 50 min to a 240 mosm kg^{-1} standard hyposmotic saline supplemented with glucose and $CaCl_2$ (Table 1).

Hepatocyte images were obtained using a video camera (Digital Hyper HAD Sony) connected to the microscope and stored on a PC computer. The dilution of the cell suspension was adjusted to obtain at least six hepatocyte on the screen. Hepatocytes images were captured in isosmotic conditions 10 and 5 min before hyposmotic shock ($t = 0$), then each minute from $t = 0$ to $t = 6$ min, and every 5 min from $t = 10$ min to $t = 60$ min. When cells were maintained under isosmotic conditions for 60 min, hepatocyte images were captured at the same

Table 1 Composition of solutions used to test the effect of gradual hyposmotic stimulation on RVD in isolated turbot hepatocytes

	Isosmotic solution 320 mosm kg^{-1}	Hyposmotic solution 220 mosm kg^{-1}	Hyposmotic solution 240 mosm kg^{-1}	Hyposmotic solution 260 mosm kg^{-1}	Hyposmotic solution 280 mosm kg^{-1}
NaCl	152	99	109	120	131
KCl	3.4	3.4	3.4	3.4	3.4
$MgSO_4$	0.8	0.8	0.8	0.8	0.8
Na_2HPO_4	0.33	0.33	0.33	0.33	0.33
KH_2PO_4	0.44	0.44	0.44	0.44	0.44
$NaHCO_3$	5	5	5	5	5
Hepes	10	10	10	10	10
Glucose	5	5	5	5	5
$CaCl_2$	1.5	1.5	1.5	1.5	1.5
pH	7.63	7.63	7.63	7.63	7.63

Concentrations are given in mmol l^{-1}

times as previously described. At each time, six perimeter measurements were performed for each cell using the image analysis software Cyberview. As isolated hepatocytes take spherical shape, volume changes estimated from perimeter measurements [volume = $4/3\pi(\text{perimeter}/2\pi)^3$] were expressed as percentage of increase or decrease from the initial isosmotic volume ($t = -10$ min).

To test the effect of hyposmotic stimulations of gradual intensity, cells were exposed to isosmotic (320 mosm kg^{-1}) then to hyposmotic solutions at either 280, 260, 240 or 220 mosm kg^{-1} (Table 1).

To assess the involvement of Cl^- efflux in RVD, hepatocytes were bathed in chloride-free isosmotic and hyposmotic solutions to increase Cl^- efflux gradient. In these experiments, Cl^- was substituted for gluconate both in iso- and hyposmotic solutions (Table 2). Other experiments were performed in which the Cl^- concentration of the hyposmotic solution was increased from 85.4 mmol l^{-1} (hyposmotic control for high [Cl^-], Table 2) to 115.4 mmol l^{-1} (hyposmotic high [Cl^-], Table 2). In these experiments, Na^+ concentration was kept constant (157.7 mmol l^{-1} in isosmotic solution and 114.7 mmol l^{-1} in hyposmotic solution) by using Na^+-gluconate (Table 2).

To determine the main effectors of RVD in turbot hepatocytes, effects of Cl^- channel inhibitors 5-nitro–2-(3-phenylpropylamino)-benzoic acid (NPPB, 40 μM) and 9-anthracene carboxylic acid (9-AC, 1 mM), carbonic anhydrase inhibitor acetazolamide (1 mM), anion transport inhibitor 4,4′-diisothiocyanatostilben-2,2′-disulfonic acid (DIDS, 100 μM), K^+/Cl^- cotransporter inhibitors furosemide (180 μM) and [(dihydro-indenyl)oxy] alkanoic acid (DIOA, 100 μM), K^+/Cl^- activator *N*-ethylmaleimide (NEM, 2 mM), K^+ channel inhibitor barium (10 mM), stretch-activated channel inhibitor gadolinium (Gd^{3+}, 200 μM) were tested. Involvement of several signalling pathways was assessed using general protein kinase inhibitor staurosporine (1 μM), p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 (1 μM), extracellular signal-regulated kinase (ERK) inhibitor PD98059 (15 μM), tyrosine kinase inhibitor genistein (30 μM), phospholipase A_2 (PLA2) inhibitor quinacrine (1 μM), phospholipase C (PLC) inhibitor U73122 (1 μM), 5-lipoxygenase inhibitor NDGA (10 μM) and cycloxygenase inhibitor indomethacin (1 μM). Except for NEM added at time = 0, all pharmacological substances were added at least 15 min before experiments and both to iso- and hyposmotic bath solutions.

To determine whether calcium was involved in RVD in turbot hepatocytes, cells were exposed at least for 30 min to calcium-free isosmotic solution supplemented with calcium chelator ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 1 mM) and Ca^{2+}-ATPase inhibitor thapsigargin (1 μM) to reduce extracellular and intracellular calcium concentration to zero. Hyposmotic shock, using calcium-free 240 mosm kg^{-1} solution supplemented with EGTA and thapsigargin, was applied.

All chemicals were purchased from Sigma. Stock solutions of EGTA and gadolinium were dissolved in standard isosmotic solution. Stock solutions of the other pharmacological agents were prepared in DMSO (dimethylsulfoxyde) except for NDGA prepared in ethanol. DMSO or ethanol concentrations did not exceed 1 μl ml^{-1} of cell suspension. Moreover, when DMSO or ethanol were solvents, they were added to control experiments at the same concentration.

Statistical analyses

Statistical analyses were conducted using Sigmastat for Windows (Jandel Scientific). All data are expressed as mean ± SEM. When variance of measured parameters was not homogeneous, they were tested by Kruskal–Wallis one way analysis of variance. Significant Kruskal–Wallis tests were followed by a post hoc multiple comparisons test (Dunn's method). When variance of measured parameters was homogeneous, they were compared using an one-way ANOVA. Significant

Table 2 Composition of solutions used to test the role of a potential Cl^- efflux on RVD in isolated turbot hepatocytes

	Isosmotic Cl^-free	Hyposmotic Cl^-free	Isosmotic control for high [Cl^-]	Hyposmotic control for high [Cl^-] (85.4 mM)	Hyposmotic high [Cl^-] (115.4 mM)
NaCl	–	–	122	79	109
KCl	–	–	3.4	3.4	3.4
$MgSO_4$	0.8	0.8	0.8	0.8	0.8
Na_2HPO_4	0.33	0.33	0.33	0.33	0.33
KH_2PO_4	0.44	0.44	0.44	0.44	0.44
$NaHCO_3$	5	5	5	5	5
Hepes	10	10	10	10	10
Glucose	5	5	5	5	5
$CaCl_2$	–	–	1.5	1.5	1.5
$CaSO_4$	1.5	1.5	–	–	–
Na^+ gluconate	152	109	30	30	–
K^+ gluconate	3.4	3.4	–	–	–
Osmolality	320	240	320	240	240
PH	7.63	7.63	7.63	7.63	7.63

Concentrations are given in mmol l^{-1} and osmolalities are expressed in mosm kg^{-1}

ANOVA were followed by a post hoc multiple comparison (Tukey test). Differences were considered significant at $P < 0.05$.

Results

Cell volume changes following hyposmotic stress

As shown in Fig. 1, presence of DMSO or ethanol at a concentration of 1 µl per ml of cell suspension induced no significant change in cell response compared with hyposmotic control. Consequently, all hyposmotic control experiments were pooled ($n = 63$ cells).

As shown in Fig. 2, continued bathing of turbot hepatocytes in isosmotic solution (320 mosm kg^{-1}) induced no significant change in relative volume whereas sudden exposure of cells to hyposmotic medium (240 mosm kg^{-1}) induced first a swelling by 19 ± 1% followed by a RVD. At time 60 min, 79 ± 5% of the initial volume increase was compensated.

Hyposmotic-induced volume changes were related to the osmotic gradient between intra- and extracellular fluid since cellular swelling gradually enhanced after exposure to osmolalities ranging from 280 to 220 mosm kg^{-1} (Fig. 2). About 280 and 260 mosm kg^{-1} stimulations induced significant lower volume increase (11 ± 1% and 13 ± 2%) compared with the 240 mosm kg^{-1} stimulation (19 ± 1%). Exposure to 220 mosm kg^{-1} led to a significant greater increase in relative cell volume (29 ± 3%) compared with that measured at 240 mosm kg^{-1}. RVD pattern following initial swelling was not significantly modified after exposure to 280, 260 or 240 mosm kg^{-1}: at time 60 min, mean cell volume reached similar values whatever the osmolality was. On the contrary, when cells were exposed to 220 mosm kg^{-1}, higher mean cell volume was observed all along the experiment compared with the osmolality conditions of three others and, at time 60 min, only 65 ± 4% of the initial volume increase was compensated (Fig. 2).

As increase in cell fragility and lysis were observed at 220 mosm kg^{-1} and lowest osmolalities, all additional experiments were performed at 240 mosm kg^{-1}.

Involvement of Cl^- and K^+ efflux during RVD

Compared with hepatocyte responses observed after exposure to the standard iso- (320 mosm kg^{-1}) and then hyposmotic solutions (240 mosm kg^{-1}), no significant change both in swelling and RVD occured when 30 mmol l^{-1} NaCl was substitued for 30 mmol l^{-1} Na^+-gluconate both in iso- and hyposmotic solutions, respectively, corresponding to isosmotic control for high [Cl^-] and hyposmotic control for high [Cl^-] solutions (Fig. 3). At time 60 min, RVD was accomplished by 78 ± 9% when cells were exposed to hyposmotic control for high [Cl^-] solution and it was accomplished by 79 ± 5% after exposure to standard hyposmotic solution. On the contrary, as shown in Fig. 2, volume recovery from swelling was prevented when the Cl^- efflux gradient was reduced by increasing the Cl^- concentration in the hyposmotic solution from 85.4 (hyposmotic control for high [Cl^-] solution) to 115.4 mmol l^{-1} (hyposmotic high [Cl^-] solution). In this condition, only 31 ± 4% of the initial increase were compensated 60 min

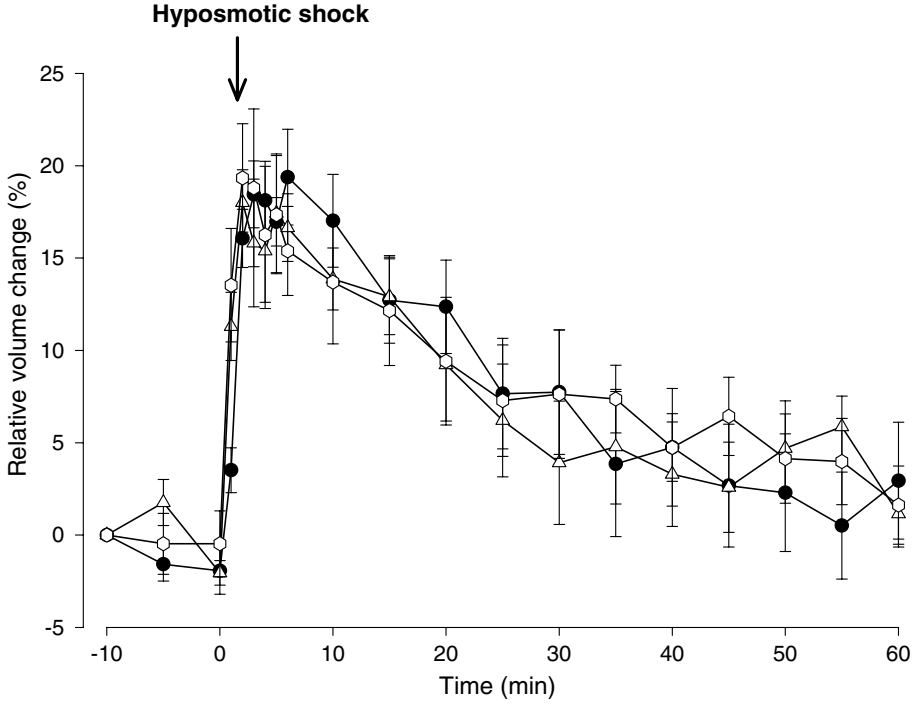

Fig. 1 Effects of DMSO and ethanol on cell response. Turbot hepatocytes were exposed to isosmotic solution (320 mosm kg^{-1}) for 10 min, then at time 0 (*arrow*), the solution was exchanged for hyposmotic solution (240 mosm kg^{-1}, *filled circles*, n = 12 cells). When effects of DMSO (*open triangle*, 1 µl ml^{-1}, $n = 6$ cells) or ethanol (*open hexagon*, 1 µl ml^{-1}, $n = 6$ cells) were tested, they were added to both iso- and hyposmotic solutions. Values are means ± SEM

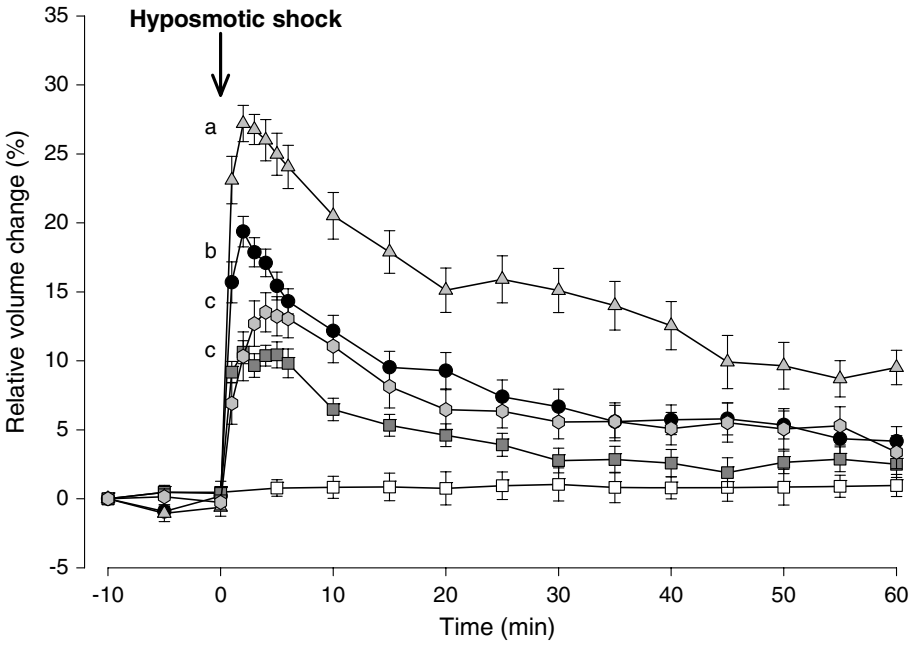

Fig. 2 Changes in cell volume following hyposmotic shock. Turbot hepatocytes were exposed to isosmotic solution (320 mosm kg^{-1}) for 10 min, then, at time 0 (*arrow*), the solution was exchanged for hyposmotic solution of either 280 (*shaded square*, $n=9$ cells), 260 (*shaded hexagon*, $n=7$ cells), 240 (*filled circle*, $n=63$ cells) or 220 mosm kg^{-1} (*shaded triangle*, $n=9$ cells). Control experiments were performed with cells exposed for 60 min to isosmotic solution (320 mosm kg^{-1}, *open square*, $n=9$ cells). Values are means ± SEM. a, b and c indicate significant difference in cell swelling at time 2 min ($P<0.05$)

after the shock, corresponding to a significant inhibition of the RVD compared with the high [Cl^-] hyposmotic control. Moreover, exposure to iso- and then hyposmotic Cl^--free solutions, to increase the Cl^- efflux gradient, accelerated significantly the regulatory process compared with standard hyposmotic control and RVD was accomplished by 96 ± 6% at time 40 min after the hyposmotic shock.

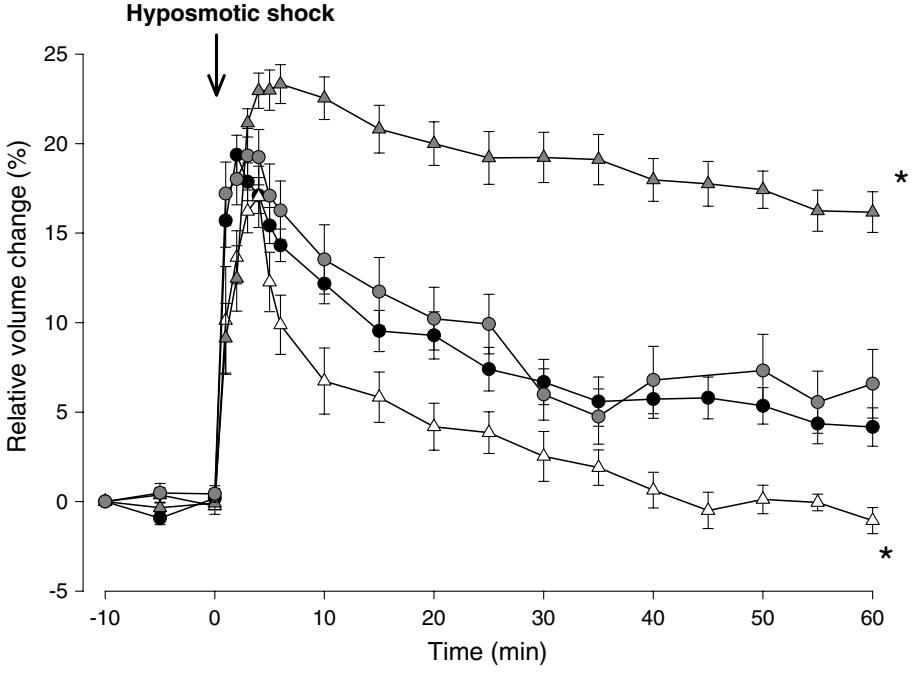

Fig. 3 Involvement of Cl^- efflux in regulatory volume decrease (RVD) of turbot hepatocytes. Turbot hepatocytes were exposed to different isosmotic solutions (320 mosm kg^{-1}, compositions despicted in Table 2) for 10 min, then at time 0 (*arrow*), the solution was exchanged for different hyposmotic solutions (240 mosm kg^{-1}, compositions despicted in Table 2) to test the involvement of Cl^- efflux in RVD response. Exposure of cells to hyposmotic control for high [Cl^-] solution (*shaded circles*, $n=16$ cells) induced no significant change in RVD compared to cells exposed to standard hyposmotic solution (*filled circle*, $n=63$ cells). At the opposite, when cells were bathed with hyposmotic high [Cl^-] solution (*shaded triangle*, $n=18$ cells) to decrease Cl^- efflux gradient, RVD was partially abolished. Increase in Cl^- efflux gradient by bathing cells with Cl^- free hyposmotic solution (*open triangle*, $n=17$ cells) resulted in a faster RVD compared to the hyposmotic control. Values are means ± SEM. *Asterisks* indicate significant difference in relative volume from the hyposmotic value ($P<0.05$) at time 60 min

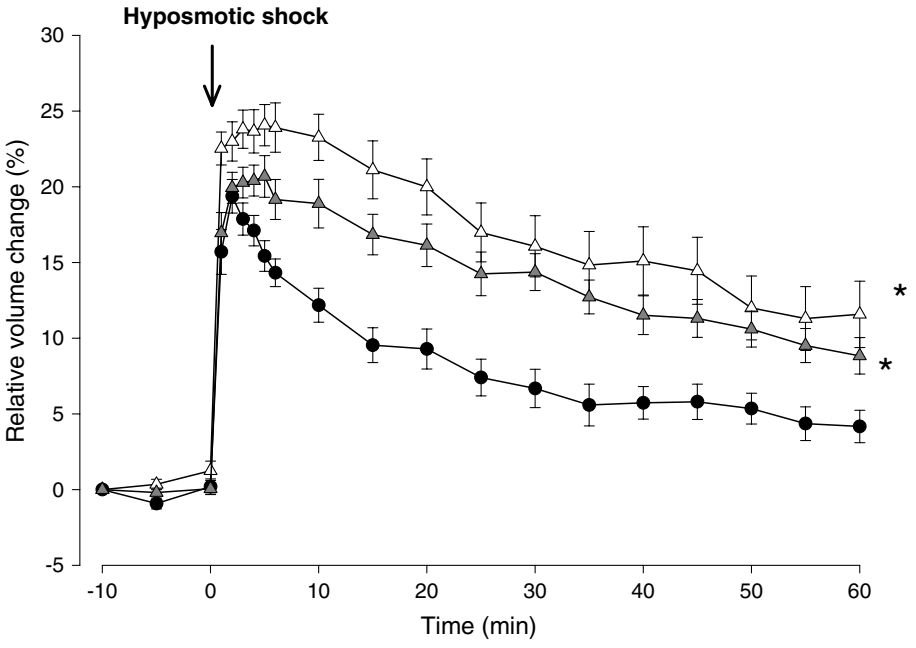

Fig. 4 Effect of NPPB and 9-AC on regulatory volume decrease. Exposure of turbot hepatocytes to a hyposmotic solution (*arrow*, time 0) resulted in a cell volume increase followed by RVD (*filled circle*, $n = 63$ cells) that was significantly inhibited in presence of Cl^- channel inhibitors NPPB (*open triangle*, 40 μM, $n = 19$ cells) or 9-AC (*shaded triangle*, 1 mM, $n = 10$ cells). Values are means ± SEM. *Astrerisks* indicate significant difference in relative volume from the hyposmotic value ($P < 0.05$) at time 60 min

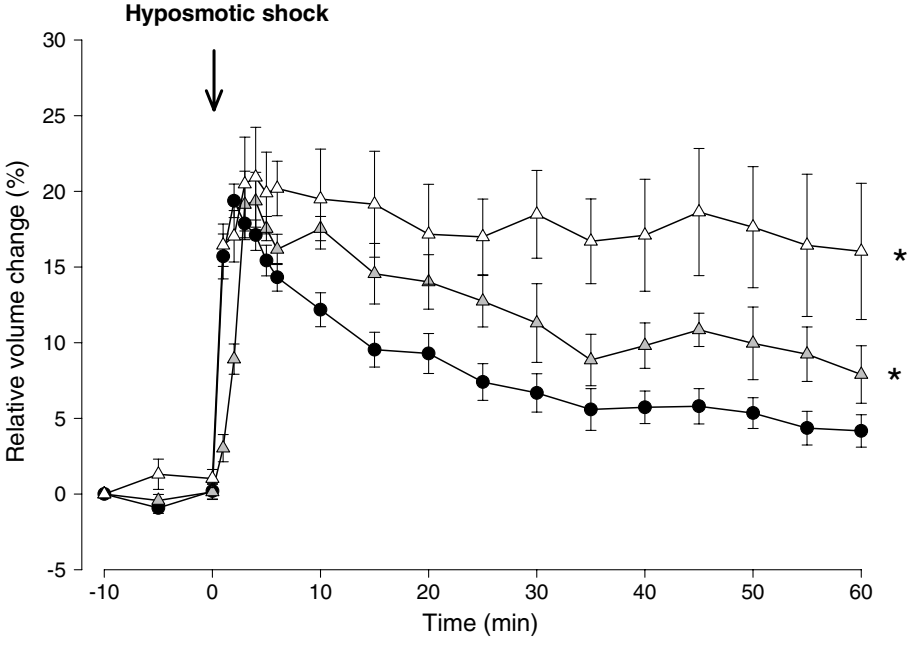

Fig. 5 Effect of acetazolamide and DIDS on regulatory volume decrease. Exposure of turbot hepatocytes to a hyposmotic solution (*arrow*, time 0) resulted in a cell volume increase followed by RVD (*filled circle*, $n = 63$ cells) that was significantly inhibited in presence of carbonic anhydrase inhibitor acetazolamide (*shaded triangle*, 1 mM, $n = 7$ cells) or anion transport inhibitor DIDS (*open triangle*, 100 μM, $n = 6$ cells). Values are means ± SEM. *Asterisks* indicate significant difference in relative volume from the hyposmotic value ($P < 0.05$) at time 60 min

The chloride channel inhibitors NPPB and 9-AC caused a significant inhibition of RVD compared with the hyposmotic control since only 52 ± 8% and 57 ± 5% of the initial increase, respectively, were compensated 60 min after the hyposmotic stimulation (Fig. 4).

Exposure of cells to acetazolamide, a carbonic anhydrase inhibitor, and DIDS, an anion transport inhibitor, led to a significant inhibition of RVD compared with the hyposmotic control. RVD was accomplished only by 59 ± 9% and 23 ± 20% in presence of acetazolamide and DIDS respectively at time 60 min after the hyposmotic stimulation (Fig. 5). Taken together, these results could suggest the involvement of a Cl^-/HCO_3^- exchanger in RVD in turbot hepatocytes.

The K^+/Cl^- cotransporter activator NEM had a significant effect on resting cell volume. The addition of the agent to the isosmotic solution resulted in a 15 ± 1% cellular shrinkage of cells compared with the isosmotic value. Exposure of cells to cotransporter inhibitors furosemide and DIOA under hyposmotic conditions strongly prevented RVD since only 25 ± 10% and 6 ± 5% of the regulatory process, respectively, were accomplished 60 min after the shock (Fig. 6).

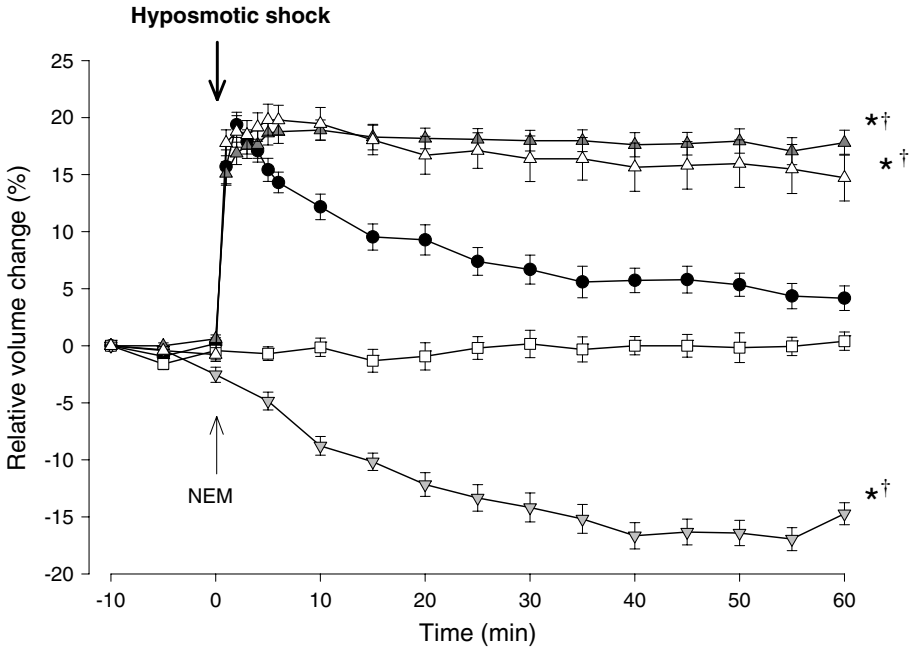

Fig. 6 Effect of furosemide and DIOA on regulatory volume decrease and effect of NEM on cell volume. Exposure of turbot hepatocytes to a hyposmotic solution (*bold arrow*, time 0) resulted in a cell volume increase followed by RVD (*filled circle*, $n = 63$ cells) that was abolished in presence of K^+/Cl^- cotransporter inhibitors furosemide (*open triangle*, 180 μM, $n = 9$ cells) or DIOA (*shaded triangle*, 100 μM, $n = 12$ cells). Addition of K^+/Cl^- cotransporter activator NEM (2 mM, *arrow*) under isosmotic conditions caused a significant decrease in cell volume (*shaded inverted triangle*, $n = 18$ cells) compared to the isosmotic control (*open square*, $n = 9$ cells). Values are means ± SEM. *Asterisks* indicate significant difference in relative volume from the hyposmotic value ($P < 0.05$) at time 60 min. *Daggers* indicates significant difference in relative volume from the isosmotic value ($P < 0.05$) at time 60 min

As shown in Fig. 7, RVD was significantly inhibited by the potassium channel inhibitor barium and the stretch-activated ion channel inhibitor gadolinium (Gd^{3+}) since only 18 ± 11% and 28 ± 14%, respectively, of the initial swelling was compensated 60 min after the hyposmotic stimulation (Fig. 7).

Involvement of protein kinases in RVD

As shown in Fig. 8, the general protein kinase inhibitor staurosporine, the p38 MAP kinase inhibitor SB203580 and the tyrosine kinase inhibitor genistein significantly inhibited RVD since, at time 60 min, cell volume

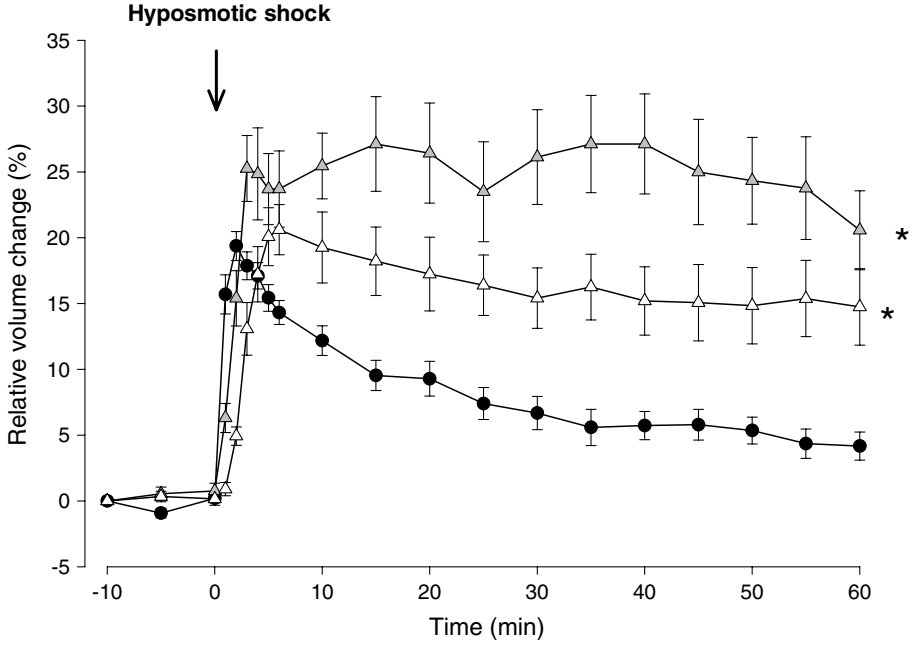

Fig. 7 Effect of barium and gadolinium (Gd^{3+}) on regulatory volume decrease. Exposure of turbot hepatocytes to a hyposmotic solution (*bold arrow*, time 0) resulted in a cell volume increase followed by RVD (*filled circle*, $n = 63$ cells) that was abolished in presence of K^+ channel inhibitor barium (*shaded triangle*, 10 mM, $n = 7$ cells) or SAC inhibitor gadolinium (*open triangle*, 200 μM, $n = 7$ cells). Values are means ± SEM. *Asterisks* indicate significant difference in relative volume from the hyposmotic value ($P < 0.05$) at time 60 min

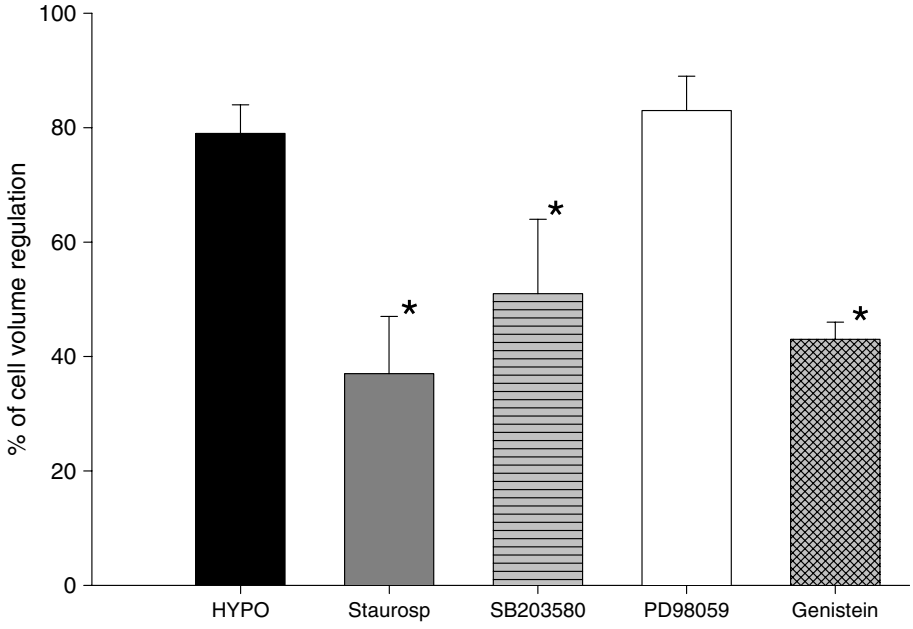

Fig. 8 Effect of staurosporine, SB203580, PD98059 and genistein on percentage of cell volume regulation 60 min after the hyposmotic stimulation. At time 60 min after the hyposmotic stimulation, turbot hepatocytes attained a volume close to the original value (*filled bar*, $n = 63$ cells). In presence of general protein kinase inhibitor staurosporine (*shaded bar*, 1 μM, $n = 7$ cells), p38 MAP kinase inhibitor SB203580 (*bar with horizontal line*, 1 μM, $n = 7$ cells) or tyrosine kinase inhibitor genistein (*hatched bar*, 30 μM, $n = 7$ cells) but not ERK1/2 MAP kinase inhibitor PD98059 (*open bar*, 15 μM, $n = 10$ cells), cells failed to return towards their initial volume. Values are means ± SEM. *Asterisks* indicate significant difference in % RVD at time 60 min from the hyposmotic value ($P < 0.05$)

regulation from swelling was accomplished by only 37 ± 10%, 51 ± 13% and 43 ± 3%, respectively, instead of 79 ± 5% under hyposmotic control conditions. In contrast, PD98059, an ERK inhibitor, failed to affect RVD and no significant difference was observed compared with the hyposmotic control.

Involvement of arachidonic acid and eicosanoids in RVD

No significant change in RVD was induced when hyposmotic stimulation was performed in the presence of the PLC inhibitor U73122 or the cycloxygenase inhibitor indomethacin. In contrast, the PLA2 inhibitor quinacrine and the 5-lipoxygenase inhibitor NDGA strongly prevented volume regulation since only 54 ± 12% and 32 ± 9% of the RVD was accomplished 60 min after the hyposmotic stimulation (Fig. 9).

Involvement of calcium in RVD

As shown in Fig. 10, when cells were exposed to Ca^{2+} free hyposmotic solution supplemented with the calcium chelator EGTA and the Ca^{2+}-ATPase inhibitor thapsigargin, RVD was strongly affected since only 32 ± 13% of the RVD was accomplished 60 min after the hyposmotic stimulation.

Discussion

The present study was aimed at identification of channels and underlying mechanisms involved in cell volume regulation following hyposmotic shock in isolated hepatocytes of a marine teleost fish, turbot, *S. maximus*. Besides providing novel informations on marine teleostean cellular physiology, our investigation was of interest from a comparative point of view. The main difficulty to work on species other than mammals is the few specific tools at disposal. In particular, pharmacological profile of a given substance, while sometimes reasonably well established in mammalian model systems, is entirely unexplored in species that are only rarely found in laboratory such as the turbot. This implies a large amount of caution in results interpretation and necessitates to test as much as possible the effects of several potential agonists or antagonists of a given target system. The results of this study have to be considered as the first step to identify the main mechanisms of RVD in turbot hepatocytes and our hypothesis have to be confirmed by further studies based on molecular identification.

As most animal cell types (Lang et al. 1998; Wehner et al. 2003), turbot hepatocytes can face transient osmotic swelling by activating intracellular signalling pathways and specific membrane transporters. When they were exposed to a decrease in extracellular osmolality, turbot hepatocytes initially behave as almost

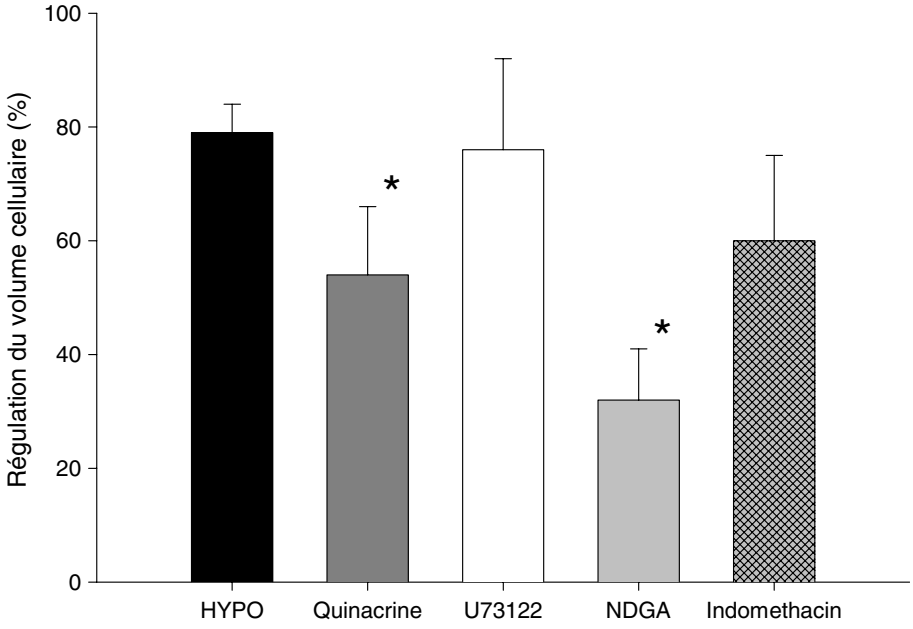

Fig. 9 Effect of quinacrine, U73122, NDGA and indomethacin on percentage of cell volume regulation 60 min after the hyposmotic stimulation. At time 60 min after the hyposmotic stimulation, turbot hepatocytes attained a volume close to the original value (*filled bar*, $n = 63$ cells). No significant change in RVD was observed when cells were incubated with PLC inhibitor U73122 (*open bar*, 1 μM, $n = 7$ cells) or cycloxygenase inhibitor indomethacin (*hatched bar*, 1 μM, $n = 7$ cells). At the opposite, in presence of PLA2 inhibitor quinacrine (*shaded bar*, 1 μM, $n = 8$ cells) or 5-lipoxygenase inhibitor NDGA (*shaded bar*, 10 μM, $n = 8$ cells), cells failed to return towards their initial volume. Values are means ± SEM. *Asterisks* indicate significant difference in % RVD at time 60 min from the hyposmotic value ($P < 0.05$)

perfect osmometers and the following swelling extent was dependent on the osmotic gradient between intra- and extracellular fluid. Then, cells have the ability to exhibit an RVD to counteract this hyposmotic-induced volume increase. Our data support the hypothesis that recovery of normal cell volume was dependent on K^+ and Cl^- efflux followed by the loss of osmotically obligated water. Indeed, increase in Cl^- efflux gradient led to a faster RVD whereas it was inhibited by reduction of Cl^- efflux gradient. In turbot hepatocytes, as in many cell types (Rubera et al. 1997; Mignen et al. 1999; Fernandez-Fernandez et al. 2002; Junankar et al. 2004), K^+ and Cl^- efflux occured via separate channels sensitive to barium or NPPB and 9-AC, respectively. Moreover,

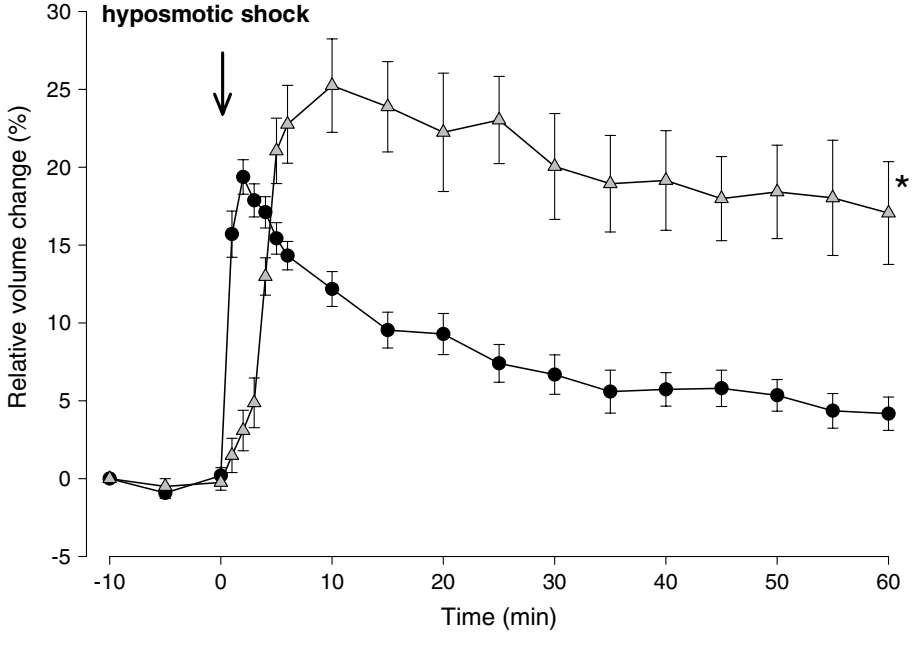

Fig. 10 Effect of calcium removing on regulatory volume decrease. Exposure of turbot hepatocytes to hyposmotic solution (*arrow*, time 0) resulted in a cell volume increase followed by RVD (*filled circle*, $n = 63$ cells) that was abolished when cells were incubated in Ca^{2+}-free iso- then hyposmotic solutions supplemented with the calcium chelator EGTA (1 mM) and the Ca^{2+}-ATPase inhibitor thapsigargin (*open triangle*, 1 μM, $n = 6$ cells). Values are means ± SEM. *Asterisks* indicate significant difference in relative volume from the hyposmotic value ($P < 0.05$) at time 60 min

inhibition of RVD process by DIDS could indicate the involvement not only of Cl^- channels but also of anion transporters, such as Cl^-/HCO_3^- exchangers, since acetazolamide, a carbonic anhydrase inhibitor, also prevented RVD. This Cl^-/HCO_3^- exchanger could operate in parallel with a K^+/H^+ exchanger inducing a net loss of KCl as in *amphiuma* erythrocytes (Cala 1980) and/or could allow the loss of organic osmolytes as in skate, *Raja erinacea*, erythrocytes (Goldstein et al. 1996).

In this study, addition of NEM under isosmotic conditions induced a significant decrease in cell volume whereas hyposmotic shock performed in the presence of furosemide or DIOA strongly inhibited RVD. Taken together, these results indicated that RVD in turbot hepatocytes could also involve activation of a K^+/Cl^- cotransporter as in human esophageal epithelial cells (Orlando et al. 2002) and rat cardiomyocytes (Taouil and Hannaert 1999). Gadolinium, a potential stretch-activated channel (SAC) inhibitor, strongly prevented RVD in turbot hepatocytes indicating a possible role of SAC in this process. These channels may be selective for K^+ or for anions to serve RVD and could have the role of cell volume perturbation sensor to trigger intracellular signalling events (Sackin 1994).

According to our results, protein kinases could play a fundamental role in the signal transduction mechanisms following hyposmotic stimulation in turbot hepatocytes since addition of staurosporine, a general protein kinase inhibitor, induced a significant decrease in cell volume regulation. In particular, protein tyrosine kinases (PTKs) could contribute to RVD in turbot hepatocytes, predominantly in the early phase of volume regulation by triggering mechanisms sensing volume perturbation and cascades of enzymes activations (Hoffmann 2000). However, PTKs could also directly phosphorylate ion channels including Cl^- channels as shown in human intestinal 407 cells (Tilly et al. 1993). MAP kinases p38 but not Erk1/Erk2 could be involved in RVD in turbot heptocytes since SB203580 but not PD98059 strongly inhibited the process. Protein p38 could contribute to K^+ efflux as in perfused rat liver (Vom Dahl et al. 2001) and/or to decrease in membrane Na^+ permeability favouring RVD as in rat hepatoma cells (Feranchak et al. 2001). Moreover, because the function of MAP kinases in the regulation of gene expression is well established, a potential role of p38 in restoring cellular homeostasis and promoting long-term survival could be proposed (Van der Wijk et al. 2000).

In turbot hepatocytes, inhibition of arachidonic acid production from membrane phospholipids by phospholipase A2 but not phospholipase C led to prevention of volume recovery from swelling. Arachidonic acid could have direct actions leading to activation of RVD effectors as in rat neuronal cells (Kim et al. 1995). Besides, it could also act mostly via its eicosanoid derivates. In turbot hepatocytes, 5-lipoxygenase but not cycloxygenase pathways could be involved in RVD since NDGA but not indomethacin strongly prevented the process. In many cell types, arachidonic acid and derivates take part in the generation of the rapid and transient rise in intracellular cytosolic-free Ca^{2+} widely observed following hyposmotic stimulation (Jorgensen et al. 1996; Tinel et al. 1997). Further experiments are necessary to state such an event in turbot hepatocytes. Ca^{2+} seems to be a fundamental factor in RVD process of this fish cell type since our results show that cell volume recovery from swelling was strongly inhibited in the absence of extra- and intracellular calcium.

In conclusion, this work demonstrates the involvement of K^+ and Cl^- loss in RVD following hyposmotic shock in turbot hepatocytes. This net efflux of KCl could occur via K^+ and Cl^- separated channels, K^+/Cl^- cotransporters and Cl^-/HCO_3^- exchangers. The regulatory volume process appeared to be under control of calcium, protein kinases, arachidonic acid and derivates. These data provide informations about the net transport of ions and the volume regulation process for an experimental material which remains to date not investigated. Finally, the present study in a recently evolved teleost, the turbot, seems to indicate a highly conserved adaptative mechanism between mammals and early vertebrates. Preservation of such a physiological process during evolutionary, all the more in osmoregulating organisms as fish and mammals, is representative of its importance for cell survival.

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