Swelling-activated transport of taurine in cultured gill cells of sea bass: physiological adaptation and pavement cell plasticity.

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ABSTRACT

We have investigated volume-activated taurine transport and ultrastructural swelling response of sea bass gill cells in culture, assuming that euryhaline fish may have developed particularly efficient mechanisms of salinity adaptation. In vivo, when sea basses were progressively transferred from seawater to freshwater, we noticed a decrease in blood osmotic pressure. When gill cells in culture were subjected to 30 % hypotonic shock, we observed a 5-fold stimulation of [3H]taurine efflux. This transport was reduced by various anion channel inhibitors with the following efficiency: NPPB>niflumic acid>DIDS=DPC. With polarized gill cells in culture, the hypotonic shock produced a 5-fold stimulation of apical taurine transport, whereas basolateral exit was 25 times higher. Experiments using ionomycin, removal of extracellular calcium, thapsigargin or BAPTA-AM suggested that taurine transport was regulated by external calcium. The inhibitory effects of lanthanum and streptomycin support Ca++ entry through mecano-sensitive Ca++ channels. Branchial cells also showed hypotonically-activated anionic currents sensitive to DIDS and NPPB. Similar pharmacology and time course suggested the potential existence of a common pathway for osmosensitive taurine and Cl- efflux through VSOAC channels. A three-dimensional structure study revealed that respiratory gill cells began to swell only 15 s after hypoosmotic shock. Apical microridges showed membrane outfoldings: the cell surface became smoother with a progressive disappearance of ridges. Therefore, osmotic swelling may not actually induce membrane stretch per se, inasmuch as the microridges may provide a reserve of surface area. This work demonstrates mechanisms of functional and morphological plasticity of branchial cells during osmotic stress.

Key words: regulatory volume decrease, hypotonic shock, branchial epithelium
INTRODUCTION

Whereas most mammalian epithelial cells do not normally undergo changes in apical and basolateral osmolality of the magnitude tested in most cell volume changes in vitro studies described in the literature, cells of euryhaline fish actually are commonly affected by large osmotic stresses. This may occur in the wild or in fish farming. Hypotonic shock is physiologically relevant to the European sea bass *Dicentrarchus labrax*, since, although it is a marine teleost, it naturally encounters fluctuations of environmental salinity. *D. labrax* are euryhaline organisms at all developmental stages, reproducing in estuarine water and can grow and thrive in freshwater (FW) (9, 10, 45, 46). Most vertebrate cells react to hypo-osmotic challenge by cell swelling followed by an active decrease in cell volume, the latter process being known as regulatory volume decrease (RVD). This RVD response occurs via efflux of $K^+$, $Cl^-$ and organic osmolytes accompanied by osmotically obligate water loss (20, 28). The cytosol of all organisms contains high concentrations of small, organic and osmotically active solutes such as polyols (*e.g.* sorbitol or myo-inositol), amino acids (*e.g.* taurine) and methyl amines (*e.g.* betaine). In most phyla investigated, from bacteria to humans, it has been observed that such solutes are lost in response to cell swelling, demonstrating their key role in cell volume homeostasis. The results accumulated in the last few years are consistent with the hypothesis that the volume-activated transport of organic osmolytes, especially taurine, may occur via a single pathway that resembles channel which is blocked by a range of anion transport inhibitors and is therefore termed VSOAC (volume-sensitive organic osmolyte and anion channel) (23, 25, 48, 57). However, the nature of the volume-activated taurine transport pathway is still controversial (55). The purpose of the present investigation was to determine 1) whether gill cells of a marine teleost species possess a transporter with VSOAC-like properties, and 2) whether the branchial epithelium, naturally subjected to large osmotic changes, has developed particularly efficient cellular mechanisms to maintain ionic homeostasis.

It is reasonable to think that the teleostean branchial epithelium, in direct contact with the external medium, could be very sensitive to environmental osmolarity and that the RVD response be more significant than in mammals. The gill respiratory (pavement) cells represent more than 80% of the teleost branchial epithelium (21, 30). They exhibit, on their surface, a complex system of microridges, reflecting expansion of outer membrane responsible for respiratory gaseous exchanges, which increases the surface area of teleosts gills by 2.5 times (42). We have shown previously that respiratory cells *in vivo* were also involved in salinity...
Gill swelling-activated transport of taurine adaptation (4), in addition to the well-described gill mitochondria-rich cells (known as chloride cells). Moreover, we found that gill pavement cells in culture were important for ionic transport and more specifically Cl⁻ transport (3). We also described the relative impermeability of the apical membrane of sea bass gill epithelium during hypotonic shock as a possible adaptation of this tissue to limit its overwhelming by water (13). Hence, gill cells (and more specifically pavement cells) proved to be involved in osmotic regulation can represent a very good model for investigating RVD mechanism and molecular adaptations to salinity changes. In fishes and cyclostomes, observations related to amino acid-regulated cell volume homeostasis have been made mostly in erythrocytes (flounder, skate, eel, trout, hagfish and lamprey), liver cells (skate, trout) and renal tubules (trout, goldfish, flounder) (44). To date, one group has followed hypo-osmotic shock in trout gill epithelium with observations on changes in cell volume and intracellular calcium signaling (32). We chose to focus on sea bass gill cells in culture which can form a highly polarized epithelium and has formerly proven to be a good model for ion transport in high-resistance epithelia (3, 5).

We show, in cultured gill cells, that the transport of [³H]taurine increased during periods of hypotonicity was regulated by extracellular Ca²⁺ and may occur through VSOAC-like transporter. Concomitant structural modifications revealed a peculiar osmotic swelling involving outfoldings of the surface pavement cell microridges. This work contributes significantly to our understanding of the rapid and efficient functional and morphological adjustments that occur in gill cells shortly after salinity changes.
MATERIALS AND METHODS

Fish
European sea bass (*D. labrax*; approximately 40 g) were obtained from a local sea farm (Cannes Aquaculture, Cannes, France) and kept at ambient temperature (14-18°C) with natural lighting in a semi-open circuit (water completely renewed every 6 h) in a 1m³ tank containing Mediterranean sea water (SW) (36 g l⁻¹ salinity). Fish were fed daily *en masse* with Aqualim pellets (Biomar, Nersac, France) in amounts equivalent to 2.5% of total body weight.

Primary cell culture
Prior to cell culture preparation, fish were held for 2 hours in a 10 liter tank of aerated SW containing antibiotics and the fungicides Furaltaladone (0.02%; Sigma, St Louis, MO, USA) and Temerol (0.02%; Francodex, France). Fish were stunned by a blow on the head, decerebrated and decapitated in accordance with French ethical guidelines (CREEA). The gills were then excised and the filaments prepared to yield a single-cell suspensions as described in Avella *et al.* (2). Cells were seeded 1) directly in 35 mm diameter Nunc Petri dishes at a density of 6x10⁵ cells cm⁻² or 2) on permeable filters at a high density (2x10⁶ cells cm⁻²) in Costar-Transwell 0.45 µm-pore plastic inserts (25 mm diameter, 4.8 cm² surface) laid in 6-well Costar plates (Costar, MA, USA). All cells were maintained at 18°C, in a humidified air atmosphere (*i.e.* atmospheric pCO₂). Medium was changed every other day, and cells were used from day 3 to day 10 after seeding at ambient temperature not exceeding 23°C. Cells were deprived of any antibiotics (see below) 48h before all experiments.

Solutions and reagents
*Primary cultures*: The culture medium consisted of Leibovitz L15 supplemented with 10% fetal bovine serum (FBS), 20 mM NaCl, penicillin (100 IU ml⁻¹), streptomycin (100 µg ml⁻¹ = 70µM) and gentamycin (200 µg ml⁻¹). The final pH was adjusted to 7.8. All culture reagents were supplied by Sigma.

*Drugs and other chemicals*: N-methyl-D-glucamine (NMDG), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), niflumic acid (NFA), diphenylamine-2-carboxylic acid (DPC), tamoxifen, ionomycin, thapsigargin, BAPTA-AM, lanthanum, nifedipin, verapamil, gadolinium, forskolin, adenosine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and arginine-vasotocin
were all purchased from Sigma. We tested that solvents (DMSO, ethanol, saline) had no significant effect on their own on gill cell response.

**Isotonic and hypotonic solutions used for taurine transport**: Iso-osmotic medium was isotonic to sea bass plasma and culture medium (350 mOsm/l). Iso-osmotic medium contained (in mM): KCl 5, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, Glucose 10, NaCl 160. Hypoosmotic medium (245 mOsm/l) contained (in mM): KCl 5, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, Glucose 10, NaCl 110. Thus, the only difference between the two media was the NaCl concentration. For both solutions, the pH was adjusted to 7.8 with NaOH.

**Physiological studies following fish transfer from seawater to freshwater.**
Twenty one approximately 100 g SW acclimated sea bass were put in 200 liter SW tanks at 18°C under natural photoperiod for three weeks. They were progressively challenged with FW by gradually decreasing the external osmotic pressure. This was accomplished by slowly introducing FW to the tanks and releasing SW regularly during 5 hours. Changes in external salinity were frequently monitored with a refractometer (Atago, Japan). To follow the corresponding modifications in sea bass internal osmotic pressure, the fish were collected at 0 h (controls), 0.5 h, 2.75 h and 4.75 h during the hypoosmotic challenge. Sampled fish were rapidly netted and killed by immersion in 200 mg.l$^{-1}$ ethyl m-aminobenzoate methane-sulfonate (MS 222). Their blood was drawn from the caudal vein with heparinazed seringa and centrifuged. The plasma collected was immediately analysed in duplicate to determine the osmotic pressure with an Automatik osmometer (Roebling, Illkirch, France). All procedures involving animals were approved by the local animal ethic committee (CREEA) in accordance with the guidelines provided by the French Agricultural Office and the legislation governing animal studies.

**Taurine transport measurements**
Gill cells were loaded with $[^{3}H]$-taurine (2.5 µCi ml$^{-1}$) (PerkinElmer, Courtaboeuf, France) for 4 h at 18-20°C in culture medium without FBS and antibiotics. They were washed 4 times by replacement of the medium (Petri dishes) or by dipping the filters in fresh medium. Cells were allowed to equilibrate for a control period (isotonic medium) and then were challenged with hypotonic solutions. The medium contained in each well (1.0 ml supernatant for experiments in Petri dishes, or 1.0 ml apical plus 1.0 ml basolateral for experiments in permeable filters) was replaced every 5 min. The radioactivity remaining in the cells at the end of the experiment was determined by disrupting the cells with 1 ml of 2% SDS solution. The
collected samples were placed in vials and supplemented with 4 ml of Ionic Fluor (Packard, Rungis, France) for beta scintillation counting (Packard Instrument, Frankfurt, Germany).

Outward movement of labelled taurine was calculated as the fraction of the total radioactivity lost per unit of time according to the equation \( k_i = \frac{cpm_i}{\text{sum}[cpm_{cells} + \text{sum cpm}_{n->i}]/t_i} \), where \( k_i \) is the efflux rate constant, \( cpm_i \) is the radioactivity released in counts per minute at time \( i \) where \( n \) samples are taken, \( cpm_{cells} \) is the radioactivity remaining in the cells in counts per minute at the end of the experiment, and \( t_i \) is the time interval for sample \( i \) in minutes (31). The sampling period occurred every five minutes. For comparing the different experimental conditions, results were expressed as percent of the initial transport rate constant (% of basal taurine release), the latter calculated during iso-osmotic control conditions (mean of control values).

**Electrophysiology studies**

*Transepithelial electrical measurements:* Each culture insert was checked for basic electrophysiological parameters known to reflect a functional polarized epithelium, prior to use in taurine transport experiments (3, 5). Cells were seeded onto 25-mm-diameter Costar-Transwell 0.45-µm-pore culture plate inserts (filters). Cells were allowed to grow to confluence, and transepithelial voltage (\( V_{te} \)) and resistance (\( R_{te} \)) were routinely checked using an epithelial volt-ohm-meter (EVOM) with "chopstick" electrodes (Millipore, World Precision Instruments, Sarasota, FL). The chopstick electrodes were immersed in the cell culture medium bathing the apical (2 ml) and basolateral (4 ml) sides of the cultures under sterile conditions. Using this technique, cell cultures could be monitored repeatedly for several days until reaching physiological reported values validating their use in the subsequent transport experiments.

*Whole-cell clamp:* The ruptured-patch whole-cell configuration of the patch-clamp technique was used. Whole-cell currents were recorded from the apical membranes of 5 to 10-day-old cultured cells. Borosilicate glass pipettes (1.5 mm OD, 1.1 mm ID, Clay Adams, New York, NY, USA) were pulled using a vertical puller (PP-83, Narishige, Japan) and filled with appropriate solution (Table 1). Pipettes with resistances ranging from 2 to 3 MOhms were connected via an Ag-AgCl wire to the headstage of an RK 300 patch-clamp amplifier (Biologic, Grenoble, France). Gigaseals were achieved spontaneously or by applying a slight suction to the patch pipette. The fast compensation system of the amplifier was used to compensate for the intrinsic input capacitance of the head stage and of the pipette. Cell membrane was ruptured by additional suction to achieve the conventional whole-cell
configuration. Settings available on the amplifier were used to compensate for cell capacitance. Extracellular solutions were perfused into the bath using a 4-channel glass pipette, whose tip was placed near the clamped cell. To achieve the hypoosmotic shock, the patch pipette solution (cell medium) was hyperosmotic (390 mOsm/Kg H$_2$O) as in Rubera et al. (49) and the bath solution was hypoosmotic (290 mOsm/Kg H$_2$O). To eliminate cationic currents, experiments were carried out after replacing Na$^+$ in the pipette solution by NMDG$^+$ (Table 1).

Voltage-clamp commands and data acquisition were controlled by a computer equipped with a DigiData 1200 interface driven by pCLAMP 6.0 software (Axon instruments, Foster City, CA, USA) which was also used to generate and analyse current-voltage (I-V) relationships. For chloride current measurement, cells were maintained at a holding potential ($V_{\text{hold}}$) of −40 mV and 400 ms pulses from −80 to +80 mV (or from −100 mV to +60 mV for DIDS experiments) were applied in 20-mV increments every 2 s. Membrane currents were filtered at 1.0 kHz and sampled at 2.5 kHz.

**Morphology**

Electron microscopy was performed in cells grown on permeable filters to get a polarized differentiated epithelium (3). Both apical and basolateral sides were rinsed with serum-free medium and challenged as necessary with hypotonic medium at various time intervals (from 15 sec to 30 min).

For transmission electron microscopy (TEM), cells were fixed in a solution of 1.6 % glutaraldehyde in 0.1 M phosphate buffer. Filters were rinsed in buffer and then post-fixed in osmium tetroxide (1% in 0.1 M phosphate buffer) during 30 min. After rinsing with distilled water, cells were dehydrated in ethanol and embedded in Epon resin. The 80-nm ultrathin sections were contrasted with uranyl acetate and lead citrate and then observed in a CM12 Electron Microscope operating at 100 kV (Philips, Eindhoven, Netherlands).

Filters were treated similarly for scanning electron microscopy (SEM) save osmium post-fixation. After rinsing with distilled water, cells were dehydrated in a graded series of ethanol and dried using hexamethyldisilazane. After drying, cells were mounted on aluminium stubs with adhesive tabs, sputter coated with AuPd for 4 minutes (Polaron, UK) and examined in a JEOL 6700F microscope (Japan).
Data analysis

Results are mean ± standard error of the mean (SE). The number of observations, n, refers to the number of cultures. Whenever possible, test experiments were assigned their own controls. Test versus control comparisons were performed with Student’s paired or unpaired t-test as appropriate. Differences between various treatments exceeding two groups were evaluated with analysis of variance followed by the Bonferroni post hoc test.
RESULTS

Physiological hypotonic shock of the fish
In order to ensure the physiological relevance of our in vitro hypoosmotic shocks, we submitted a group of sea bass to a stepwise decrease in external salinity from SW to FW over 5 hours (Fig.1A). During this hypoosmotic challenge, the plasma osmotic pressure showed a significant 6% decrease followed by an 11% decrease after 2.75 and 4.75 hours, respectively (Fig.1B). The lowered osmoregulation capacity (however transient) of challenged fish in these experimental conditions was revealed by the positive correlation observed between changes in external and internal osmotic pressure (Fig. 1C).

Hypotonic release of taurine from cultured gill cells and its pharmacology
We investigated whether or not taurine behaves as an organic osmolyte in gill respiratory cells. Taurine transport was studied under isoosmotic conditions (control) and after application of a 30 % hypoosmotic shock in primary cultures of gill cells grown in Petri dishes (Fig.2A). We observed a significant stimulation (5 fold) of basal transport, which became maximal 10 min after the osmotic challenge. The effect was sustained for the entire duration of hypoosmotic challenge. Activated transport was reversible upon return to isotonic conditions. A second shock application showed that this effect was fully reproducible.

To characterize the taurine transport pathway, we investigated the effects of various anion channel and transporter inhibitors (Fig.2B). After a 15-min control period in iso-osmotic medium, the inhibitor was introduced into the isotonic medium for an additional 15 minutes to test its own effect on basal taurine transport. At the end of this period, a hypoosmotic shock was applied in the presence of the inhibitor. The values given in Fig. 2B correspond to the flux levels reached 10 min after the osmotic challenge in the presence or absence of inhibitors. Most of the drugs investigated had significant inhibitory effects. The sensitivity to anion-selective channel blockers was NPPB>niflumic acid>DIDS>DPC. Tamoxifen (20 µM) and DPC at low concentration (300 µM) had no effect.

Permeability of apical and basolateral membranes to taurine
To dissociate apical from basolateral components of taurine transport, gill respiratory cells were grown on permeable filters. The transepithelial potential, Vt, was 18 ± 1 mV and the resistance, Rt, was 3035 ± 106 Ω x cm² (n=13). These specifications are characteristic of a functional tight epithelium (3). Apical and basolateral taurine transports were measured as a
function of time, under isoosmotic followed by hypoosmotic conditions with both sides containing the same medium (Fig. 3A). The hypoosmotic shock produced a large increase (25-fold) in taurine permeability at the basolateral side but only a moderate increase at the apical side (5-fold). Therefore, after hypoosmotic shock, large differences could be observed between both membranes, so that permeability of the basolateral membrane was 5 times greater than that of the apical membrane. It should be noted that the rate reached by the apical taurine exit corresponded to the hypotonically stimulated taurine transport in cells grown in Petri dishes (Fig.2A). To investigate the taurine transport pathway involved in these events, 1 mM DIDS was applied to the system. A significant reduction in both apical and basolateral transports was observed (Fig. 3B).

In natural conditions, fish gill epithelium forms a physical barrier between the two compartments, external water and blood, which contain very different osmotic pressures and can vary according to environmental conditions. Therefore, we investigated the effect of asymmetrical osmotic shock by exposing only the apical or the basolateral membrane to hypotonic stress. Surprisingly, application of hypotonic shock to the apical compartment elicited an increase in apical taurine movement only (Fig. 4A). Likewise, applying the shock to the basolateral compartment induced a large increase in basolateral taurine transport only (Fig. 4B). The apical response was 5 times greater than that of control whereas the basolateral osmotic shock resulted in a 35-fold increase in taurine transport. As with symmetrical challenge (Fig.3A), the basolateral response was 5 to 7 times higher than the apical response. In both experimental conditions (Figs. 3A and 4), the levels of basolateral and apical membrane stimulation were very different with the apical response being much less substantial than the basolateral one.

**Calcium regulation of taurine transport**

To investigate the regulation of taurine transport, we first sought to increase intracellular calcium [Ca$$^{++}$$], directly, to see whether this would trigger taurine exit (27). This was accomplished with the addition of ionomycin, a Ca$$^{++}$$ ionophore (t=15 min), which indeed induced a substantial transitory outward movement of labelled taurine in isotonic medium (Fig. 5A, 1$$^{st}$$ peak at 25 min). Upon subsequent hypotonic challenge in the presence of ionomycin (t=45 min; Fig. 5A, 2$$^{nd}$$ peak), the observed effects of both stimuli were not additive: no significant difference in taurine movement was observed compared to concurrent, hypotonically-challenged controls.
To elucidate the role of external Ca\(^{++}\) in taurine transport, experiments with zero Ca\(^{++}\), 0.55 µM thapsigargin or 10 µM BAPTA-AM in the external medium were performed (Fig. 5B). Application of a hypoosmotic shock in a calcium-deprived medium resulted in an 83% significant inhibition of the stimulated-taurine isotopic flux, confirming a major role of extracellular calcium in taurine transport. To test further that the hypotonically activated-taurine transport was not due to a release of Ca\(^{++}\) from internal stores, cells were pre-incubated with 0.55 µM thapsigargin for 1 hour prior to exposure to a hypotonic solution. Thapsigargin depletes intracellular Ca\(^{++}\) stores and induces a rapid and pronounced increase in the concentration of cytosolic free Ca\(^{++}\) (58). In the presence of this agent, we did not observe any change in shock-induced taurine transport, ruling out the release of calcium from intracellular stores as a signal. Moreover, when the intracellular calcium chelator BAPTA-AM was added to fish gill cell culture for 1 hour prior to exposure to hypotonicity, a 46% significant reduction of the shock-activated taurine transport was observed and can be explained by chelation of calcium ions entering the cells from outside.

The mechanism of calcium entry during hypoosmotic shock was further examined. Addition of lanthanum chloride, which blocks Ca\(^{2+}\) channels non-specifically, produced a complete inhibition of the hypotonically activated taurine transport (Fig. 6A). To determine which type of Ca\(^{++}\) channel was involved, treatment of cultures with the L-type-specific blockers nifedipin (20 µM) or verapamil (20 µM) was undertaken. In the same conditions of osmotic challenge, these agents did not modify the stimulated taurine transport; the percentage of basal taurine release 15 min after the shock was 598 ± 35% for control, 627 ± 9% for nifedipin, and 698 ± 48% for verapamil (Student’s unpaired t-test, two-tail, no significant difference with \(p=0.61\) for nifedipin and \(p=0.14\) for verapamil, \(n=3-6\)) (not shown). Finally, the question arose as to the possible recruitment of stretch-activated calcium channels permitting calcium entry during the hypoosmotic shock. To elucidate this question, gadolinium (100 µM) was used with the same protocol, and did not inhibit osmotically-activated taurine transport since the percentage of basal taurine release 20 min after the shock was 1059 ± 60% for control, and 1079 ± 130% for gadolinium (no significant difference with \(p=0.86, n=6-9\)) (not shown). However, the addition of streptomycin (500 µM), another known blocker of stretch-activated and mechano-sensitive ion channels (54), significantly reduced (24 ± 5% decrease; \(p<0.005\)) hypotonic stress-stimulated transport (Fig. 6B).

To examine further intracellular signalling related to calcium, we used the cAMP-elevating agent, forskolin, an adenylcyclase activator. When forskolin (10 µM) was added to the isotonic medium during the initial phase, it did not mimic the hypotonic shock. No
spontaneous stimulation of basal taurine exit was observed. Subsequent hypoosmotic challenge did not modify significantly the stimulated response in the presence of forskolin compared with corresponding controls. The following percentages of basal taurine release 15 min after osmotic shock were observed for control and forskolin-treated cells, respectively: 598 ± 35%, for control; 604 ± 11%, for forskolin; no significant difference with \( p=0.88, n=6 \) (not shown). These results suggest that increased cAMP does not contribute to \( \text{Ca}^{++} \) mobilization during hypotonic shock. We also did not observe any effect of adenosine, DPCPX (1,3-dipropyl-8-cyclopentylxanthine), an \( \text{A}_1 \) adenosine receptor blocker, or arginine-vasotocin treatment on volume-activated taurine transport (data not shown).

**Cl\(^-\) conductance of gill cells challenged by hypoosmotic shock**

To study the effects of osmotic pressure on Cl\(^-\) conductance in primary cultures, currents were induced by osmotic shock. After successful gigaseal formation, the whole-cell configuration of the patch-clamp technique was obtained in 15% of cases. Figure 7, A-C, shows families of currents observed immediately (A), 3 min (B) and 7 min (C) after the beginning of the whole-cell recording and the application of the osmotic shock. A volume-dependent current developed rapidly and reached a steady state maximum value within 7 min (Fig.7C). At this time, the current at +80 mV had an amplitude 2.2 times that of the current at -80 mV (+80 mV=975±165 pA, -80 mV =-449±47 pA, \( n=7: p<0.005 \), Student’s paired t-test), thereby showing outward rectification. Since these currents were time-independent (Fig.7B and C), their I/V relationships were calculated over their initial phase of activation, i.e. 13 ms after the onset of the voltage pulse (Fig.7D). These data were obtained from experiments performed in symmetrical Cl\(^-\) concentrations. The I/V curve crossed the potential line at 0 mV, the reversal potential (\( E_{\text{rev}} \)) of Cl\(^-\) in those conditions. Therefore, in the absence of permeant cations in the pipette, this outward current was likely carried by Cl\(^-\). To further characterize this potential Cl\(^-\) current, we tested two anion channel blockers added separately to the bathing solution 7 min after whole-cell recording, when typical traces of maximal hypotonically-activated current were obtained. Addition of 100 \( \mu \)M NPPB or 1 mM DIDS to the bathing solution inhibited the volume-stimulated whole-cell Cl\(^-\) current within 5-10 min. The I/V relationships are given in Fig.7E and 7F respectively. NPPB totally inhibited both inward and outward volume-activated currents. DIDS inhibited the hypotonically activated currents in a voltage-dependent manner, with the block decreasing from to 72±16 % at -60 mV to 100% at +60 mV (n=5). The ranking order for inhibition was NPPB>DIDS.
Ultrastructural observations of gill cell swelling during hypoosmotic shock

SEM observations of the three-dimensional structure of control gill cells in culture revealed that they were organized in a regular epithelial-like structure composed of a mosaic of polygonal cells with the longest axis measuring 10 to 30 µm (Fig. 8A, A1 and A2). This arrangement of cells has been described previously (2, 3). The apical cell surface was formed by a complex system of microridges (Fig. 8A1). These morphological structures are characteristic of respiratory (pavement) cells and consistent with in vivo observations of teleost gill epithelium (21, 30), indicating that the cells studied in these experiments were polarized and highly differentiated. As early as 15 s after application of the hypotonic shock, we observed swelling in approximately 30% of cells from the monolayer (Fig. 8B). Their apical surface became smoother with varying degrees of ridge disappearance (specific focus on round swollen cells: Fig. 8B1, B2 and B3). The swelling effect intensified with time within the cell monolayer so that, during hypoosmotic challenge, we simultaneously observed various proportions of flat gill cells with microridges and round swollen cells with a smooth surface. This phenomenon started to reverse 10 min after the sustained hypotonic shock, at which time cells showed a progressive return to a flat morphology with marked apical surface microridges. The time-course of cell-swelling and reversion depended on the cell monolayer tested but all cultures always completely reverted within 30 min post-challenge, demonstrating the cell adaptation to hypotonicity and the RVD phenomenon (Fig. 8C, C1 and C2).

In TEM observations, cells grown on permeable filters presented a polarized structure. Transversal sections revealed microridges at the cell surface (Fig. 9A) and tight junctions located at the apical side (Fig. 9G), thus confirming previous SEM observations (Fig. 8). Figure 9B shows the simultaneous presence of flat pavement cells with marked microridges and round cells with a smooth surface 15 s after the application of the hypotonic shock. As stated above, during osmotic challenge we observed an increasing number of round swollen cells in the monolayer (Fig. 9C, D, E, F), a phenomenon that progressively subsided 10 to 30 min after the shock was applied (Fig. 9G, H).
DISCUSSION

During osmotic challenge, it may be essential for fish to activate emergency systems with rapid modulations of solute transport across the gill epithelium in order to maintain ion and water balance. Modifications of ion transport, as well as rapid changes in gill morphology, in accordance with ambient salinity have been observed in euryhaline teleost (4, 19, 29). The present work examines the physiological response to hypotonic challenge in primary gill cell culture which form a salt-secreting epithelium. In most vertebrate cells, hyposmotic shock causes cell swelling followed by the RVD response. RVD occurs through efflux of K⁺, Cl⁻ and organic osmolytes accompanied by osmotically obligate water loss (20, 28). We have previously demonstrated the presence of stretch-activated K⁺ channels in sea bass gill cells and discussed their mechano-sensitivity during hypotonic-induced cell swelling (12, 13). This study focuses taurine permeability in response to hypoosmotic shock. It demonstrates a stimulation of taurine exit more important at the basolateral side and questioned taurine transport pathway. Taurine movement is regulated by extracellular Ca++. These physiological events accompany substantial morphological remodelling.

Hypoosmotic shock stimulates Cl⁻ conductance

Cell swelling has been shown to activate an outwardly rectifying anion-selective conductance in all mammalian cells studied (41). The present work in gill cells shows the development of a volume-dependent current likely carried by Cl⁻, activated by a 30 % hypotonic shock. This conductance observed here also rectifies outwardly. This type of current has not been described in fish gills before and does not appear to be present in killifish opercular epithelium (36). Contrary to mammalian cells, this volume-activated current does not become inactivated with time during membrane depolarisation. The absence of inactivation of a swelling-activated anion conductance has also been observed in skate hepatocytes (22). NPPB (100 µM), a carboxylate analogue and Cl⁻ channel blocker, reduced the volume-stimulated current by 100% with the outward and inward currents equally affected. The effect of the stilbene derivative, DIDS (1 mM), was a little less efficacious but still inhibited the developed current by 70 to 100%, depending on the potential applied. In many cell lines, DIDS inhibition was also voltage-dependent and the swelling-activated outwardly rectifying Cl⁻ currents were blocked using lower doses of DIDS (< 500 µM) (1, 22, 41, 56). The pharmacological properties observed here with a potency order of NPPB>DIDS were also described in other mammalian cells (1, 40, 41).
Characteristics of the taurine pathway

The ability of cells to release amino acids (as well as inorganic ions) in response to hypoosmotic stress has been demonstrated in many species from bacteria to humans. In most marine organisms, cells contain large concentrations of taurine and other organic osmolytes that fill the osmotic gap between external and internal ion concentrations. Intracellular taurine concentrations are known to range from 20 to 65 mM in various fish (7, 22, 38).

Sea bass gill cells constitute a likely source of taurine release during osmotic shock as measured by isotopic fluxes. Upon hypoosmotic challenge, we observed a 5 to 10-fold increase in taurine release above basal levels. The effect of hypotonically-induced taurine transport is sustained and can be fully reversed by returning to an isotonic condition, as also observed in glial and HeLa cells (8, 18). The branchial epithelium may use its capacity for taurine transport during hypotonic challenge *in vitro*, and very likely during saline variations *in vivo*. Together, this suggests an important physiological role of taurine at in maintaining cellular homeostasis, which is compatible with the reported taurine concentration in gill cells of 27 mM in catfish *Ictalurus punctatus* and 35 mM in trout *Salmo gairdneri* (respectively 16, 59).

For many cell types, it is widely accepted that, upon swelling, taurine exits the cell through permeable volume-regulated anion channels referred to as volume-sensitive organic anion channels (VSOAC) (6, 57). We have shown here that hypotonically stimulated taurine transport was significantly reduced by anion channels inhibitors with the potency order of NPPB>NFA>DPC>DIDS. This pattern of inhibition is similar to that found in rat glial cells (NPPB>NFA>DPC>DIDS) (8). Despite the relative non-specificity of the different inhibitors, pharmacological inhibition of taurine transport is the most used method involving the contribution of anion channels. Therefore, considering the selectivity for different inhibitors it can be suggested that this pathway is a swelling-activated anion channel-like structure. When comparing electrophysiological (Cl⁻ transport) and kinetic (taurine transport) data, we observed that the maximum activation of the outwardly rectifying volume-activated anion current was reached within 7 min, and that maximum taurine release was accomplished within 10 min, corresponding to similar activation time courses. Moreover, the pharmacological agents NPPB and DIDS inhibited both chloride current and taurine transport to the same extent, nearly 100%. These observations suggest the existence of a single common pathway for osmosensitive taurine and Cl⁻ efflux under hypoosmotic conditions, possibly *via* a VSOAC channel (24, 26). None of the pharmacological agents investigated significantly
reduce taurine transport in isotonic conditions (results not shown), suggesting that this pathway is quiescent when cells are at steady state. A potential candidate could be the anion exchanger AE1 (band 3 protein) which is also sensitive to DIDS and the only channel described in volume regulation of fish cells (17, 43). However, one cannot rule out a non-specific effect of anion channel blockers, nor that there may be two types of channels in gill cells in culture, one responsible for swelling-induced Cl⁻ current, and another for DIDS- and NPPB-sensitive amino acid transport, both having the same “trigger” for activation.

**Taurine permeability of basolateral membrane increases during hypoosmotic challenge**

The present work shows that apical and basolateral membranes have the same permeability to taurine in isotonic medium, whereas in hypotonic medium basolateral membranes developed a considerably higher permeability than apical membranes. Therefore, the hypotonically-activated taurine transport pathways are distributed in a polarized way in gill cells. Similar findings have been observed in goldfish renal cells (14) and in amphibian A6 cells (51), but not in flounder renal cells (7). The increased gill secretion of taurine through the apical membrane in a diluted environment will help the fish excrete any excess of osmotically active material and obligated water more rapidly, thus facilitating the RVD, as observed by Benyajati and Renfro (7) in flounder renal tubules. When asymmetrical osmotic shocks were applied, we observed higher permeability and sensitivity of the basolateral membrane compared to the apical membrane. In a previous study, we described the relative impermeability of the apical side of sea bass gill epithelium in culture towards Cl⁻ when measuring ¹²⁵I flux (reflecting chloride flux) during asymmetrical hypoosmotic shock (13). Moreover, Gilmour et al. (15) found a reduction of the transcellular permeability and an increase in transepithelial resistance when the apical membrane of gill cells in culture was exposed to hypotonicity (freshwater). In view of these results, the relatively low permeability of the apical membrane observed in our study during hypoosmotic shock may have physiological relevance for fish which need to limit an overwhelming load of water into the cells in response to a rapid decrease in salinity.

**Calcium entry stimulates taurine release**

Calcium signaling pathways play a major role in activating and controlling RVD mechanisms in most cell types (37). Osmotic swelling can increase intracellular calcium by activation of Ca⁺⁺-permeable channels in the cell membrane and Ca⁺⁺ release from intracellular stores. Trout gill cells in culture display intracellular Ca⁺⁺ elevation when subjected to a hypoosmotic
Our initial findings were that taurine can be released by an external calcium signal independent of a change in osmotic pressure (Fig. 5A); no additional transport mechanisms were observed in response to osmotic shock. Thus, whereas an increase in cytosolic Ca\(^{++}\) of external origin may appear as a weak permissive factor in hypotonically evoked taurine release, it may also constitute a full signal by itself. Our results in the absence or in the presence of thapsigargin or BAPTA-AM (Fig. 5B) strongly supports our hypothesis that the hypotonic shock activates an influx of external Ca\(^{++}\), leading to increased cytosolic Ca\(^{++}\) levels and stimulation of taurine release. Similar findings were described for human HeLa cells and rat pituicytes (27, 47).

The mode of Ca\(^{++}\) entry in response to a hypotonic shock has been investigated. The abolition of taurine release upon lanthanum addition suggests the stimulation of hypotonically Ca\(^{++}\)-permeable channels. For most cells, stretch-activated ion channels (SAC) are the putative pathways for Ca\(^{++}\) entry (P-type, L-type and TRP channels) in hypotonic conditions (34, 50). In our study, the absence of taurine transport inhibition in response to verapamil and nifedipin indicates that L-type Ca\(^{++}\) channels are not involved in Ca\(^{++}\) entry. Similar results with verapamil were observed in amphibian renal A6 cells challenged by hypotonic shock (51). Although gadolinium had no effect, taurine transport was significantly reduced by streptomycin, another blocker of stretch-activated ion channels (54). This specific inhibition of hypotonically induced Ca\(^{++}\) entry suggests the existence of stretch-activated Ca\(^{++}\)-channels in gill cells. In trout gills, the presence of an epithelial Ca\(^{2+}\) channel (ECaC) regulated by environmental salinity has been demonstrated (52, 53). More studies are needed to elucidate these findings and to further analyze the taurine signaling pathway(s) in gill cells.

**Gill pavement cell plasticity controls RVD through unfolding and wrinkling of surface microridges**

The surface of sea bass gill respiratory cells in primary culture exhibits a complex system of microridges with various degrees of wrinkling. It was considered until now that this remarkable morphology reflects expansion of outer membrane for respiratory gaseous exchanges, increasing the surface area of teleost gills 2.5 times (42). However, the exact function of these microridges has remained unclear since they are not restricted to respiratory structures like gills, but can also be found in association with other epithelial cells that are subjected to mechanical challenge. Our present SEM and TEM ultrastructural changes of gill pavement cell microridges during acute salinity changes or RVD are the first to be described in the literature.
Our results reveal a striking process of unfolding of the apical membrane ridges of gill respiratory cells with the outer surface area becoming smoother during osmotic stress. This morphological change demonstrates that the cell membrane has reserve unfolding available and could explain the report that neither membrane tension nor capacitance could be affected by cell-swelling (44). Therefore, we propose that osmotic swelling may not actually induce membrane stretch per se inasmuch as the microridges may provide reserve surface area for swelling during mechanical stress. Okada (41) suggested that cell-swelling probably results mainly from loss of cell membrane invaginations that are supported by the F-actin-based meshwork. Although this function has not been described for gill cell microridges in the literature, our study reveals that the topography of the branchial epithelium is clearly involved in the RVD process, providing mechanical adaptation during the osmoregulation process.

On a kinetical point of view, we can state that gill respiratory cells were very reactive to hypotonicity since the significant structural response started as early as 15 s after the challenge. This enables euryhalinity in fish, which need to osmoregulate quickly considering the enormous osmotic constraints that they encounter during their life. When comparing the time course of morphological and physiological events examined here, it is reasonable to postulate that early initial cell swelling (occurring in seconds) may trigger taurine and Cl⁻ transport which become activated after several minutes. Moreover, the cell volume decrease was initiated 10 min after hypotonic insult, at which time taurine and Cl⁻ permeabilities were at their maximum activation rate. This observation reflects the efficiency of taurine and Cl⁻ transport and is consistent with the proposed key role of these solutes in the RVD. The total duration of RVD kinetics (up to 30 min) is similar to what has been described in a single cell suspension of trout gill cells (33).

The volume-regulatory response in a number of cell models was found to be sensitive to pharmacological disruption of the cytoskeleton, and was accompanied by small changes in total cellular F-actin. Daborn et al. (11) and Okada (41) stated that microridges are structurally supported by polymerised actin cords. Therefore, a role for the cytoskeleton in regulating swelling-activated ion and osmolyte channels can be proposed. Together, these structural observations indicate a high degree of morphological modelling in gill respiratory cells in response to an acute stress induced by environmental salinity changes.

**Conclusion**

When sea bass move from sea water to estuaries, their gill epithelium are exposed to hypotonicity both on the apical side which is in direct contact with a diluted medium, but also
on the basolateral side, because of decreased plasma osmolarity in hypotonic medium. This osmolality decrease is transient for euryhaline species. It is likely that gill cells respond specifically to blood parameters such as reported for chloride cells in killifish operculum epithelium (60). Our results suggest that decreased plasma osmolarity may be the decisive factor that can trigger ion and osmolyte excretion. The control of epithelial Cl\(^{-}\) secretion by basolateral hyposmolarity has been described previously in the killifish (35). Moreover, plasma cation variations have been shown to signal internal polyvalent cation receptors proteins (CaRs) serving as salinity sensors in gills and other organs (39).

The higher exit of taurine on the basolateral side may contribute to increased reabsorption into the blood circulation, limiting the loss of this valuable to the external environment. Investigators have reported that physiological concentrations of plasma taurine increased from 0.1-0.8 mM to 1.3-2.5 mM when marine fish were transferred from isosmotic to hyposmotic conditions (7). We suggest that the high rate of taurine secreted into the circulation (i.e. at the basolateral side) may contribute to the restoration plasma osmotic pressure in fish, but this effect will need to be further investigated.

**Perspectives and significance**
Understanding cell volume regulation is a challenge because of the large variety of mechanisms with which different species and cells translate osmotic stress into plasma membrane modifications. Few studies have examined the RVD response in organisms physiologically confronted to osmotic challenges in their natural environment. We predicted that euryhaline fish had developed very efficient mechanisms fundamental to adaptation to extreme osmotic conditions encountered during their life. Here we have described major mechanisms of functional and morphological plasticity occurring in a salt-transporting epithelium. This study supports the idea that maintenance of a constant cell volume in the face of osmotic stress is among the most evolutionarily ancient cellular homeostatic mechanisms. In the future, to identify key proteins involved in fish salinity adaptation, as membrane transporters, an interesting approach will be the comparison of protein or mRNA banks established from gills of fish adapted to FW and SW.

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REFERENCES


FIGURE LEGENDS

**Figure 1**: Physiological correlates of a hypotonic challenge. (A) Measurement of salinity decrease in the external medium (t=0: SW to t=4.75 h: FW). (B) Follow-up of the simultaneous reduction in plasma osmolarity. Statistical comparisons were made with Student’s unpaired t-test (one-tail). * p<0.01 (the number of animals analyzed is 5 to 6 for each time point). (C) Positive correlation between external and internal osmotic pressures, correlation coefficient r=0.96, p<0.001.

**Figure 2**: Hypotonically induced [³H]taurine efflux in gill cells in primary cultures on Petri dishes and its inhibition. A) Cells were preloaded with [³H]taurine, and the efflux was measured as a function of time. Medium bathing cells was alternated between iso- and hypotonicity as indicated above trace (n=11, 3 animals). B) Effects of anion channel inhibitors on hypotonically-stimulated taurine transport. “Basal” corresponds to data obtained from cells during the initial iso-osmotic period. “Shock” corresponds to control response 10 min after the hypoosmotic shock. Other data were recorded in the presence of various inhibitors under similar conditions. Statistical comparisons between inhibitors and “shock” data were estimated with Student’s unpaired t-test: ** p<0.001; * p<0.01, (n=4 for NPPB, NFA, Tamoxifen and DPC; n=7 for DIDS; n=11 for shock, 3 animals).

**Figure 3**: Dissociated taurine movements measured in gill respiratory cells grown on permeable filters (symmetrical conditions). The apical and basolateral [³H]taurine effluxes were followed under iso-osmotic or hypoosmotic conditions without (A) or with (B) 1mM DIDS introduced at both the apical and basolateral sides (n=3-4, 2 animals). ♦: apical; ■: basolateral.

**Figure 4**: Effect of an asymmetrical hypotonic shock measured in gill respiratory cells grown on permeable filters. [³H]taurine effluxes were monitored as a function of time through the apical and basolateral membranes. A) Cell monolayers were subjected to an apical shock, while the basolateral medium remained iso-osmotic. B) Cell monolayers were subjected to a basolateral shock, while the apical medium remained iso-osmotic. (n=3, 2 animals) ♦: apical; ■: basolateral.
**Figure 5:** Calcium regulation of hypotonically stimulated taurine transport measured in gill respiratory cells grown in Petri dishes. A) Effect of ionomycin on taurine transport (n=6, 2 animals): ♦ control; ■ 1 µM ionomycin. B) Effect of calcium-depleted medium (0 Ca²⁺ext), thapsigargin (0.55 µM; 1h incubation prior to hypotonic shock) or BAPTA-AM (10 µM; 1h incubation prior to hypotonic shock) on taurine transport. Data were collected 15 min after application of the hypoosmotic shock (n=3-9, 3 animals). Statistical comparisons were performed with one-way analysis of variance followed by Bonferroni’s post-hoc test for all pairwise multiple comparisons. 0 Ca²⁺ and BAPTA were significantly different from hypotonic “shock” (*p<0.05), but thapsigargin was not.

**Figure 6:** Characterization of Ca²⁺ channels involved in hypotonically developed taurine transport measured in gill respiratory cells grown in Petri dishes. A) Effect of lanthanum (n=3, 2 animals): ♦ control; ■ 1 mM lanthanum. B) Effect of streptomycin (n=3, 2 animals): ♦ control; ■ 500 µM streptomycin.

**Figure 7:** Swelling-activated Cl⁻ currents recorded in primary cultures of sea bass gill cells and their inhibition. A, B and C: Whole-cell currents were recorded at the time of hypoosmotic shock indicated above traces. D, E and F: Average current-voltage relationships measured 13 ms after pulse onset (n=5-7, 2 animals). D: Swelling-activated whole-cell Cl⁻ current (♦ control; ● 3-min hypoosmotic shock; ■ 7-min hypoosmotic shock). E: Effect of NPPB on hypotonically developed currents recorded at t=0 (♦), and at maximum of the volume-activated response (t=7 min) in the absence (■) or presence (▲) of 0.1 mM NPPB for 6 min to reach maximal inhibition. F: Effect of DIDS on hypotonically developed currents. Whole-cell currents were recorded at t=0 (♦), and at maximum of the volume-activated response (t=7 min) in the absence (■) or presence (▲) of 1 mM DIDS for 5 to 10 min to reach maximal inhibition.

**Figure 8:** Sea bass gill cells cultured on permeable filters during hypoosmotic challenge observed by SEM (observations from 3 animals). (A) Control cells in isotonic medium: the culture was composed of a monolayer of “flat” gill cells. Insets (A1 and A2): details of contiguous epithelial polygonal cells showing well-defined intercellular junctions and irregular arrangements of surface microridges characteristic of gill respiratory cells; (B) 15 s to 2 min after the hypotonic shock, numerous round cells appeared within the monolayer.
Insets (B1, B2 and B3): specific focus on round swollen cells with a smooth surface; (C) 30 min after the hypotonic shock: return to control shape with flat cells showing surface microridges, reflecting the RVD phenomenon. Insets (C1 and C2): details of the monolayer with flat cells. Abbreviations: rc, round cells; fc, flat cells; mr, microridges; pm, plasma membrane. Scale bar=100 µm for A, B, C and 10 µm for all other pictures.

**Figure 9**: Sea bass gill cells cultured on permeable filters during hypoosmotic challenge observed by TEM (observations from 3 animals). (A): control: pavement cell with developed surface microridges (x13,860); (B): 15 s after the hypoosmotic shock. Note the juxtaposition of a round swollen cell with a smooth surface (rc) and a flat cell with superficial microridges (fc) (x7,220); (C), (D), (E) and (F): specific focus on round cells, 15 sec (C), 2 min (D), 5 min (E) and 10 min (F) after hypoosmotic shock. Note that the intracellular organization is intact (respectively x8,200; x8,000; x 10,133; x12,100); (G): Detail of a tight junction between respiratory cells, 5 min after the hypoosmotic shock (x72,800); (H): 10 min after the hypoosmotic shock. Note the beginning of recovery, flat cells presenting a wrinkled surface (x12,000). Abbreviations: A, apical side; B, basal side; fc, flat cell; rc, round cell; mr, microridges; t, tight junctions; n, nucleus; rer, rough endoplasmic reticulum; m, mitochondria; g, Golgi apparatus.
**TABLE 1**: Composition of solutions used in whole cell clamp experiments

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<th>Pipette (mM)</th>
<th>Bath (mM)</th>
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<tr>
<td>NMDG$^+$</td>
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<td>Cl$^-$</td>
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<td>Na$^+$</td>
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<td>Ca$^{++}$</td>
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<tr>
<td>Mg-ATP</td>
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<td>EGTA</td>
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Mannitol was added to the pipette and bath solutions in adequate concentrations to reach the respective osmotic pressures of 390 and 290 mOsm/kg. Solutions were kept at room temperature (20°C). The pipette solution was titrated to pH 7.4 with 6N HCl and the bath solution to pH 7.8 (similar to sea bass blood pH) with 1N NaOH.
Figure 2

(A) Graph showing the change in % of basal urine release over time (min) under isosmotic and hypotonic conditions.

(B) Bar graph comparing basal and shock urine release with various treatments (DIDS, NPPB, NFA, Tamo, DPC) in different concentrations (1 mM, 100 μM, 100 μM, 20 μM, 300 μM, 1 mM).
Figure 4

A

B

% of basal tauire release

Time (min)
Figure 9